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PHOSPHOLIPID PEROXIDES ARE INTERMEDIATES IN THE ENZYMIC OXIDATIVE CLEAVAGE OF MICROSOMAL PHOSPHOLIPIDS

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BILLY KAI SHU TAM

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PHOSPHOLIPID PEROXIDES ARE INTERMEDIATES

IN THE ENZYMIC OXIDATIVE CLEAVAGE

OF MICROSOMAL PHOSPHOLIPIDS

APPROVED BY

Paul B. McCay

Thomas Riggs

R. Carnell

Jordan J. H. Tang

Simon H. Wender

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

The occurrence of enzymes in liver microsomes was first investigated by Hogeboom and Schneider (1, 2). The identification of microsomes as fragments of the endoplasmic reticulum of the cell was achieved by Palade and Siekevitz (3, 4). These investigators performed a systematic electron microscopic study of intact rat liver, liver homogenates and isolated microsomes. As a result of their studies, they concluded that microsomes can be divided into two groups, one with smooth-surfaced vesicles, and the other with rough-surfaced vesicles.

The composition of smooth and rough liver microsomes has been reported (5); however, because of the composite nature of the preparations, the values have limited interest. The presence of copper, manganese, heme, flavins, cytochrome b_5, non-heme iron, sodium, potassium, calcium, zinc, NADH, NAD^+, NADPH and $NADP^+$ has been established. The presence of coenzyme Q, FAD, FMN and carbon monoxide-binding pigment was established also (6, 7); the general composition of liver microsomes appears to be similar to that of mitochondria insofar as redox substances

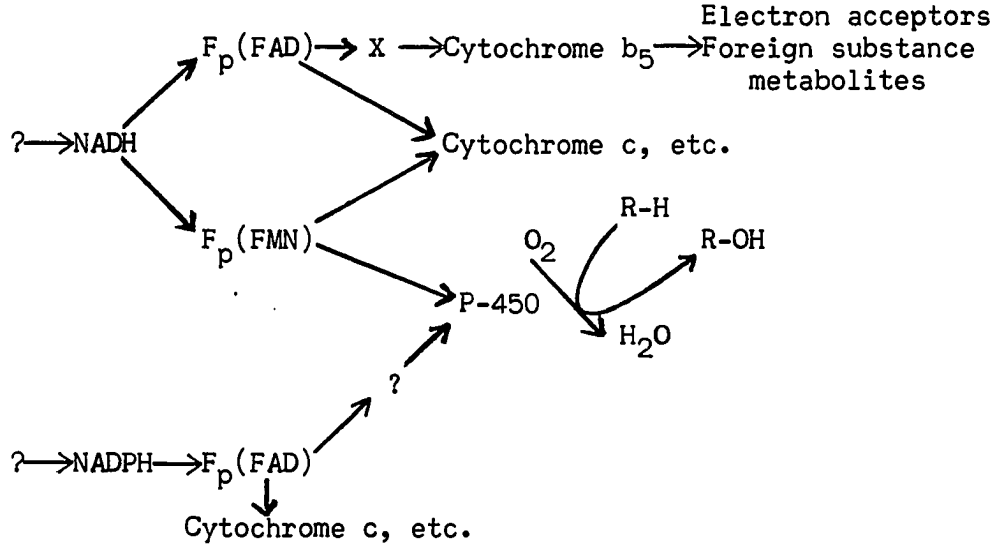
are concerned. In addition, all of the components of smooth microsomes also occur in rough microsomes. NADH-cytochrome b_5 reductase (8) and NADPH-cytochrome c reductase (9) are FAD enzymes. But NADH-cytochrome c reductase may be an FMN enzyme (10). Cytochromes P_{-450} and P_{-420} have been partially purified and characterized (11). Another heme iron studied by Mason et al., (12), called "Fe-x" has been isolated and is believed to function in a manner analogous to that of cytochrome a or a_3 of the mitochondrion. It is present in both P_{-420} and P_{-450} in the microsomes.

The enzymic activities of microsomes from various tissues have been summarized (13, 14, 15); all principal classes of enzymes have been found: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. These enzymes support major energy-requiring and metabolic processes such as biosynthesis of proteins, steroids, lipids and polysaccharides as well as transporting processes. The microsomal oxidoreductases include various component enzymes of the metabolic network and mixed-function oxidases, which either form or require reduced coenzymes for action. Among them are enzymes which metabolize carcinogens, drugs and other xenobiotic substances. These mixed-function oxidases conform to the general reaction:

$$NADPH + H^+ + R\text{-}H + O_2 \longrightarrow NADP^+ + R\text{-}OH + H_2O$$

These oxidases are believed to involve an electron transport chain found in the membrane of the endoplasmic reticulum (16). The relationships between the components of the microsomal electron transport system have been established by: (a) studies of isolated components; (b) studies of the effects of various electron acceptors and inhibitors;

(c) studies of the rate of induction of the components by drugs; and (d) studies of the appearance of various enzymic activities in the developing liver. These studies may be accounted for by the following scheme which is a composite of those given by Siekevitz, Mason, Ernster and Sato (17, 18, 5, 19, 20):

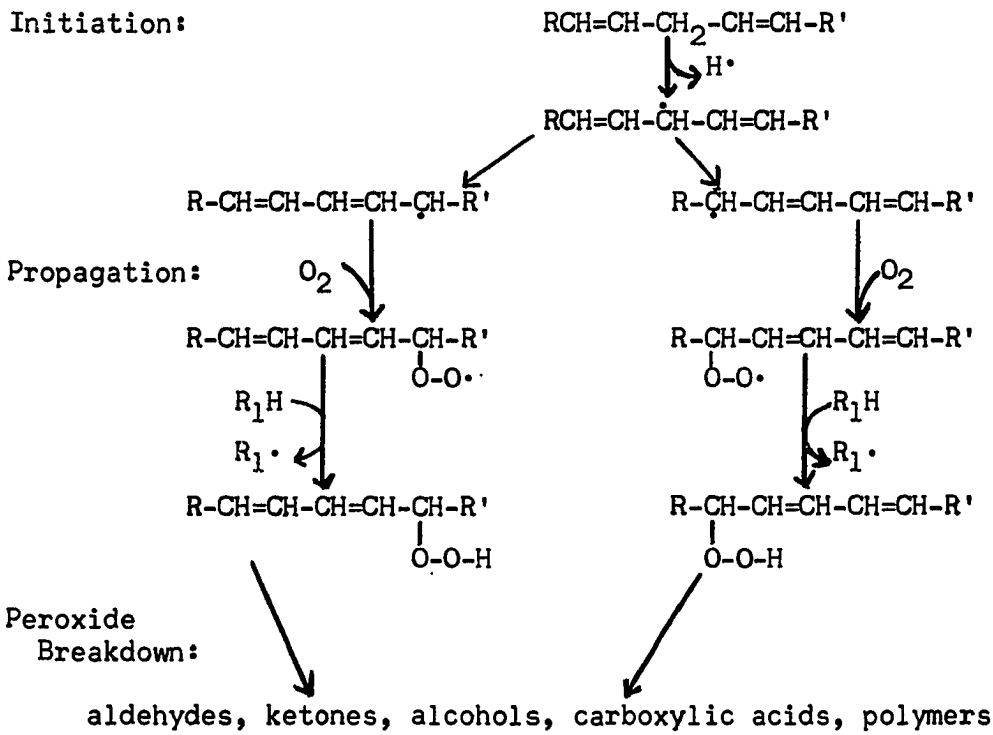


where NADH and NADPH are di- and triphosphopyridine nucleotides, respectively; F_p(FAD) and F_p(FMN) are flavoproteins. Previously, there appeared to be only two electron transport chains, one for the oxidation of NADH and another for the oxidation of NADPH. Gillette et al., (21) found that oxidative deaminations and dealkylations, as well as hydroxylation of aromatic hydrocarbons, and oxidation of side chains and sulfoxide formation, were initiated by a NADPH oxidase system present in microsomes. NADPH-cytochrome c reductase probably involves part of this NADPH oxidase system (22, 23). Hochstein and Ernster (24) later demonstrated that rat liver microsomes, in the presence of NADPH, oxygen, and ADP or other nucleoside diphosphates, catalyze the rapid formation of a 2-thiobarbituric acid (TBA)-reacting compound which they assumed to be

derived from the peroxidation of lipids in the enzyme preparation. The reaction can be followed readily by the formation of this chromogen (which Hochstein and Ernster believed to be primarily malondialdehyde) which is known to be the product formed in the autoxidation of unsaturated lipids. The formation of this chromogen could be prevented by ethylenediaminetetraacetate (EDTA), parachloromercuribenzoate (PCMB), several antioxidants such as diphenylphenylenediamine and α-tocopherol. Its formation could also be prevented by inactivation of microsomes by heat prior to incubation with NADPH.

Lipid peroxidation in vitro has been studied in model systems for several years and many, though not all, of the basic mechanisms and properties of such systems are understood. It is believed by Lundberg (25) and Ingold (26) that lipid peroxidation is an autocatalytic series of reactions involving a free radical mechanism. The process can be divided into four phases:

1) initiation --- formation of a free radical on the lipid;
2) propagation --- formation of a peroxide by reaction of the lipid free-radical with O_2; abstraction of H from other lipids to form the hydroperoxide and more free radicals;
3) hydroperoxide breakdown --- formation of more free radicals and carbonyl compounds and other substances;
4) chain termination --- formed radicals react with each other or other substances to form stable products.



Chain Termination:

$$\text{R-CH=CH-CH=CH-CH-R'} \quad (\text{O-O}\cdot) \quad + \quad \text{X} \longrightarrow \text{Inactive products}$$

$$\text{R}\cdot \quad + \quad \text{X} \longrightarrow \text{Inactive products}$$

R_1H may be an unsaturated fatty acid with a methylene group of a methylene interrupted (1,4-)pentadiene portion present, and X is either another compound, or free radical, which may react with a free radical to form another substance which may then be stable and inactive.

Among other kinds of lipid-derived products which are formed by this process (27), carbonyl derivatives are usually formed in relatively larger quantities. Aldehydes are by far the predominant products. When polyunsaturated fatty acids, such as arachidonic, docosapentenoic or docosahexaenoic acids, undergo autoxidation, a 2-thiobarbituric acid-reacting chromogen is formed. It is presumed to be malondialdehyde and is

used as an index to measure the extent of lipid peroxidation (28).

Lipid peroxidation *in vivo* has been reviewed by Tappel (29). Enzyme-associated lipid peroxidation has been reported to occur with the rat liver microsomal enzyme L-gulonolactone oxidase in α-tocopherol-deficient animals (30, 31). This was the first report of lipid peroxidation related to the function of an enzyme. However, it has been difficult to demonstrate directly that lipid changes occurred during the function of this enzyme (32).

In a study of another microsomal oxidase, Beloff-Chain *et al.*, (33) reported that oxygen consumption accompanied the oxidation of NADPH by rat liver microsomes. The oxygen uptake was stimulated by ADP. Hochstein and Ernster (24) indicated that oxidation of NADPH by liver microsomes resulted in the uptake of excess oxygen and the production of a substance reacting with 2-thiobarbituric acid to form a colored compound with a maximum absorption at 532 mμ. The substance was believed to be malondialdehyde and its identity was established (34, 35). The NADPH in NADPH-dependent oxidation of ethanol by liver microsomes could not be replaced by H_2O_2 but could be replaced by glucose and glucose oxidase. It is believed that microsomal oxidation of ethanol is H_2O_2 dependent. However, the contribution, if any, of the microsomal mixed function oxidase system to the total oxidation of ethanol in mammals appears to be insignificant (36). The rate of methanol oxidation *in vivo* was found to bear a direct relationship to the amount of particulate catalase in the livers of rat, mouse and guinea pig (37). Hepatic oxidases were studied in rat and monkey liver homogenates and in subcellular fractions for their capacity to provide hydrogen peroxide for the cata-

lase-dependent peroxidative oxidation of methanol. These studies indicate that hydrogen peroxide generation is rate-limiting in the peroxidation of methanol in both rat and monkey liver (38).

Recently, May and McCay (39, 40) demonstrated that the microsomal fraction of rat liver tissues contains an enzyme system which catalyzes the oxidative cleavage of certain polyunsaturated fatty acids (PUFA) situated at the β-position of the phospholipids of phosphatidyl-ethanolamine and phosphatidyl-choline in the microsomal membrane. The system, requiring NADPH and molecular oxygen, is highly active in freshly isolated microsomes. Measurable structural alteration of the membrane results. The reaction consumes 1 mole of phospholipid polyunsaturated-fatty acid and 4 moles of oxygen for each mole of NADPH oxidized. The process is very heat-labile and is inhibited by Mn^{2+}, Co^{2+}, EDTA and PHMB. The products of the reaction include water-soluble aldehydes (presumably derived from the methyl end of the cleaved polyunsaturated-fatty acid chain) and phospholipids whose β-acyl groups contain carbonyl functions. Additional evidence makes it appear almost certain that at least part of the function of α-tocopherol is involved with the regulation of this membrane-altering activity (41).

The purpose of the work described in this dissertation has been to elucidate further the events occurring during the reaction described above. An outline of the experimental approach follows:

1) Detection of a probable intermediate in the reaction.
2) Determination if its formation is enzymic.
3) Determination of the course of its formation and disappearance during this enzymic reaction.

4) Isolation of some of the reaction products.

5) Demonstration of the substitution of H_2O_2 for NADPH in the reaction system.

6) Determination if the H_2O_2-dependent peroxidative cleavage of phospholipids is catalase-dependent.

CHAPTER II

MATERIALS AND METHODS

Materials

Reagents

All chemicals and solvents were reagent grade and were used as obtained except where specified otherwise.

Adenosine 5'-Diphosphate, sodium (ADP) was obtained from P-L Biochemicals, Inc., Milwaukee, Wisconsin.

Trichloroacetic acid (TCA), Tris (Hydroxymethyl) amino methane (purified), hydrogen peroxide (30%), sodium azide, aniline, and potassium iodide were obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

Potassium chloride, manganese chloride, and soluble starch (suitable for iodometry) were purchased from Merck, Rahway, New Jersey.

Lyophilized Crotalus adamanteus venom was a product of Ross Allen Reptile Institute, Silver Springs, Florida. It was used as a source of phospholipase A (α-phosphatide-β-acyl-hydrolase).

Ninhydrin was purchased from Pierce Chemical Company, Rockford, Illinois.

Rhodamine-6-G and tetranitromethane were obtained from K & K Laboratories, Plainview, New York.

PHMB (Para-hydroxymercuribenzoate) was a product of Sigma Chemical Company, St. Louis, Missouri.

Boron trifluoride-methanol was a product of Applied Science Laboratories, State College, Pennsylvania.

Hexane (Chromatoquality reagent) was obtained from Matheson Coleman & Bell Company, Norwood, Ohio.

Petroleum ether, diethyl ether (anhydrous), methanol, chloroform, monobasic potassium phosphate, dibasic potassium phosphate, ferric chloride, and sodium chloride were purchased from Fisher Scientific Company, Fair Lawn, New Jersey.

Silica gel G (containing $CaSO_4$ as binder) was obtained from Warner Chilcott Laboratories Instruments Divisions, Richmond, California.

Phosphatidylethanolamine, phosphatidylcholine, and adsorbosil-5 were obtained from Applied Science Laboratory, Inc., State College, Pennsylvania.

Lysophosphatidylcholine was a product of Koch-Light & Co., Ltd., Colnbrook, England.

Linoleic acid was purchased from California Biochemical Corporation, Los Angeles, California.

The following chemicals were obtained from Eastman Organic Chemicals, Rochester, New York: 2, 4-dinitrophenylhydrazine, sodium ethylenediaminetetraacetate, 2-thiobarbituric acid, catechol, quinone, o-phenylenediamine.

Reagent grade silicic acid, 100 mesh, was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

NADPH (Nicotinamide adenine dinucleotide phosphate, reduced

form), sodium D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase and L-thyroxine were obtained from Sigma Chemical Company, St. Louis, Missouri.

Glacial acetic acid, sodium cyanide, ferric chloride, and ammonium hydroxide were purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey.

3-Amino-1, 2, 4-triazole was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

Iodoacetic acid was a product of Nutritional Biochemicals Corporation, Cleveland, Ohio.

Instruments

Dubnoff Metabolic Shaking Incubator manufactured by Precision Scientific Company, Chicago, Illinois.

International Portable Refrigerated Centrifuge, Model PR-2, manufactured by International Equipment Company, Boston, Mass.

Short Wave Ultra-Violet Hand Lamp, Model SL 2537, made by Fisher Scientific Company, New York.

Rotary Evaporator made by A. S. Aloe Company, St. Louis, Missouri.

International Centrifuge, Size 2, Model 3 made by International Equipment Company, Boston, Mass.

Beckman Quartz Spectrophotometer, Model D.U., made by Beckman Instruments, Inc., South Pasadena, California.

Gram-Atic Balance, Model B, made in Switzerland by E. Mettler, Zurich.

Vortex Jr Mixer made by Scientific Industries, Inc., Queens

Village, New York.

Precision Balance, Model L, made by Federal Pacific Electric Company, Newark, New Jersey.

Precision Balance made by Roller Smith, Bethlehem, Pennsylvania.

Corning pH Meter, Model 12, made by Corning Glass Works.

Spinco Ultracentrifuge, Model L, made by Beckman Instruments, Palo Alto, California.

Cary Model 14 Recording Spectrophotometer, Applied Physics Corporation, Pasadena, California.

Animals

In all experiments, male albino rats bred in this laboratory from the Holtzman-Sprague Dawley strain were fed a rat pellet diet or a synthetic diet. Animals of 9 to 10 weeks of age (220 to 250 grams in weight) were selected for use.

Materials for Diets

Vitamins (except α-tocopheryl acetate), cod liver oil, casein and Alphacel (a pure, powdered cellulose added for bulk) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Stripped lard (α-tocopherol and other volatile materials removed by molecular distillation), α-tocopheryl acetate and α-tocopherol were obtained from Distillation Products Industries, Rochester, New York.

Experimental Diets

α-Tocopherol-deficient diets. The experimental diet used was that of Young and Dinning as modified by Caputto et al., (42). The method of Hubbell et al., was used to prepare the salt mixture and vitamin

mixture (43).

Composition of Vitamin Mixture

Biotin	1.1 mg
Calcium Pantothenate	225.0 mg
Choline Chloride	22.5 g
Dextrose	100.0 g
Folic Acid	112.5 mg
Inositol	22.5 g
Nicotinamide	4.5 g
2-Methylnaphthoquinone	5.6 mg
Pyridoxine.HCl	112.5 mg
Riboflavin	112.5 mg
Thiamine	112.5 mg
Vitamin B_{12}	1.0 mg

Composition of Salt Mixture

$AlK(SO_4)_2 \cdot 12H_2O$	0.017%
$CaCO_3$	54.300%
$FeSO_4 \cdot 7H_2O$	0.090%
KCl	11.200%
KH_2PO_4	21.200%
KI	0.008%
$MgCO_3$	2.500%
$MgSO_4$	1.600%
$MnSO_4 \cdot H_2O$	0.035%
NaCl	6.900%

Composition of Basal Diet

	Percent Composition
Casein, vitamin free	17.0
Sucrose	37.3
Corn starch	36.0
Cod liver oil	3.0
Lard, stripped	3.0
Salt mixture	3.0
Vitamin mixture	0.7

The basal diet was mixed with Alphacel in a ratio of 10 part diet to 1 part Alphacel.

α-Tocopherol-supplemented diets. These diets were identical to α-tocopherol-deficient diet except 10 mg α-tocopherol acetate was added to every hundred grams of diet.

Stock Diet (Pellet Diet)

Rats that were not maintained on an experimental diet were fed a commercial pellet diet from Rockland Laboratories, Teckland Incorporated, Monmouth, Illinois. This diet had the following ingredients: soybean meal, ground yellow corn, fish meal, pulverized barley, wheat midlings, ground wheat, dehydrated alfalfa meal, pulverized oats, feeding oat meal, dried skim milk, 1% animal fat, vitamin A palmitate, irradiated dried yeast, niacin, calcium pantothenate, riboflavin supplement, 0.5% dicalcium phosphate, copper oxide, traces of manganese oxide, menadione, vitamin B_{12}, 1% calcium carbonate, 1% sodium chloride, cobalt carbonate, iron carbonate, calcium iodate and zinc oxide. The manufacturers guaranteed the following analyses: crude protein, 24%; crude fat, 4%;

and crude fiber, 6%.

Microsomes

For each preparation, three rats were used. The animals were killed by a blow on the head and the livers were quickly removed. Twenty grams of liver were collected and washed at 0°C in about 100 ml of cold 0.15 M tris-HCl buffer, pH 7.5. The liver, about 20 grams, was cut into small pieces with scissors. Homogenization in tris-HCl buffer was performed in a tight-fitting glass homogenizer of the Potter & Elvehjem type. The first centrifugation was performed at 9,000 r.p.m. for 15 min. in a Spinco Model L or L2 preparative ultracentrifuge with rotor No. 30, using cellulose nitrate tubes with a volume of 30 ml. The pellet, containing nuclei, cell debris and mitochondria, was discarded. The supernatant fraction was then placed in 4 tubes and centrifuged for 90 minutes at 30,000 r.p.m. (78,000 x g). The pellet microsomes that precipitated were then washed twice with 0.15 M tris-HCl buffer, pH 7.5, and spun for two additional runs of 60 minutes each at 30,000 r.p.m. The microsomal pellets were used immediately or afterwards stored for up to 2 weeks in the deep freezer (-20°C) in the same plastic tubes covered with parafilm. The frozen pellet of microsomes was thawed immediately before use and suspended by homogenization in tris-HCl buffer (0.1 M, pH 7.5) so that 1 ml of the suspension was equivalent to the microsomes from 1 gram of liver wet weight. Once thawed, microsomes were not refrozen for later use since it was found that repeated freezing and thawing caused an increase in the 2-thiobarbituric acid (TBA) chromogen in the control incubation systems.

Methods

Enzyme Assay Procedure

Peroxide determination. Liver microsomes were prepared as previously described. Microsomes were suspended in 0.1 M tris-HCl buffer, pH 7.5, so that 1 ml of suspension contained the microsomes from 1 gram of wet liver. The incubation systems had the following compositions (unless stated otherwise): Control: 0.1 M tris-HCl buffer, pH 7.5, 4 mM ADP, 0.012 mM $FeCl_3$ (or 1.2×10^{-5} M Fe^{3+}), and 0.1 ml microsomal suspension/ml of system (containing approximately 1.2-1.5 mg microsomal protein). Experimental: same as control plus 0.3 mM NADPH. The systems were incubated in a water bath at 37°C in air (unless stated otherwise). If the buffer was previously oxygenated by bubbling O_2 through the solution, 1 minute for every 5 minutes of incubation, the incubation system is designated throughout the investigation as "oxygenated." Otherwise, it is called non-oxygenated. Incubation volumes varied from 1 to 20 ml depending on the nature of the experiment. The reaction was terminated by adding 0.2 ml of 35% aqueous TCA per ml of reaction system. The peroxide content was then determined essentially according to the method of Kokatnur, Bergan and Draper (44) by adding 0.5 ml of a 1% starch solution and 1 ml of absolute methanol (saturated with KI) for each ml of incubation system. After 5 minutes, the sample was titrated with a standardized $Na_2S_2O_3$ solution (5×10^{-3} N). Draper's method is a modification of the methods of both Wheeler (45) and Lundberg (46).

Chemical equation for iodometric titration of peroxide. The chemical reaction between phospholipid peroxide and iodide as well as the iodometric titrations between sodium thiosulfate and iodine can be sum-

marized as follows (47):

$$ROOR + 2I^- + 2H^+ \longrightarrow I_2 + ROR + H_2O$$

$$2S_2O_3^= + I_2 \longrightarrow S_4O_6^= + 2I^-$$

$$ROOR + 2S_2O_3^= + 2H^+ \longrightarrow S_4O_6^= + ROR + H_2O$$

The last equation indicates clearly that one mole of peroxide is equivalent to two moles of thiosulfate to react.

Mathematical equation for peroxide determination from titration. The mathematical equation which was used to determine the amount of peroxide formed in the enzymic oxidative cleavage of microsomal phospholipids can be written as:

$$\text{(milliliters) (Normality)} = \text{milliequivalents}$$

$$= \frac{\text{milligrams of } Na_2S_2O_3}{\text{equivalent weight of } Na_2S_2O_3}$$

$$(ml)(N) = \frac{mg}{248.18}$$

$$(248.18)(ml)(N) = \text{mg of } Na_2S_2O_3$$

$$\frac{(248.18)(ml)(N)}{248180} = \text{moles of } Na_2S_2O_3$$

Since it requires two moles of $Na_2S_2O_3$ to react with one mole of peroxide, the following equation can then be derived:

$$\text{moles of peroxide} = 10^{-3}(ml)(N) \times \tfrac{1}{2}$$

$$= 5 \times 10^{-4}(ml)(N)$$

$$\mu \text{ moles of peroxide} = 5 \times 10^{-4}(ml)(N) \times 10^6$$

$$= 5 \times 10^2(ml)(N)$$

Assay of formation of malondialdehyde in the reaction mixture.

Malondialdehyde was determined by the procedure of Ottolenghi (48). The reaction system and conditions of incubation were as described above for peroxide determination. The control system contained: 0.1 M tris-HCl buffer, pH 7.5, 4 mM ADP, 0.012 mM $FeCl_3$, and 0.1 ml microsomal suspension/ml of system (containing approximately 1.2 to 1.5 mg microsomal protein). The experimental contained the same components as the control system plus 0.3 mM NADPH. The systems were incubated in a Dubnoff incubator at 37°C in air (unless stated otherwise). If the buffer was previously oxygenated by bubbling O_2 through the solution, 1 minute for every 5 minutes of incubation, the system was designated as "oxygenated." Oxygenation was applied only because the author found it was necessary to create identical incubation conditions to show that both peroxide and malondialdehyde are oxygen-dependent. The reaction system was terminated by the addition of 0.5 ml of 35% (weight/volume; w/v) trichloroacetic acid (TCA) per ml of incubation mixture. One ml of 0.75% TBA (w/v) was then added per ml of incubation system and the mixtures were heated in a boiling water bath for 15 min. After cooling, 1 ml of 70% TCA (w/v) per ml of incubation system was added to each tube and swirled gently. The samples were then centrifuged and the optical density of the clear pink supernatant fraction was determined at a wavelength of 532 mμ. If the sample color was too intense to be read directly, suitable dilutions were prepared by adding a mixture with the same composition as that of the sample. Samples that were turbid were extracted with chloroform (to remove dispersed lipid) and the optical density of the clear aqueous layer was then determined.

Determination of protein. Protein was determined by the method

of Lowry et al., (49). A sample containing 20 to 100 μg of protein was diluted to 1.0 ml with distilled water. To the diluted sample 1 ml of an alkaline copper sulfate solution was added and the mixture allowed to stand at room temperature for 10 minutes. One-tenth ml of 1.0 N phenol reagent was then added and the tubes were mixed with a vortex mixer. After 30 minutes at room temperature, the sample was read at a wavelength of 500 mμ. Bovine serum albumin was used as a standard.

Lipid Analyses

Total lipids extraction. In some of the experiments the total lipids were extracted from the incubation systems with 20 volumes of chloroform-methanol (2:1, v/v). To this one-phase system was added 0.2 volume of (0.5%, w/v) NaCl solution, and the mixture was shaken. After separation of the phases, the chloroform layer, which contained the lipids, was recovered and evaporated to dryness in a rotary evaporator. The lipid residue was redissolved in a small but known volume of chloroform and stored in the refrigerator under nitrogen until analysis. This procedure of extraction of the lipids is essentially that of Folch et al., (50).

Rubber membrane dialysis. The solvent (chloroform) was evaporated from the total lipid and the latter was redissolved in n-hexane. This lipid solution was transferred to a latex rubber membrane (Perry Rubber Company, Massillon, Ohio) which was placed in 200 ml of the same solvent in a stoppered flask. The gas phase was flushed with nitrogen. The hexane dialysate was changed 3 times. Dialysis was completed after 24 hours at room temperature in a ventilated hood (51). The dialysate was analyzed for phosphorus to be certain that no leakage had occurred.

The phospholipid fraction was pipetted from the rubber membrane, dried in vacuo and stored in chloroform under nitrogen. The dialysate was evaporated to dryness and the neutral lipids thus recovered were stored in chloroform under nitrogen. After determination of lipid phosphorus, the purity of the isolated phospholipids and neutral lipids was checked by thin-layer chromatography with lipid standards for positive identification.

Determination of lipid phosphorus. After mineralization, the method of Fiske and SubbaRow (52) was used to determine inorganic phosphorus derived from the lipid extracts. An aliquot from the total lipid extract was transferred to a pyrex tube and the chloroform was evaporated. Then, 1.2 ml of 5 N sulfuric acid was added; the mixture was heated and, when the contents became charred, 4 drops of concentrated nitric acid were added and the heating was continued until the solution was clear and colorless. After some cooling, water was added and the solution heated again to boiling in order to hydrolyze any pyrophosphate formed during the charring procedure. The sample solutions were then washed into a 10 ml volumetric flask. One ml of 2.5% ammonium molybdate reagent and 0.4 ml of the reducing solution were added. The solutions were made up to 10 ml with distilled water, mixed well and allowed to stand about 20 minutes. The optical density was measured at 660 mμ. A standard phosphate sample containing one μmole of inorganic phosphate was carried through the entire procedure in each assay.

The reducing reagent was prepared as a powder and dissolved before use. It consisted of 0.1 gram of 1-amino-2-naphthol-4-sulfonic acid, 6 grams of sodium bisulfite and 0.2 gram anhydrous sodium sulfate,

all finely triturated together. For use, about 0.25 gram was dissolved in 10 ml of distilled water.

The standard phosphate solution was prepared by dissolving KH_2PO_4 (reagent grade, thoroughly dried) in distilled water (136.1 mg/100 ml distilled water). One μmole of inorganic phosphate should give an optical density of 0.354 at wavelength 660 mμ.

Thin-Layer Chromatography (TLC)

Thin-layer chromatography on silica gel G as adsorbent was used to compare the characteristics of lipids from control and experimental incubation systems. Silica gel G was slurried in water (30 g/52 ml) spread over glass plates in a uniform thickness (250 microns) and air dried. Plates were activated 30 minutes at 110°C prior to use. Lipid samples containing 8 to 10 μg of lipid phosphorus were spotted in compact spots (3 mm) for phospholipid separation. Spots for separation of 2, 4-dinitropheynlhydrazine derivatives contained about 12 μg lipid phosphorus. Development of the plates was carried out in one of the following solvent systems: chloroform-methanol-ammonia (80:20:2) for both phospholipids and phospholipid peroxides, and petroleum ether-diethyl ether-glacial acetic acid (90:10:1) first, and (70:30:1) later, was used for separation of 2, 4-dinitrophenylhydrazine derivatives. After the solvent front had moved 15 cm, the plate was removed, air dried and sprayed with a suitable reagent solution depending on the analysis of interest. Standards were used for positive identification on each plate.

Detection of Phospholipid Peroxide by Thin-Layer Chromatography

Thin-layer chromatography was performed with the lipid extracted

from the incubation systems on glass plates coated with silica gel G. Chloroform-methanol-ammonia (80:20:2) (v/v/v) was the solvent system. Lipid spots which contained peroxides were detected by spraying the developed plates with a solution containing 5 parts of 1% soluble starch and 10 parts of methanol saturated with KI. Subsequently, the plates were sprayed with 35% trichloroacetic acid. Spots containing lipid phosphorus were detected by spraying with Zinzadze reagent. Other lipid spots were then detected by charring. Phospholipid standards were run on each plate for positive identification of sample lipids.

Detection of Epoxide by Thin-Layer Chromatography

The method used is that of Fioriti et al., (53, 54). Thin-layer chromatography was carried out using silica gel G plates. The developing system was chloroform-methanol-ammonia (80:20:2, v/v/v). After development, the plate was sprayed thoroughly with 0.05 M picric acid in 95% ethyl alcohol and immediately placed in a tank saturated with the vapor of a diethyl ether-95% ethyl alcohol-acetic acid solution (80:20:1, v/v/v). Thirty minutes later, the plate was removed and exposed to ammonia fumes for 1-2 min. The epoxides appeared as orange spots on a yellow background. After these spots had been marked, the plate was then sprayed with Zinzadze or any other suitable reagent solution.

Spray Reagents for Thin-Layer Chromatography

Potassium iodide reagent. Lipid spots which contained peroxides were detected by spraying the developed plates with a solution containing 5 parts of 1% soluble starch and 10 parts of methanol saturated with KI. Immediately following, the plates were sprayed with 35% trichloroacetic

acid. A mixture of purple and brown spots of phospholipid peroxides appears after one to two minutes. Following this, a Zinzadze molybdenum reagent for detection of lipid phosphorus can still be applied without any adverse effect.

Rhodamine-6-G reagent. 0.005% rhodamine-6-G in 95% ethanol was sprayed, and neutral lipids were detected using ultraviolet light. Yellow fluorescent or dark purple spots corresponding to various lipids were obtained (55).

Ninhydrin reagent. 0.5% ninhydrin in n-butanol was used for free lipid amino group detection. After spraying, the plates were warmed in an oven at 110°C for 5 to 10 minutes in order to develop the pink color characteristic of amino groups. The reagent was used for detection of phosphatidyl ethanolamine and phosphatidyl serine.

Modified Zinzadze molybdenum reagent for detection of lipid phosphorus (56). This reagent was used to detect lipid phosphorus and was sensitive to 4 μg of phosphorus per spot on the developed plates. Solution I: 1 liter of 25 N sulfuric acid and 40.1 gm of molybdenum oxide were boiled gently. Solution II: 500 ml of solution I and 1.78 gm of powdered molybdenum were boiled for 15 minutes. Equal volumes of solutions I and II were mixed and two volumes of water were added. The bright blue spots of phosphorus-containing lipid appear immediately after spraying. The background color is pale blue.

Preparation of Methyl Esters

Methyl esters were prepared by use of a boron-trifluoride-methanol reagent (57). To a dry lipid sample (300 μg of lipid P), 1 ml of boron-trifluoride-methanol reagent was added. The mixture, sealed in a

screw cap culture tube, was heated for 10 minutes in a boiling water bath. The tubes were cooled and one ml of water and 2 ml of n-hexane were added. Fatty acid methyl esters were extracted in the n-hexane portion.

Gas-Liquid Chromatography of Fatty Acid Methyl Esters

Fatty acid methyl esters derived from the total lipid were separated and analyzed qualitatively and quantitatively by gas-liquid chromatography (GLC). Separations were effected on packed columns of diethylene glycol succinate polyester (EGSP). A known amount of internal standard (methyl arachidate or methyl ester of heneicosanoate) was added in the sample at the time of extraction of the lipid from the incubation mixture. Known standards were also run periodically and under instrument conditions identical to those used for the samples. Methyl esters were identified by comparison of their relative retention times (relative to palmitate) with those of the standard ester mixtures. Detector response was always linear with weight in the range of sample sizes used.

Perkin Elmer Model 881

Column	Aluminum length 6 ft. inside diameter 4.0 millimeters
Packing	15 percent w/w EGSP on gas chrom P 60/80 mesh
Temperatures	Column 180°C Injector 230°C Detector 230°C
Carrier gas	Nitrogen 60 ml/min

Sample size 0.35 µl dry lipid

Preparation of 2, 4-Dinitrophenylhydrazone of Phospholipids

The microsomal incubation systems had a final volume of 10 ml each. They had been incubated for one hour before extraction was performed with 20 volumes of chloroform-methanol (2:1, v/v) containing 15 mg% dinitrophenylhydrazine. The control and experimental systems were treated alike. Then 0.2 volume of 0.5% NaCl was added and the mixtures were allowed to stand overnight at 5°C. The chloroform layer was removed and evaporated to dryness. A small volume of chloroform (10 ml) was used to dissolve the lipid residue which was then subjected to column chromatography on 2 gm of silicic acid to remove neutral lipids and unreacted 2, 4-dinitrophenylhydrazine. The 2, 4-dinitrophenylhydrazones of the phospholipids were then eluted from the column with an excess of methanol and were treated later on with snake venom.

Treatment of the 2, 4-Dinitrophenylhydrazone of Phospholipids with Snake Venom

Rattlesnake venom was used as a source of phospholipase A which hydrolyzes the β-fatty acid of phosphatidylethanolamine and phosphatidylcholine (58) resulting in the formation of lysophosphatides and free fatty acids. Dry lipid sample, which had been treated with 2, 4-dinitrophenylhydrazine, containing 300 µg of phosphorus, was dissolved in 3 ml of peroxide free ether, and 0.5 ml of the venom solution containing 0.5 mg of venom in 0.005 M calcium chloride was added. The ether itself was treated with ferrous sulfate and later with anhydrous sodium sulfate before it was used for extraction. The lysophosphatides precipitated at 5°C in

2 to 24 hours depending on the lipid source. The ether-soluble free fatty acids were separated from the lysophosphatides by means of a Pasteur pipette after centrifugation. More ether was added for thorough extraction and separation. The lysophosphatides could be further purified by Folch extraction. Completeness of the venom action was monitored by thin-layer chromatography.

Determinations of Peroxides

In incubation system. To 1 ml of the incubation system was added 1 ml of saturated potassium iodide solution in methanol (11.1 gm KI in 40 ml MeOH) and 0.5 ml of 1% starch solution. The potassium iodide solution should be made fresh each day and stored in the dark at all times. Then 0.2 ml of 35% trichloroacetic acid was added, and the mixture was allowed to stand for 5 minutes. The mixture was then shaken thoroughly. The sample was then titrated with a standardized sodium thiosulfate solution (10^{-2} N).

In total lipid extract. Ten ml of microsomal incubation system were incubated for 15 minutes and then the total lipids were extracted by the Folch procedure. After separation, the sample was evaporated to dryness and dissolved in a known volume of methanol. Determination of lipid phosphorus was carried out and a known quantity of total lipid (approximately 30 μg lipid phosphorus) was pipetted into a test tube. Evaporation of the solvent was done by flushing the sample with pure nitrogen gas. One ml of 0.1 M tris-HCl buffer solution was then added. The sample was stirred thoroughly with a pointed glass rod and a Vortex mixer was used to make the suspension more homogeneous. One ml of saturated potassium iodide solution, 0.5 ml 1% starch solution and 0.2 ml 35% trichloro-

acetic acid were added. The sample was shaken, allowed to stand for 5 minutes, and then titrated with standardized sodium thiosulfate solution.

In phospholipids. The total lipid obtained from 10 ml of the incubation system was subjected to rubber membrane dialysis. The phospholipid fraction was retained inside the rubber membrane and recovered therefrom. Determination of lipid phosphorus was performed. An aliquot of a known quantity of phospholipid (approximately 30 μg lipid phosphorus) was used for the determination of peroxide content, according to the method described in the preceding paragraph.

In the β-position fatty acids of phospholipids. The phospholipids obtained from rubber membrane dialysis were subjected to phospholipase A action by adding Crotalus adamanteus venom according to the procedure of Long and Penny (58). The free fatty acids hydrolyzed from the β-position of the glycerophosphatides were recovered from the supernatant after the lysoglycerophosphatides, which precipitate during the hydrolysis procedure, were sedimented. Peroxide determination was carried out on the hydrolyzed phospholipid fractions by the same method described previously.

Turbidity Measurements

Changes in turbidity of microsomal suspension were determined as described by Robinson (59). The incubation volume was 2 ml containing 1.0 mg microsomal protein and also containing the NADPH-generating system. The measurements were made in quartz cuvettes with a 1.0 cm light path and the optical density was determined at 520 mμ. Readings were made between zero and 60 minutes of incubation.

Experiments Using Hydrogen Peroxide (H_2O_2) as a Substrate for the Enzyme Reaction Instead of NADPH

The experimental conditions were the same except oxygenation of the buffer was not applied. After experiments with various concentrations of hydrogen peroxide in the system, it was found that a minimum concentration of 90 mM of hydrogen peroxide per ml incubation was required to produce maximum activity. This was the concentration used thereafter in the other experiments. A solution of H_2O_2 was prepared each day by diluting 1 ml H_2O_2 (30%) to 10 ml with 0.1 M tris-HCl buffer, pH 7.5. One-tenth of this solution, containing 90 μmoles, was added to the experimental system instead of NADPH. The diluted stock solution was stored in the refrigerator at 5°C before use.

NADPH-Regenerating System

In some experiments, it was considered desirable to maintain a constant level of totally reduced NADP. This was achieved by including in the 1 ml incubation system 6 mM of glucose-6-phosphate and 0.5 units of purified glucose-6-phosphate dehydrogenase. NADPH was determined from the optical density at 340 mμ after deproteinization with ethyl alcohol (3 ml per milliliter of incubation system).

CHAPTER III

RESULTS

Detection of Peroxide in the Microsomal Incubation System

Earlier studies had indicated that little, if any, peroxide could be detected in the altered lipids extracted from the microsomal enzyme system at the end of a one-hour incubation period. In the present study, a sequential analysis of the total reaction system disclosed that significant amounts of peroxide were formed only transiently (Fig. 1). In this experiment, microsomes (containing 1.4 mg protein) were incubated in a final volume of 1 ml of oxygenated tris-HCl buffer. The reaction was carried out in a Dubnoff apparatus. The other incubation systems and conditions were as described under "Methods." On starting the reaction, peroxide formation reached a peak level within 10 to 15 minutes after which time the level rapidly fell to a low value. The process is heat labile. The course of malondialdehyde formation during this same reaction is also shown in Fig. 1. The total amount formed is small compared to the peak level of phospholipid peroxide formation. Since the course of NADPH-dependent peroxide formation indicated that this product was transitory in nature, it appeared that the peroxides detected might be intermediates in the NADPH oxidase system which catalyzes the cleavage of polyunsaturated-fatty acids in membrane-bound phospholipids. Although

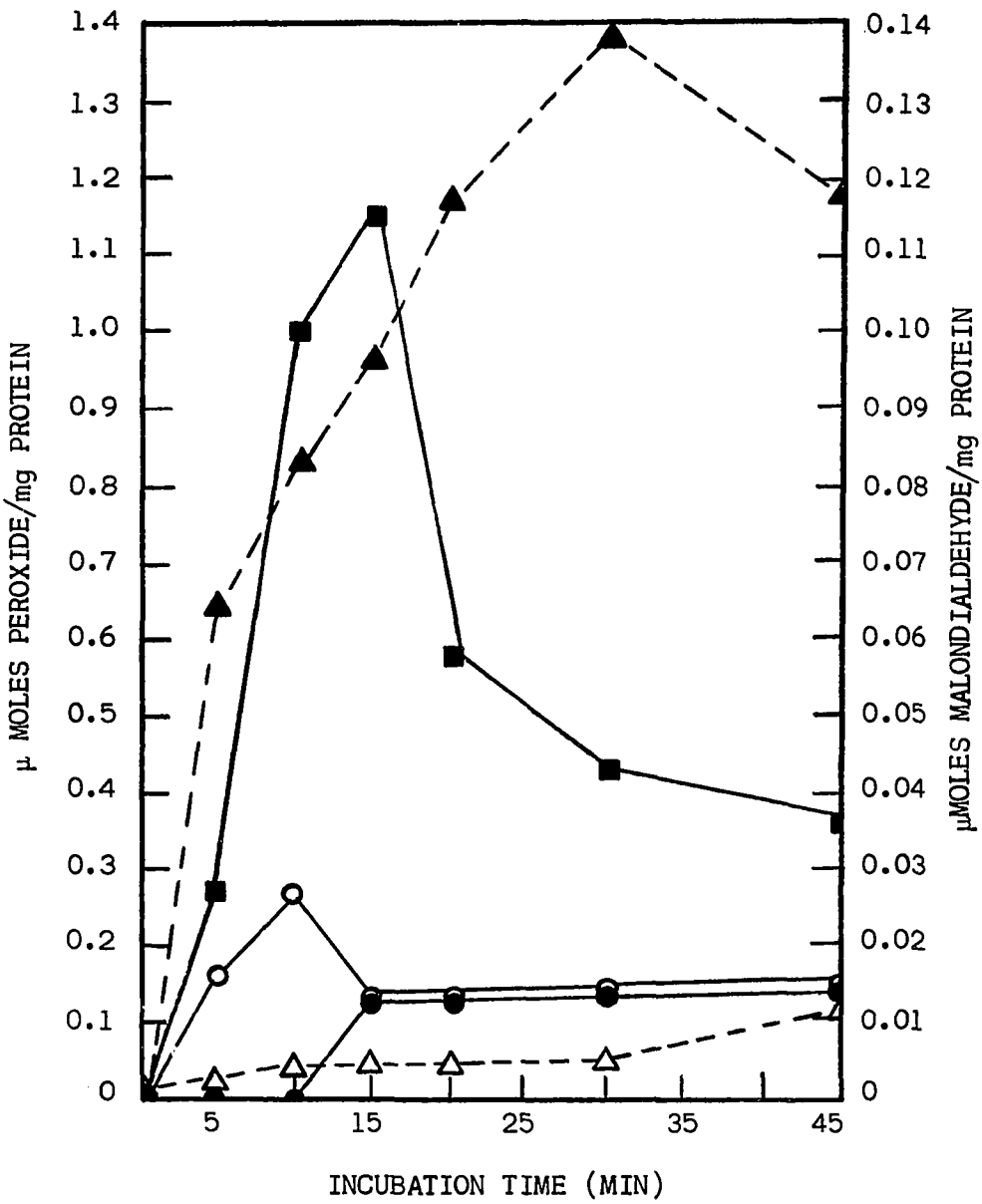


Figure 1. Progress curve of peroxide formation and of malondialdehyde formation during enzymic oxidation of NADPH by liver microsomes.

Incubation systems and conditions were as described under "Methods." ■, peroxide formation in experimental system; O, peroxide formation in control system; ●, peroxide formation in an experimental system in which the microsomes had been heated at 65°C for one minute before addition to the system; ▲, malondialdehyde formation in experimental system; △, malondialdehyde formation in control system.

the total amount of peroxide formed in the system could not be determined because of the degradation reaction, the amounts present at the peak of the formation suggested that sufficient peroxide had formed to account for the disappearance of phospholipid-bound polyunsaturated-fatty acids during the reaction. For example, in a typical experiment (Table 1), both control and experimental systems contained microsomes equivalent to 15 mg of protein. Oxygenation of the buffer was carried out because the rate of the reaction is such that oxygen in solution is otherwise rapidly depleted, and the rate of the reaction then becomes dependent on the rate of diffusion of oxygen into the medium. Incubations were performed at 37°C in a Dubnoff shaking water bath. At the end of 15 minutes, both systems were subjected to the Folch extraction procedure and subsequently the fatty acids in the lipid extract were converted to methyl esters. The fatty acid methyl esters from both control and experimental system were then analyzed by gas-liquid chromatography. Table 1 shows that the total losses of highly unsaturated fatty acids (arachidonic, docosahexenoic and linoleic acids) amounted to a total of 5.64 μmoles. The total amount of peroxide present in a duplicate 10 ml system at 15 minutes was 11.2 μmoles. Presumably, the total amount formed would be significantly more if all the peroxide had accumulated in the system instead of undergoing degradation. It is clear, however, that more peroxide is formed than there are polyunsaturated fatty acids utilized; thus, many of these fatty acids must undergo multiple attacks by O_2 if the peroxide formed is associated with the fatty acid fraction. This is in harmony with the previously established stoichiometry of 4 moles of O_2 taken up per mole of polyunsaturated fatty acids consumed (40).

TABLE 1

COMPARISON OF POLYUNSATURATED-FATTY ACID LOSS IN MICROSOMAL PHOSPHOLIPIDS TO PEAK LEVEL OF NADPH-DEPENDENT PHOSPHOLIPID PEROXIDE FORMATION

System	Microsomal fatty acid content					
	18:0	18:1	18:2	20:4	22:6	Total
	μ moles					
Control	6.67	3.21	4.51	5.49	2.05	21.93
Experimental	6.40	2.96	3.36	2.75	0.82	16.29
Difference	0.27	0.25	1.15	2.74	1.23	5.64

Total loss of fatty acid in experimental system	Maximum peroxide during reaction
μ moles	μ moles
5.64	11.2

Incubation systems, lipid extractions and analytical procedures are described under "Methods." The incubation was performed at 37°C for 15 minutes. Incubation volumes were 10 ml.

Identification of the Peroxide as a Phospholipid

Most, if not all, the peroxide formed in the system is associated with the β-position fatty acids of the phospholipids. This was established first by demonstrating that the peroxide was extracted along with the lipid fraction of the experimental system. (In well-washed extracts the peroxide moiety is stable for days if stored in the dark at 0°.) Table 2 shows that of the total amount of peroxide present in the system after 15 minutes of incubation, over 80% could be accounted for in the extracted lipid fraction (Expt. 1). Fractionation of the total lipid into neutral lipid and phospholipid fractions by rubber membrane dialysis resulted in the recovery of nearly all of the peroxide in the phospholipid fraction (Expt. 2). Thin-layer chromatography of the lipid extracts followed by processing the plates with reagents for detecting peroxides demonstrated that only phosphatidylcholine and phosphatidylethanolamine in the experimental system contained detectable peroxide (Fig. 2). If the lipid extract from the experimental system is treated with snake venom phospholipase A, the peroxide moiety is then recovered in the free fatty acid fraction (Table 2, Expt. 3 and 4). Since phospholipase A selectively hydrolyzes the β-position fatty acyl groups of phospholipids, these experiments demonstrate that the β acyl groups are the substrate for the peroxide-forming reaction. These results make it reasonable to assume that peroxide formation in the incubation system is part of the mechanism that accomplishes the NADPH-dependent cleavage of the membrane polyunsaturated-fatty acids since most of these are situated at the β-position of the phospholipids. This assumption derived additional support from the subsequent studies with inhibitors.

TABLE 2

NADPH-DEPENDENT PEROXIDE FORMATION BY LIVER MICROSOMES: LOCALIZATION OF THE PEROXIDE MOIETY IN THE β-POSITION FATTY ACIDS OF THE PHOSPHOLIPID FRACTION

Expt. no.	System	Total Peroxide Content				
		Incubation system	Lipid extracted from incubation system	Neutral lipid fraction	Phospholipid fraction	β-Position fatty acids of phospholipid
		μmoles/ml of incubation system				
1	Control	0.38	0.25	----	----	----
	Experimental	1.50	1.25	----	----	----
2	Control	0.38	----	0.12	0.25	----
	Experimental	2.18	----	0.13	1.63	----
3	Control	0.38	0.25	----	----	0.30
	Experimental	1.50	1.25	----	----	1.00
4	Control	----	0.38	----	----	0.25
	Experimental	----	1.88	----	----	1.25

Experimental system contained NADPH. Experimental conditions are as described under "Methods." In each experiment the incubations were performed with 10 ml systems from which aliquots were taken for peroxide determination and for extraction of microsomal lipids on which peroxide determinations were subsequently done. Values obtained for the various components are expressed as total per ml of original incubation system. Separation of neutral lipids from phospholipid was accomplished by rubber membrane dialysis. Phospholipid β-position fatty acids were obtained by treating known quantities of the phospholipid fraction with snake venom phospholipase A (see "Methods"). Incubation period, 15 minutes.

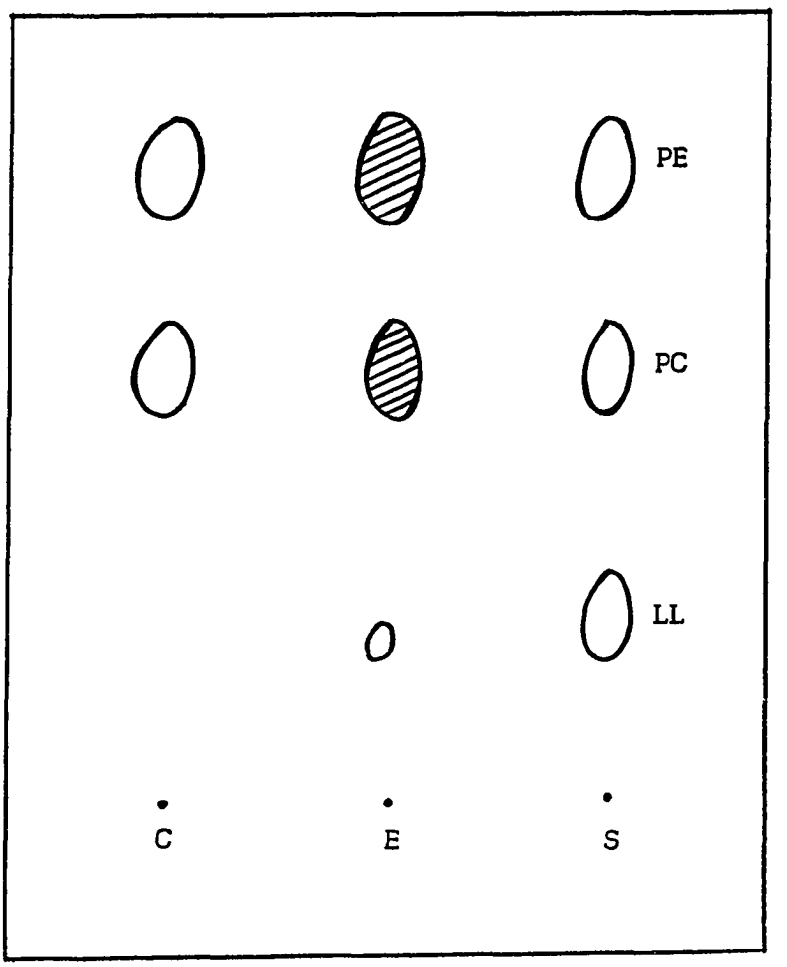


Figure 2. Thin-layer chromatography of total lipids extracted from liver microsomes after incubation for 15 min. in an experimental system (E) and in a control system (C).

Only phosphorus positive spots are shown. Cross-hatching indicates detection of peroxides. Standard markers (S) are phosphatidylethanolamine (PE), phosphatidylcholine (PC) and lysolecithin (LL). Solvent system: chloroform-methanol-ammonia (80:20:2). Support: silica gel G. Experimental conditions and detection of lipid phosphorus and peroxides are described under "Methods."

Inhibitors of Phospholipid Peroxide Formation

The utilization of membrane polyunsaturated-fatty acids by the NADPH oxidase system has been shown to be inhibited by each of the following substances: 10^{-3} M Mn^{2+}, 10^{-3} M Co^{2+}, 10^{-3} M EDTA and 1.6×10^{-4} M PHMB (40). In addition, prior heating of microsomes at 65° for one minute before initiating the reaction abolishes the oxidation of membrane polyunsaturated-fatty acids completely. If phospholipid peroxides are intermediates in this reaction, their formation should also be inhibited by these factors. Figure 3 shows that phospholipid peroxide formation in the presence of either Mn^{2+} or Co^{2+} is totally inhibited. The same results were obtained when EDTA was added to the system, and substantial reduction of activity occurred on adding PHMB (Fig. 4).

Inhibitors of Enzymic Malondialdehyde Formation

Figure 5 shows that the enzymic malondialdehyde formation in the presence of either Mn^{2+} or Co^{2+} is entirely inhibited. Similar results were obtained when EDTA was added to the system and about 60% of reduction of activity occurred on adding PHMB (Fig. 6).

Isolation of 2, 4-Dinitrophenylhydrazine Derivatives of Phospholipids

The reaction systems were incubated for one hour to permit maximum polyunsaturated-fatty acid utilization to occur. An NADPH-regenerating system was included in the incubation system as described under "Methods." In addition, 0.5 M nicotinamide was added. The final incubation volume was 10 ml and contained 14 mg microsomal protein. The systems were incubated at 37°C and without oxygenation. Twenty volumes of

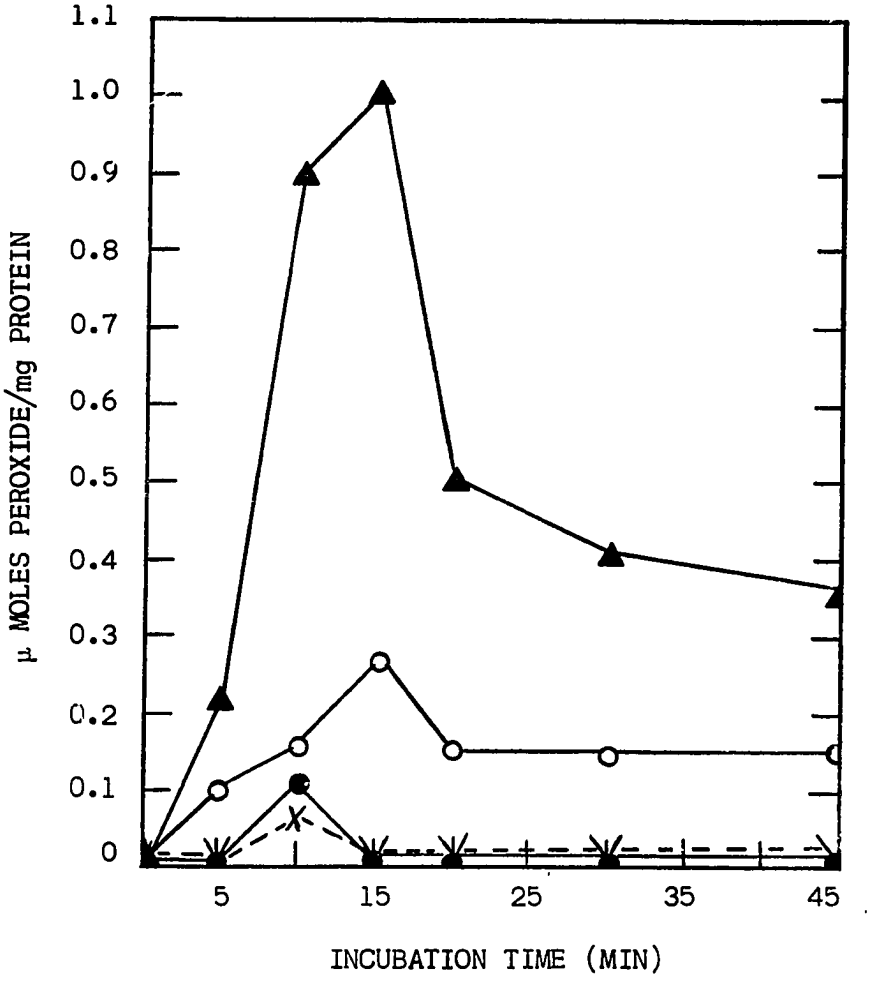


Figure 3. Inhibition of enzymic phospholipid peroxide formation in liver microsomes by Mn^{2+} and Co^{2+}.

Incubation systems and conditions are described under "Methods." Incubation volume was 1 ml containing 1.4 mg microsomal protein. Oxygenation was applied at 37°C. ▲, experimental system; O, control system; X, experimental system containing 1.0 mM $MnCl_2$; ●, experimental system containing 1.0 mM $CoCl_2$.

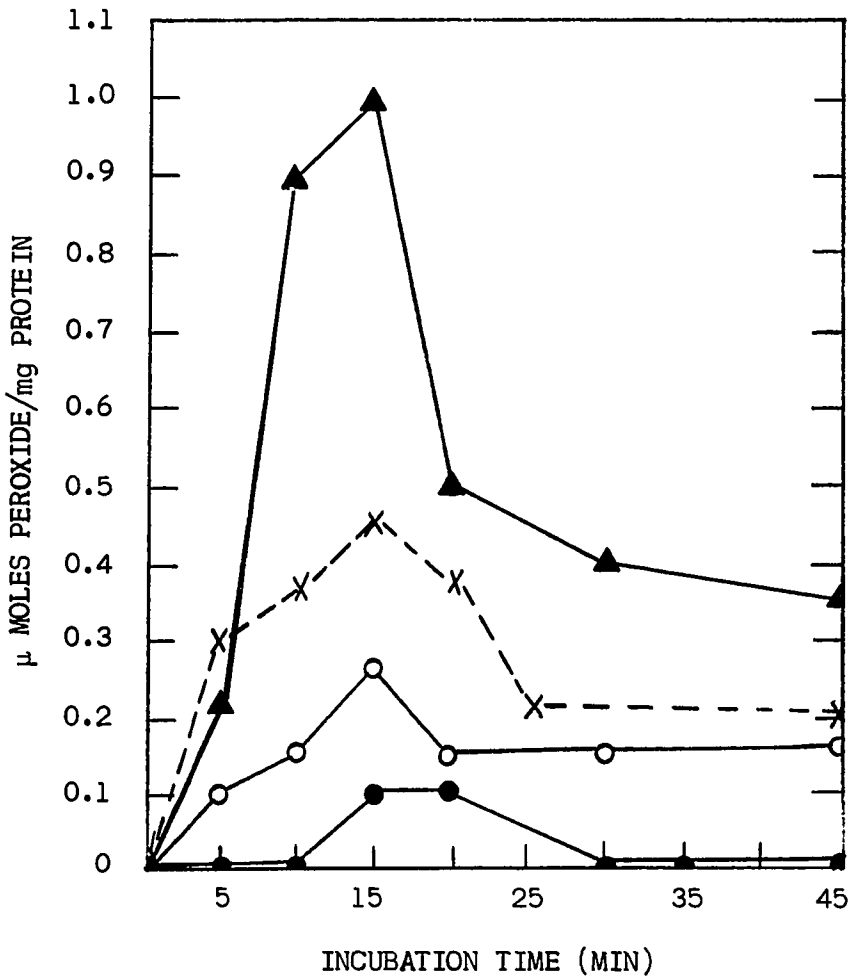


Figure 4. Inhibition of enzymic phospholipid peroxide formation in liver microsomes by PHMB and EDTA.

Incubation systems and conditions are described under "Methods." Incubation volume was 1 ml containing 1.4 mg microsomal protein. Incubation was at 37° in oxygenated buffer. ▲, experimental system; O, control system; X, experimental system containing 0.16 mM PHMB; ●, experimental system containing 1.0 mM EDTA.

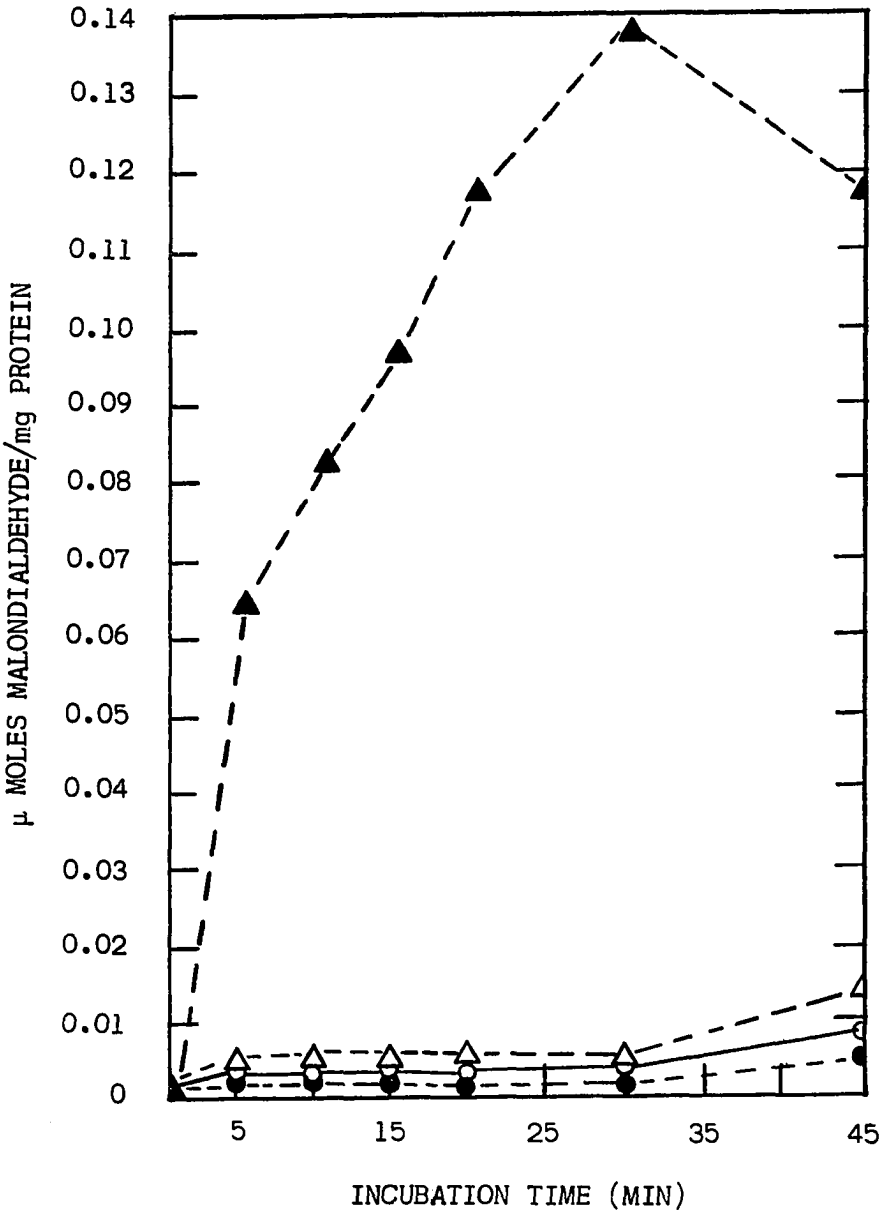


Figure 5. Inhibition of enzymic phospholipid PUFA cleavage in liver microsomes by Mn^{2+} and Co^{2+}.

Incubation systems and conditions are described under "Methods." Incubation volume was 1 ml containing 1.4 mg microsomal protein. Incubation was at 37° in oxygenated buffer. ▲, experimental system; △, control system; O, experimental system containing 1.0 mM $CoCl_2$; ●, experimental system containing 1.0 mM $MnCl_2$.

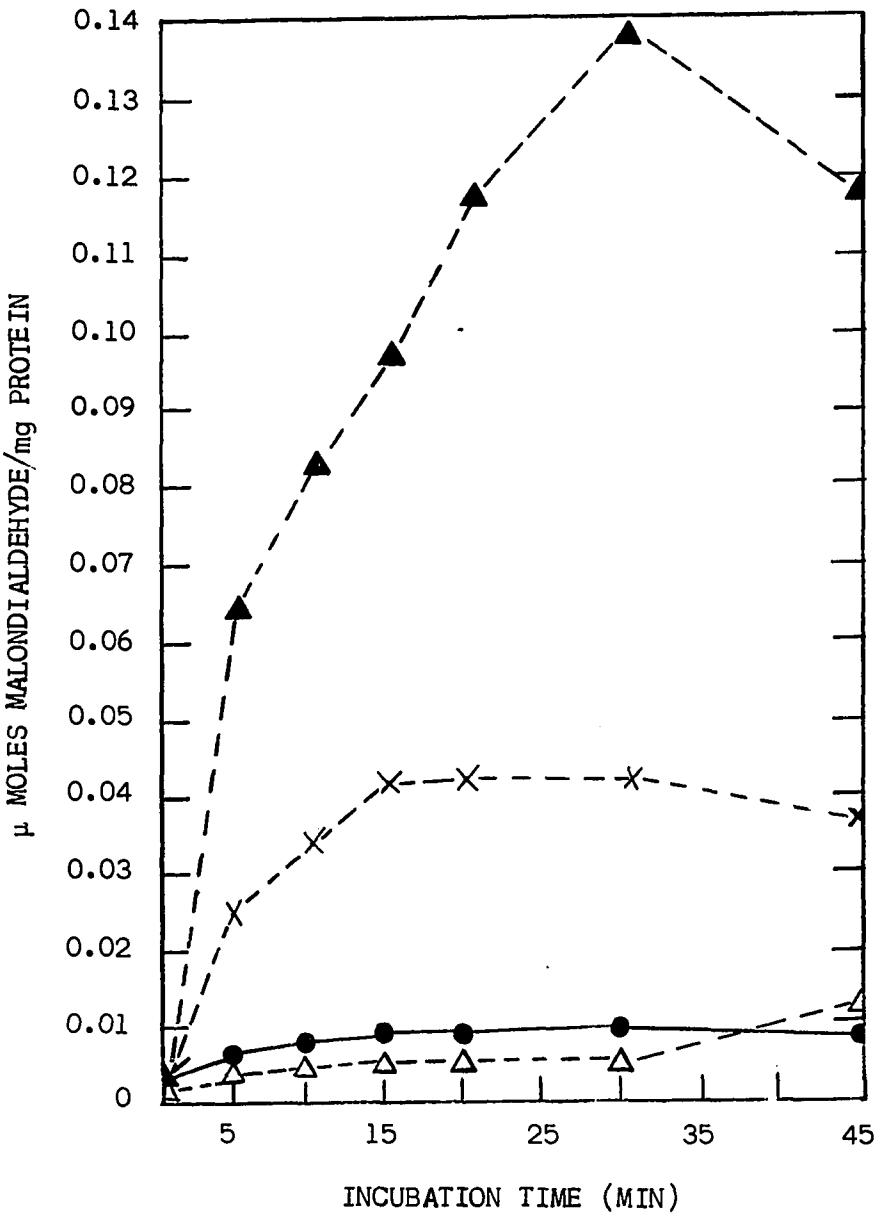


Figure 6. Inhibition of enzymic phospholipid PUFA cleavage in liver microsomes by PHMB and EDTA.

Incubation systems and conditions are described under "Methods." Incubation volume was 1 ml containing 1.4 mg microsomal protein. Incubation was at 37° in oxygenated buffer. ▲, experimental system; △, control system; X, experimental systems containing 0.16 mM PHMB; ●, experimental systems containing 1.0 mM EDTA.

chloroform-methanol (2:1) containing 15 mg% dinitrophenylhydrazine were added. The mixtures were then washed with 0.2 volume of 0.5% NaCl and subjected to column chromatography on silicic acid to remove neutral lipids and unreacted 2, 4-dinitrophenylhydrazine. The purified phospholipids from the experimental system exhibited a deep orange color, whereas the phospholipids from the control system appeared pale yellow in color. When these altered lipids were treated with phospholipase A (snake venom), the orange material was recovered in the free fatty acid fraction. Unaltered free fatty acids could be separated from the derivatives by thin-layer chromotagraphy (Fig. 7). The derivatives could also be separated into at least 13 substances with differing polarities by rechromatographing components obtained from initial thin-layer chromatographic separation (System 1) in several solvent systems. Sufficient amounts of these components have been recovered to determine that some of them occur in much greater quantities by weight than others. Identification of these many components has not been accomplished. Control system phospholipid contained very small quantities of phenylhydrazone compounds, but were not obtained in sufficient amounts to be fractioned in this manner.

Effects of Various Factors on Reaction Rate

Effect of Temperature

The rate of most chemical reactions, catalyzed or not, increases as the temperature is raised. Enzyme-catalyzed reactions follow the general rule, but with the added complication that, as protein, the enzymes are adversely affected by elevated temperatures. As a result of the two competing factors, a plot of the rate of an enzymic reaction versus the

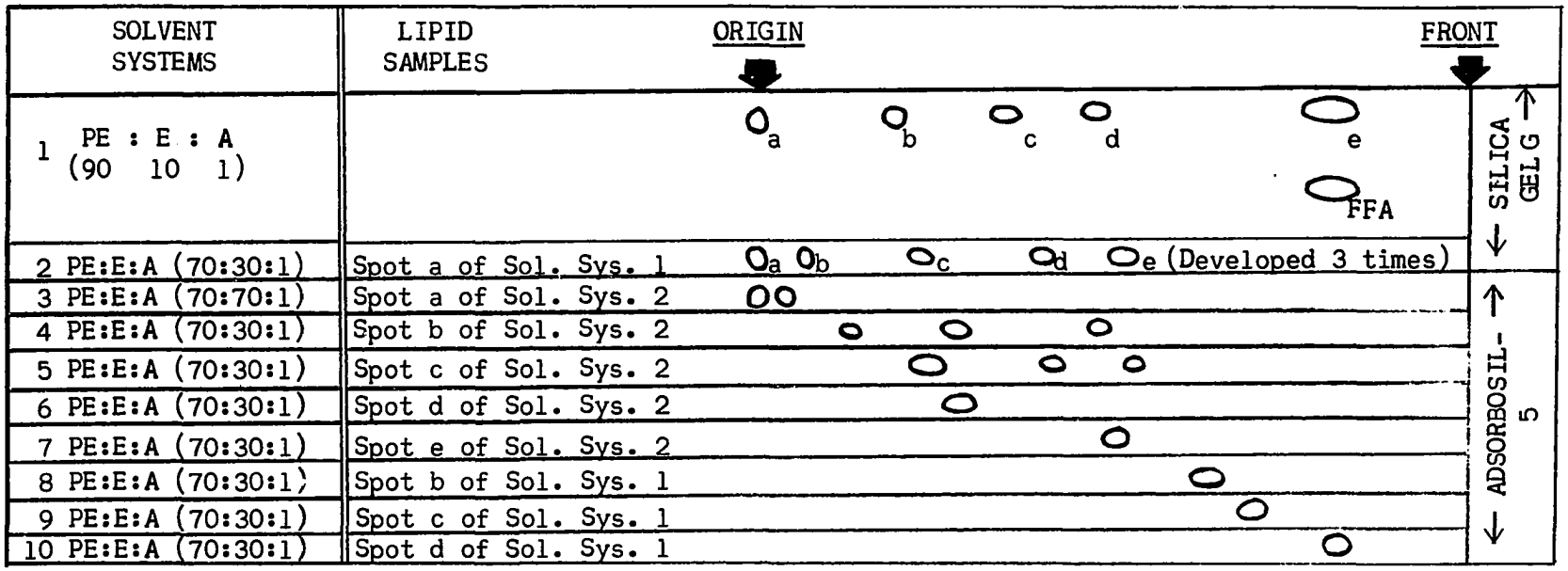


Figure 7. Diagramatic representation of thin-layer chromatographic studies: Separation of 2,4-dinitrophenylhydrazine derivatives of β-position fatty acids of microsomal phospholipids which have undergone NADPH-dependent peroxidation.

Total lipids were extracted from the experimental system with chloroform-methanol (2:1) containing 2,4-dinitrophenylhydrazine and the phospholipid fraction was isolated as described in the text. The β-position fatty acids were hydrolyzed with snake venom phospholipase A and the free fatty acid (FFA) fraction recovered. Details of this procedure are described under "Methods." The unaltered free fatty acids could be separated from those which had reacted with 2,4-dinitrophenylhydrazine by thin-layer chromatography on silica gel G with solvent system 1 and were detected with Rhodamine-6-G spray. Experimental system β FFA represents the β-position fatty acid fraction from experimental system phospholipids. Standard free fatty acid marker is indicated as FFA. The fatty acid 2,4-dinitrophenylhydrazones separated into four components. Component a was further separated into five components on silica gel G in solvent system 2. All other separations of the various components were carried out on Adsorbosil-5. Thirteen separate components were detected. All components are positioned according to their R_f values in the solvent indicated.

temperature exhibits a maximum, as shown in Figure 8. Most animal enzymes begin to be inactivated at a significant rate at temperatures over 50°C. The optimal temperature for the formation of phospholipid peroxide is at 5°C. However, between 5°C and 45°C, the decrease in peroxide formation is probably due to an increase in the rate of peroxide destruction or other structural factors in microsomal lipids. As may be expected, the reaction is very labile at temperatures above 37°C.

Effect of pH

Enzymes, being proteins, cannot withstand the action of strong acid or base. However, even over the pH range in which inactivation does not occur, enzymes exhibit optima in their activity. Figure 9 shows that the pH optimum for peroxide formation is pH 8.0, although the enzyme system retains measurable activity at pH 6.0 and at pH 11.0. Since the incubation system involves most likely more than one enzyme, the optimum in the curve must be a resultant or compromise of all the pH effect on all the reactions involving peroxide formation and destruction.

Effect of Oxygen

The microsomal oxidoreductases, including enzymes of the metabolic network and mixed-function oxidases, have been reported by many investigators to be highly oxygen-dependent and also may form or require reduced coenzymes for action. Since phospholipid peroxides are intermediates in the phospholipid cleavage reaction leading to malondialdehyde formation, the effect of oxygen on both peroxide formation and malondialdehyde formation should be similar. Incubation systems which contained in 1 ml 1.35 mg of microsomal protein each were conducted as described

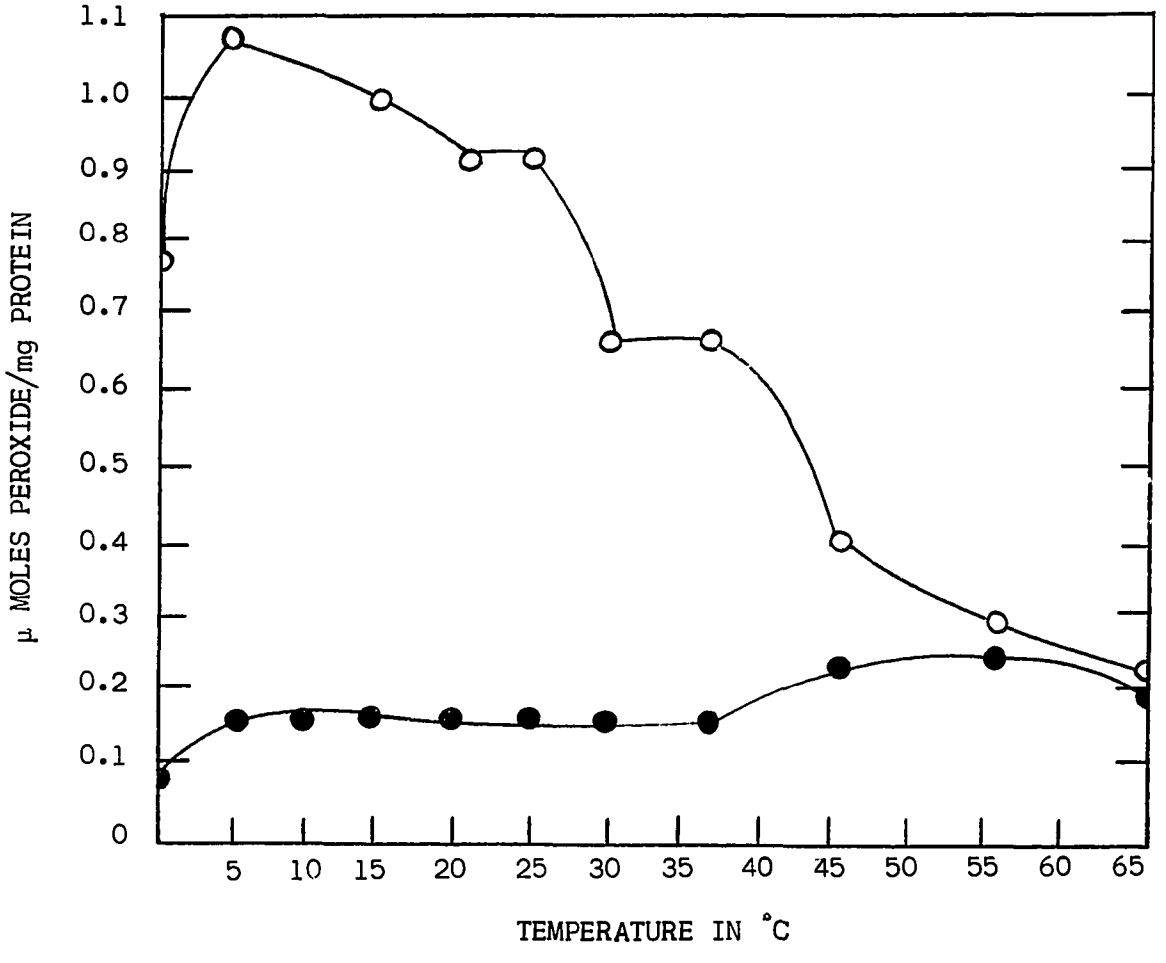


Figure 8. Effect of temperature on phospholipid peroxides formation.

Incubation systems and conditions were as described under "Methods." Microsomes containing 1.54 mg protein were incubated in a volume of 1 ml with oxygenated tris-HCl buffer solution for 15 minutes. O, peroxide formation in experimental system; ●, peroxide formation in control system.

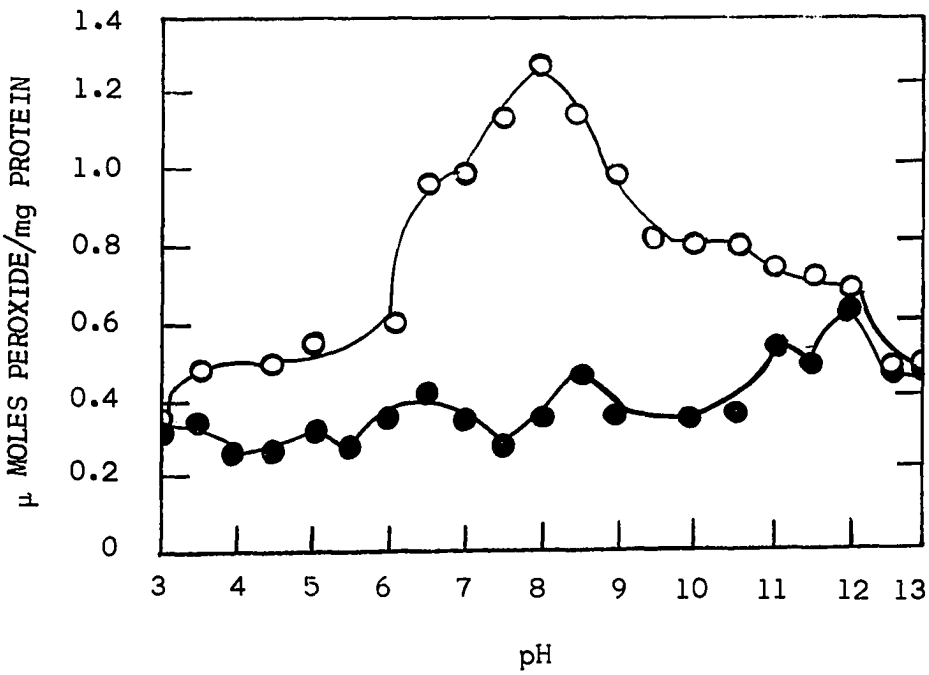


Figure 9. Effect of pH on phospholipid peroxides formation.

Incubation systems and conditions were as described under "Methods." The incubation volume was 1 ml and contained microsomes equivalent to 1.54 mg protein. The systems were incubated at 37°C for 15 minutes with oxygenated tris-HCl buffer solution. O, peroxide formation in experimental system; ●, peroxide formation in control system.

under "Methods." Figure 10 shows that peroxide formation is increased about 4-fold with oxygenation. This is in harmony with Figure 11 which shows that malondialdehyde is also correspondingly increased 4-fold.

Products of the NADPH-Dependent Phospholipid Alterations

Detection of Lipid Epoxide

Lipids extracted from incubation systems were subjected to thin-layer chromatographic analysis for the presence of epoxides. A positive test was obtained for the experimental system lipid in the form of a pinkish-orange colored spot (Fig. 12), but was not found in the lipid from the control system. Although this result may suggest the presence of lipid epoxide in the experimental system, further studies are required in order to prove its existence with certainty.

Thin-Layer Chromatographic Evidence of Phospholipid Alteration

Phospholipids extracted from liver microsomes after incubation in an experimental system and in a control system for 45 minutes at 37°C with oxygenation, were developed in a solvent system for separating phospholipids. Figure 13 shows that qualitatively the phospholipid composition of the two extracts appears to be identical and consisted primarily of phosphatidylethanolamine, phosphatidylcholine and lysolecithin. However, the relative amounts of these phospholipid components appear to be different in the two extracts. The total amount of phosphatidylethanolamine and phosphatidylcholine are decreased in the experimental system. A spot with the same R_f as lysolecithin but not identified as such is present in greater abundance in the experimental system; very little of

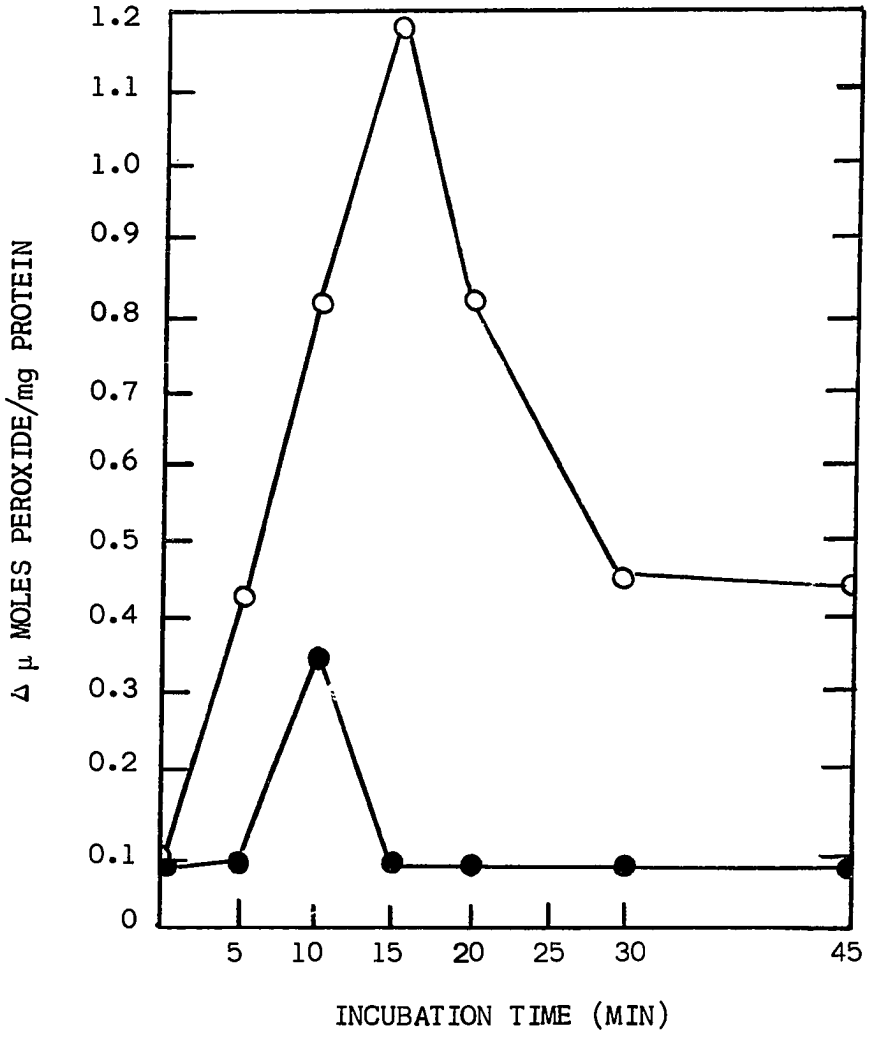


Figure 10. Progress curve of phospholipid peroxides formation at 37°C under air and O_2.

O—O, difference between experimental and control incubation systems with oxygenation. ●—●, difference between experimental and control incubation systems without oxygenation. Incubation volume was 1 ml containing microsomes equivalent to 1.35 mg protein. Incubation systems and conditions were described as under "Methods."

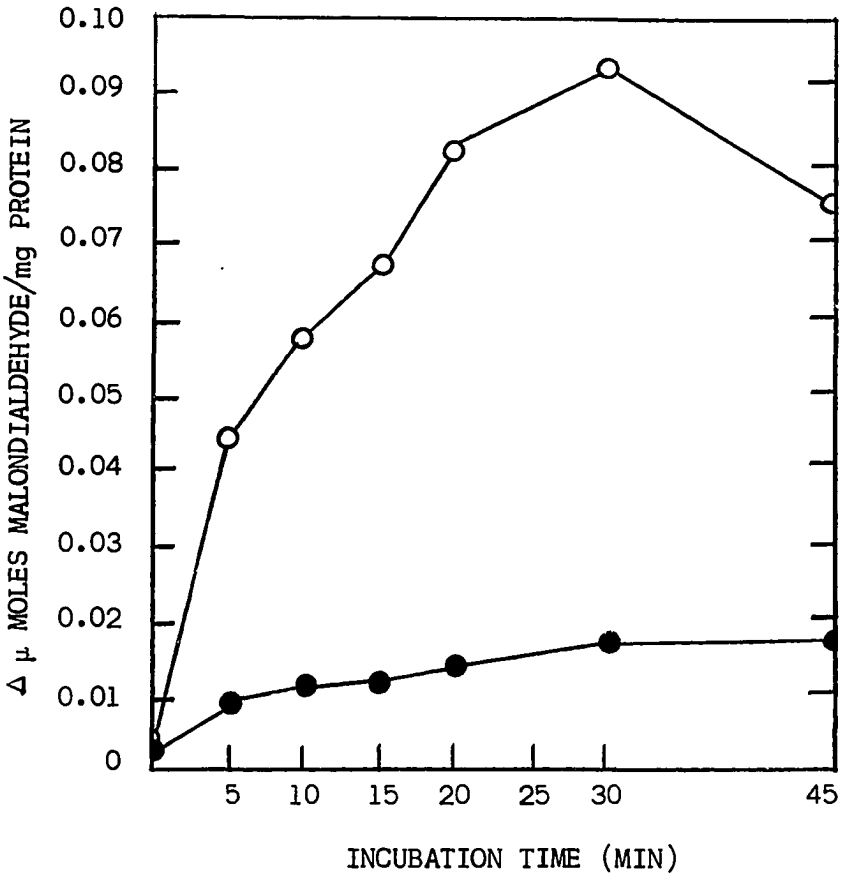


Figure 11. Progress curve of phospholipid PUFA cleavage under air and O_2 at 37°C.

Incubation volume was 1 ml containing microsomal protein 1.35 mg. Incubation systems and conditions were described as under "Methods." O—O, difference between experimental and control incubation systems with oxygenation. ●—●, difference between experimental and control systems without oxygenation.

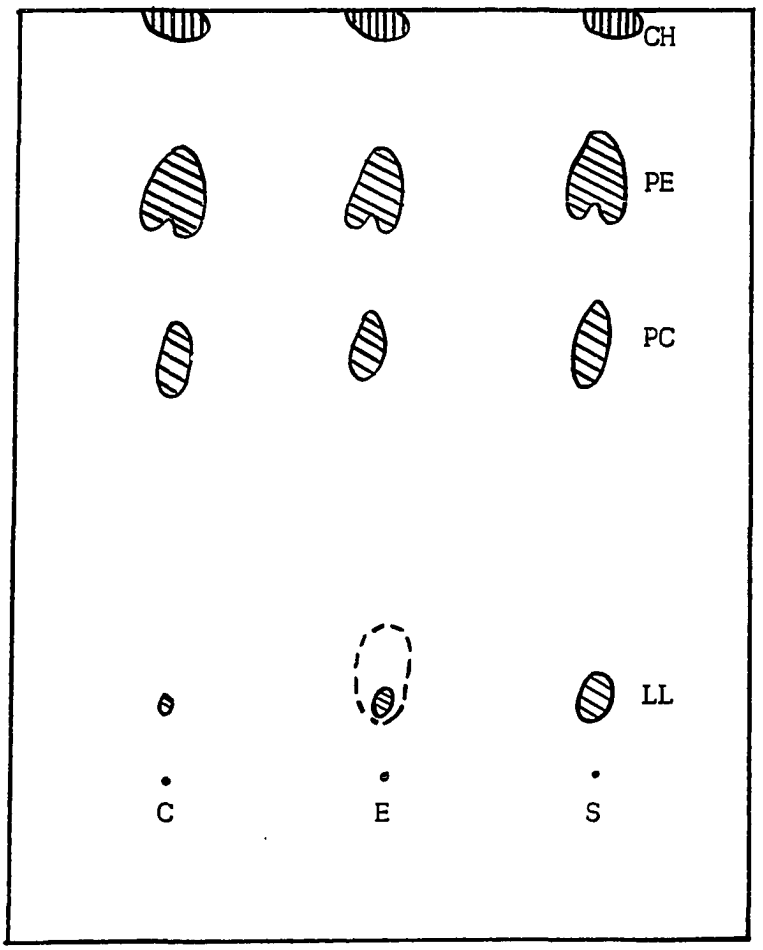


Figure 12. Detection of lipid epoxide by thin-layer chromatography.

Microsomal lipid was extracted from liver microsomes after incubation in an experimental system (E) and in a control system (C) for 15 minutes at 37°C without oxygenation. Standard markers (S) are cholesterol (CH), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and lysolecithin (LL). Solvent system: chloroform-methanol-ammonia (80:20:2). Support: silica gel G. Incubation systems and development of the plate are described under "Methods." Broken line depict epoxide positive spot. Cross-hatching indicates phosphorus positive. Vertical lines indicate other areas appearing after heavy charring.

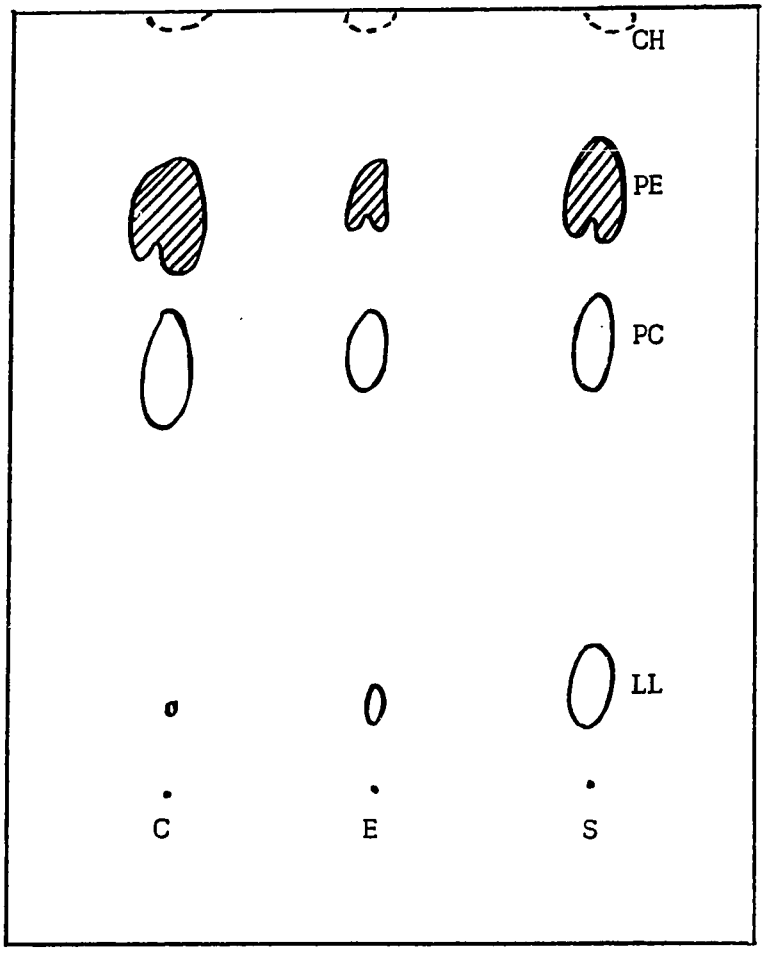


Figure 13. Thin-layer chromatography of phospholipids extracted from liver microsomes after incubation in an experimental system (E) and in a control system (C) for 45 minutes at 37°C and with oxygenation.

Standard markers (S) are cholesterol (CH), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and lysolecithin (LL). Solvent system: chloroform-methanol-ammonia (80:20:2). Support: silica gel G. Cross-hatching indicates ninhydrin positive. Circumscribed areas depict phosphorus positive. Broken lines indicate other areas appearing after charring. Experimental systems and detection of lipid spots are described under "Methods."

this material was present in the control. The results show that following the initial formation of phospholipid peroxides in the phosphatidylethanolamine and phosphatidylcholine components at 15 minutes, prolonged incubation results in a decrease of these two components in the microsomal lipid. It appears, therefore, that the peroxides formed in the phospholipids are intermediates in the enzymic oxidative cleavage of microsomal phospholipids occurring in the presence of NADPH and oxygen. The carbonyl-containing phospholipids produced by the cleavage undergo further reaction (probably polymerization) during the longer incubation periods, forming slower-running, phosphorus-positive components.

Changes in the Physical Properties of the Microsomal Membrane

Since the reaction brought about measurable changes in the chemical composition of the microsomal phospholipids, the turbidities of the suspensions were monitored during the reaction by the method of Robinson (59) in an attempt to obtain physical evidence for structural alteration in the microsomal membranes. Figure 14 shows that in the experimental system, the turbidity of the microsomal suspension decreased about 40% during the first 10 minutes of the reaction, and at 13 minutes the suspension had less than 50% of its initial turbidity. The turbidity of the control system did not change significantly. That this turbidity change was produced by the NADPH-dependent enzymic reaction, and the reaction under study is indicated by the prevention of turbidity changes in the experimental system as a result of prior heating of the microsomes at 65°C for one minute.

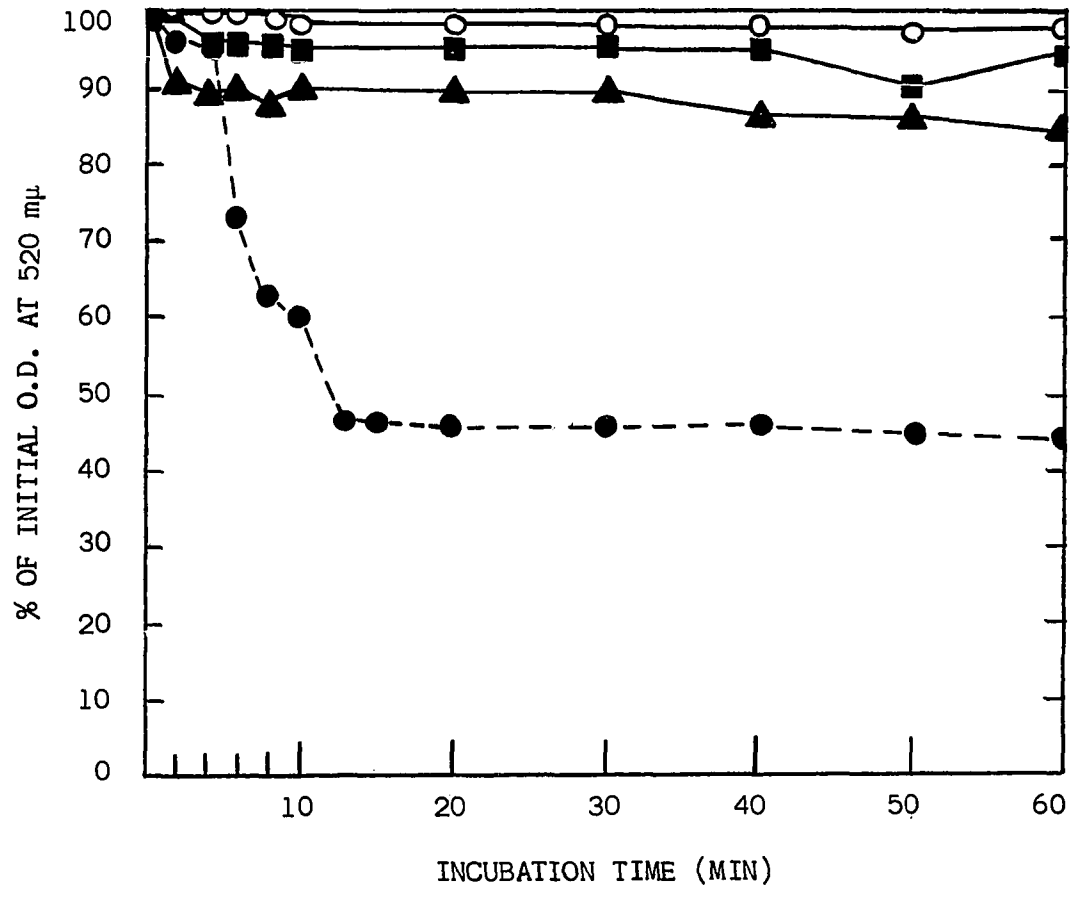


Figure 14. Turbidity change in the microsomal incubation system during enzymic phospholipid peroxide formation.

Incubation conditions and procedure were as described under "Methods." ●, experimental system; O, control system; ▲, experimental system in which the microsomes had been heated at 65°C for one minute before addition to the system; ■, control system in which the microsomes had been heated as above.

H_2O_2-Dependent Enzymic Peroxidative Cleavage of Microsomal Phospholipids

The NADPH-dependent oxidation of ethanol by the liver microsomal fraction has been studied by many investigators (36). It was concluded from their results that NADPH cannot be replaced by H_2O_2 *per se* but can be replaced by glucose plus glucose oxidase in the system. It was also suggested that ethanol oxidation by the microsomal fraction is mediated through an H_2O_2-dependent system which involves catalase. An investigation was made on the effect of H_2O_2 on the system under study, including the possibility that it could substitute for NADPH. Figure 15 shows that this phospholipid cleavage in microsomes also occurred when NADPH was replaced by H_2O_2. The peak of malondialdehyde formation occurs after the first 10 minutes of incubation. The process is enzymic as it is shown to be heat labile (Fig. 16).

Effect of H_2O_2 Concentration on the Cleavage of Polyunsaturated Fatty Acid in Phospholipids

Different concentrations of H_2O_2 in the incubation system were studied in an effort to obtain maximum enzyme activity and thus enhance the opportunity for finding measurable lipid changes in the microsomes. Liver microsomes (containing 1.24 mg of protein) were incubated at 37°C with various concentrations of H_2O_2 in a final incubation volume of 1 ml from zero time to 45 minutes of incubation. Figure 17 shows that the level of malondialdehyde formation is increased significantly when the H_2O_2 concentration rises from 0.9 mM to 90 mM.

At lower concentrations of H_2O_2 (0.9-9 mM), maximum malondialdehyde formation was observed after 30 to 45 minutes of incubation. How-

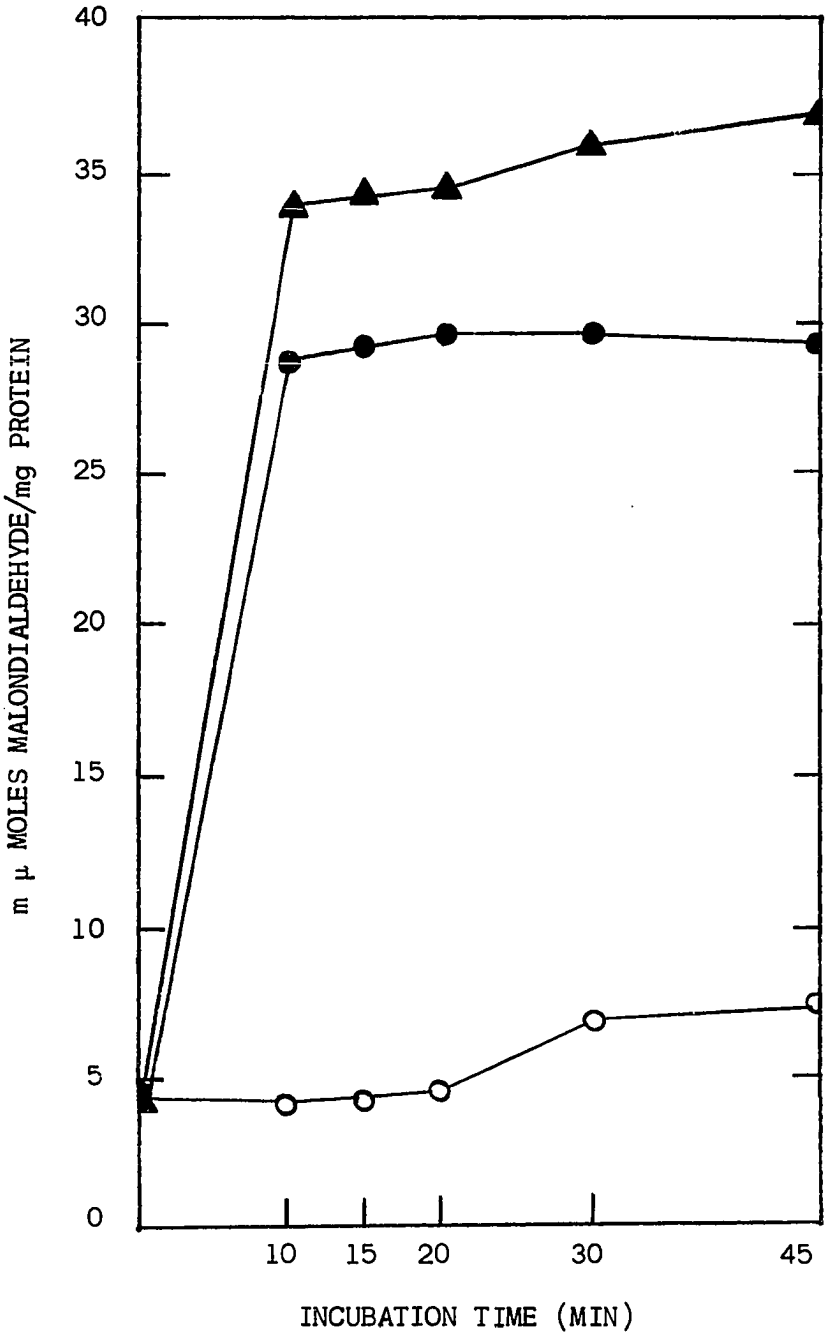


Figure 15. Progress curve of phospholipid PUFA cleavage during enzymic oxidation of NADPH or H_2O_2 by liver microsomes.

Incubation systems and conditions are described as under "Methods." Incubation volume was 1 ml containing 1.2 mg microsomal protein. ▲, experimental system containing 0.3 mM NADPH; ●, experimental system with 90 mM H_2O_2; O, control system.

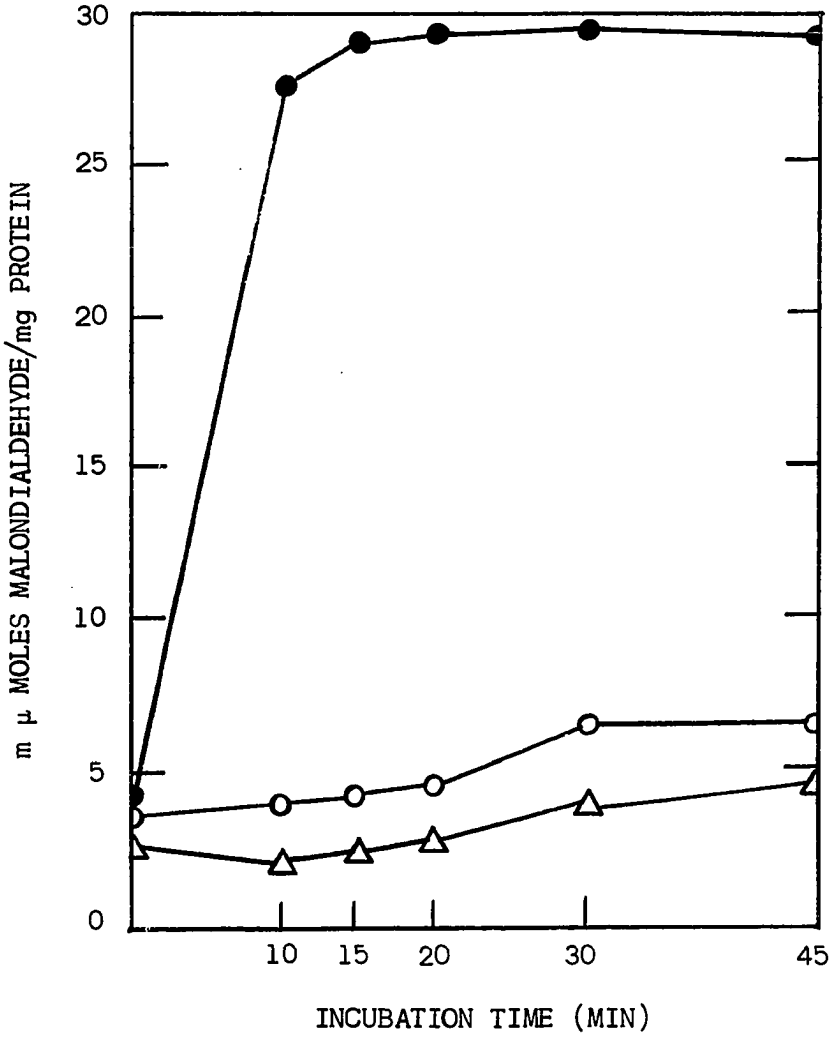


Figure 16. Heat lability of H_2O_2-dependent enzymic microsomal phospholipid PUFA cleavage.

Incubation systems and conditions are described as under "Methods." Incubation volume was 1 ml containing 1.2 mg microsomal protein. ●, experimental system with 90 mM H_2O_2 as substrate; O, control system; Δ, experimental system with 90 mM H_2O_2 but microsomes had been subjected to prior heating at 65°C for 1 minute before addition to the system.

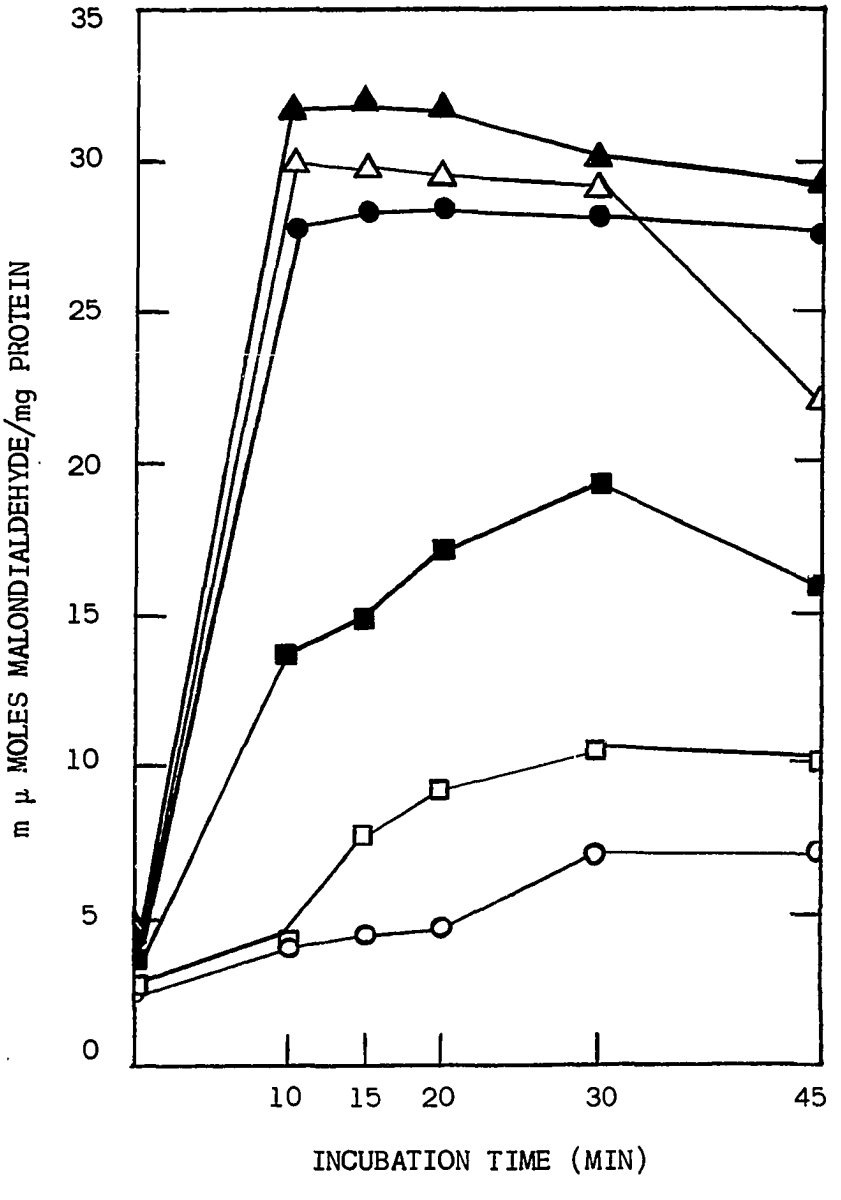


Figure 17. Effect of various concentrations of H_2O_2 on the phospholipid PUFA cleavage.

Incubation systems and conditions are described as under "Methods." Incubation volume was 1 ml containing 1.2 mg microsomal protein. O, control system; all the others are experimental systems with different concentrations of H_2O_2, such as: □, 0.9 mM; ■, 9 mM; ●, 90 mM; △, 270 mM; ▲, 180 mM.

ever, at higher concentrations (90-270 mM), maximum formation usually occurred at about 10 minutes of incubation. From the data obtained here, at least three hypotheses can be derived. The first one suggests that at higher concentrations of H_2O_2, the other substrate for the reaction (phospholipid polyunsaturated fatty acids) becomes limiting after 10 minutes of incubation. Therefore, after 10 minutes, malondialdehyde formation does not continue to increase. The second hypothesis is that the high concentration of H_2O_2 in the reaction may cause the oxidation of essential groups on the enzyme, resulting in inactivation. The third hypothesis is concerning with some other steps which become rate limiting. When the concentration of H_2O_2 is low, the step immediately after H_2O_2 is rate limiting. When the concentration of H_2O_2 is high, the concentration of the intermediate components will be high also. The step immediately after these intermediate components may become rate limiting then. The first possibility is shown below to be erroneous as a result of analyzing the composition of the microsomal fatty acids by gas-liquid chromatography at the end of the reaction. Although the polyunsaturated fatty acids such as arachidonic and docosahexenoic acids were reduced in amount significantly (Table 3), these fatty acids were still far from being exhausted as an effective substrate for the enzymic peroxidative system (assuming that the levels of depletion obtained with NADPH as a substrate can be used as a guide). The second possibility appears more likely that high concentrations of H_2O_2 damage the enzyme comparing curves with 180 and 270 mM H_2O_2 in Fig. 17. But the third possibility that some other steps become rate limiting appears most likely in the case here.

TABLE 3

FATTY ACID COMPOSITION OF MICROSOMAL LIPIDS

System	Microsomal fatty acid content					
	18:0	18:1	18:2	20:4	22:6	Total
			μ moles			
Control	5.98	3.19	4.20	5.50	1.22	20.09
Experimental (H_2O_2)	5.16	3.14	4.18	4.38	0.92	17.78
Difference	0.82	0.05	0.02	1.12	0.30	2.31

Methyl esters were prepared from the total microsomal lipid of control and experimental incubation systems and analyzed by gas-liquid chromatography. Incubation volumes were 10 ml each, containing a total of 18 mg microsomal protein per system. The experimental system contained 270 μ moles of H_2O_2 per ml of reaction system. The incubation time was 45 minutes. Table 3 shows that the losses of the highly unsaturated fatty acids, arachidonic and docosahexenoic acids, amounted to 1.42 μ moles. Although the total amount of these acids utilized is less than when NADPH is the substrate, the results with H_2O_2 as substrate are consistent with the occurrence of the same type of phospholipid cleavage. To obtain further evidence that a similar (or possibly the same) enzyme system is involved in the H_2O_2-dependent reaction, studies with known inhibitors of the NADPH-dependent reaction were performed.

Inhibition Studies on H_2O_2-Dependent Phospholipid Cleavage

Figure 18 shows that inclusion of 1.0 mM Mn^{2+} or 1.0 mM Co^{2+} in the reaction system prevented the formation of malondialdehyde. Similarly, when 1.0 mM EDTA was added to the experimental system, inhibition also occurred but it was not as pronounced as with the divalent cations. The lack of full inhibition with EDTA was interesting since it almost completely inhibited the NADPH-dependent system (40). The utilization of the phospholipid PUFA by the H_2O_2 system appears to follow the same pattern as the NADPH-requiring system and is equally heat labile as the latter. Continuing with the comparative study of inhibitors on the two systems, Table 4 summarizes the comparative effects of a number of substances on the activity of the enzyme in the H_2O_2 system and in the NADPH system.

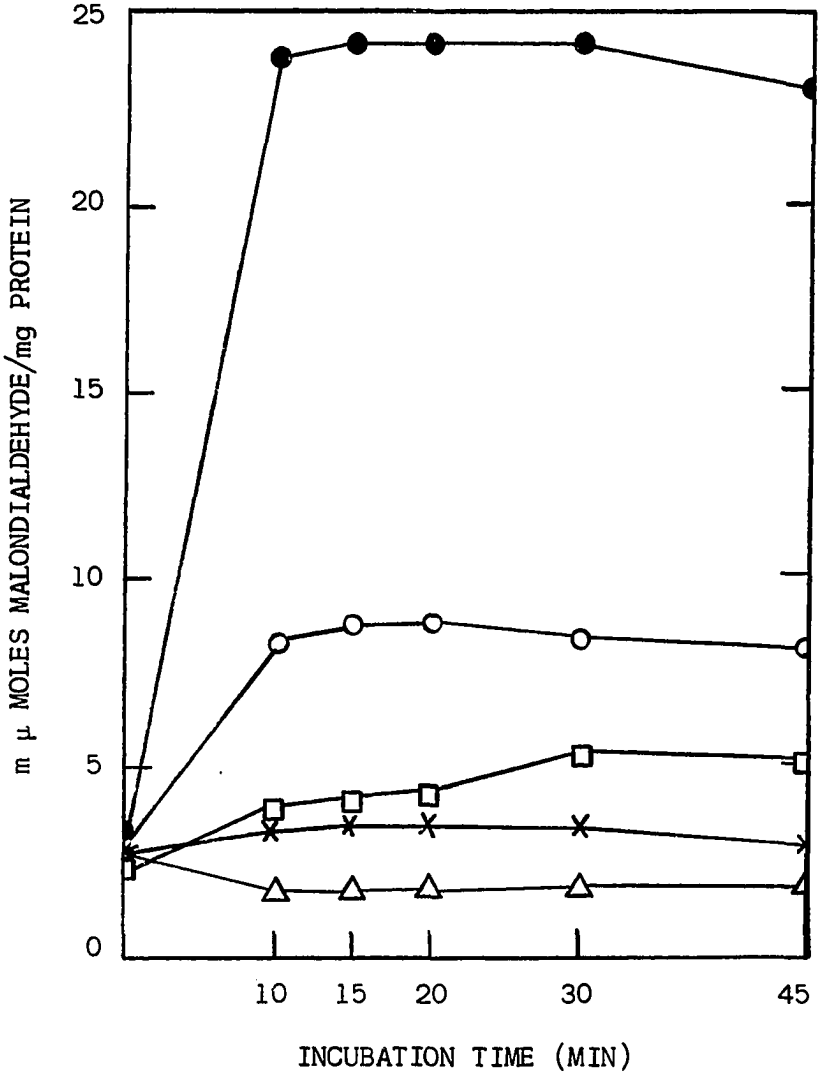


Figure 18. Inhibition of the H_2O_2-dependent enzymic phospholipid PUFA cleavage in liver microsomes by EDTA, Co^{2+} and Mn^{2+}.

The incubation systems and conditions are as described under "Methods." Incubation volume was 1 ml containing 1.5 mg microsomal protein. The experimental system contained 90 mM of H_2O_2. ●, experimental system; O, experimental containing 1.0 mM EDTA, X, experimental system containing 1.0 mM Co^{2+}; Δ, experimental system containing 1.0 mM Mn^{2+}; ■, control system.

TABLE 4

EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITIES OF THE NADPH-DEPENDENT AND H_2O_2-DEPENDENT PHOSPHOLIPID CLEAVING SYSTEMS

Inhibitors	Percent Inhibition	
	NADPH-Dependent System	H_2O_2-Dependent System
	%	%
None	0	0
EDTA (10^{-3} M)	85.8	66.2
Co^{2+} (10^{-3} M)	91.8	89.0
Mn^{2+} (10^{-3} M)	95.3	91.8
Tetranitromethane (8.16×10^{-2} M)	75.0	37.3
Formalin (3.3×10^{-4} M)	100.0	100.0
Aniline (2.68×10^{-3} M)	95.5	94.0
Quinone (2×10^{-4} M)	100.0	100.0
Catechol (4×10^{-3} M)	100.0	100.0
α-Tocopherol-supplemented diet (18 months)	73.5	79.0
Sodium cyanide (2×10^{-3} M)	10.0	100.0
Sodium azide (2×10^{-3} M)	25.0	100.0
3-Amino-1, 2, 4-triazole (2×10^{-3} M)	10.0	50.0
L-thyroxine (4×10^{-3} M)	100.0	86.4
Iodoacetic acid (2×10^{-3} M)	100.0	100.0
O-phenylenediamine (2×10^{-3} M)	0	14.0

The results of the studies on metallic ions indicate that both systems are inhibited by EDTA (10^{-3} M), Co^{2+} (10^{-3} M) and Mn^{2+} (10^{-3} M) up to or close to 90% of the activity. Tetranitromethane (8.16×10^{-2} M) inhibits the NADPH system more effectively than the H_2O_2 system. Tetranitromethane is a colorless solution at room temperature. In the presence of some electron donors, tetranitromethane can be reduced to a compound having a yellow color in solution (60, 61). If the peroxidation of the microsomal phospholipid involved electron transfer systems with components having certain redox potential values, then the reaction systems in the presence of tetranitromethane might be inhibited. The preliminary data in Table 4 show that both systems are inhibited by tetranitromethane, although the inhibition of the NADPH-dependent system seems to be far more effective than inhibition of the H_2O_2-dependent system. Some aromatic compounds and amines, such as aniline, quinone and catechol did, however, markedly inhibit phospholipid cleavage by microsomes in both systems. These compounds are metabolized by the mixed function oxidase which may also be the enzyme system responsible for phospholipid cleavage. Several investigators have suggested that these compounds may inhibit phospholipid oxidation by competing for free radicals produced by the oxidases. Another similar inhibitory effect was found in both systems when liver microsomes were obtained from α-tocopherol-supplemented rats. This compound is also a very effective free radical trapping agent.

The most notable differences in the effect of inhibitors on the two systems were those caused by sodium azide, sodium cyanide and 3-amino-1, 2, 4-triazole. Azide and cyanide completely inhibit the activ-

ity of the H_2O_2-dependent system, but had relatively small effects on the NADPH-dependent system. Similarly, the 3-amino-1, 2, 4-triazole caused 50% inhibition of the H_2O_2 system but had only slight effect on the other.

L-thyroxine, a thyroid hormone, also inhibited both systems. The chemical structure of this hormone is that of an aromatic compound which contains iodine. Whether it is the aromatic structure which acts as a free radical trapping agent or the iodine which may bind to the aromatic structure of tyrosine and the thiol groups of the enzyme will require further investigation. It has been reported that L-thyroxine binds to some proteins also.

Complete inhibition of both systems was also produced by iodoacetic acid. Since iodoacetic acid is known to be an inhibitor of enzymes which possess a sulfhydryl group in their active site, these data provide additional evidence for the involvement of sulfhydryl groups in the phospholipid cleavage reaction.

Effect of Dietary α-Tocopherol on the H_2O_2-Dependent and NADPH-Dependent Cleavage Reactions in Liver Microsomes

Male rats (150 gm) were divided into two groups. One group was placed on an α-tocopherol-deficient diet, and the other group was placed on the same diet supplemented with 10 mg% α-tocopheryl acetate. After a period of 18 months, the rats were sacrificed and microsomes were isolated as described in "Methods." Microsomes from animals fed the α-tocopherol-deficient diet and from those fed the commercial ratio had substantial phospholipid-cleaving activity, but there was significantly greater

activity in the microsomes from animals deprived of α-tocopherol (Fig. 19). Microsomes from tocopherol-supplemented animals showed no activity during the period of the experiment. Similar results were obtained for the NADPH-dependent system (Fig. 20) confirming the findings of May (62) and the report made by McCay et al., (41).

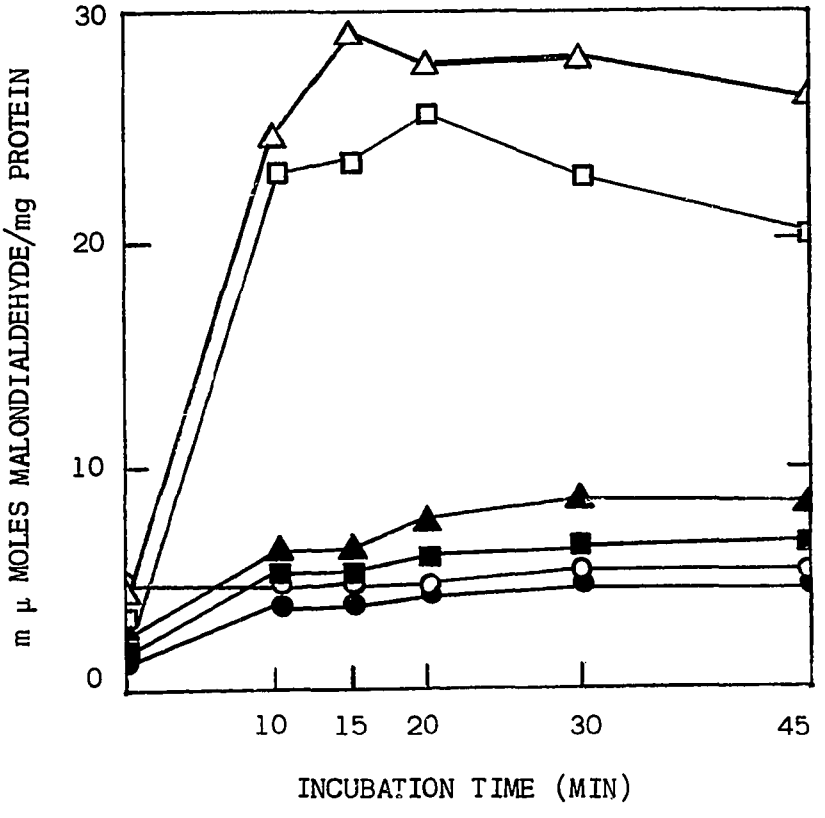


Figure 19. Progress curve of the H_2O_2-dependent malondialdehyde formation by liver microsomes of α-tocopherol-deficient and α-tocopherol supplemented rats, and rats fed commercial ration.

The systems were as described under "Methods" for the NADPH-dependent system except that 90 mM of H_2O_2 was substituted for NADPH in the experimental systems. Incubation volumes were 1 ml containing 1.24 mg microsomal protein. Δ, experimental system with microsomes from tocopherol-deficient rats; ▲, control system with microsomes from tocopherol-deficient rats; O, experimental system with microsomes from tocopherol-supplemented rats; ●, control system with microsomes from tocopherol-supplemented rats; □, experimental system with microsomes from rats fed a commercial ration; ■, control system with microsomes from rats fed a commercial ration.

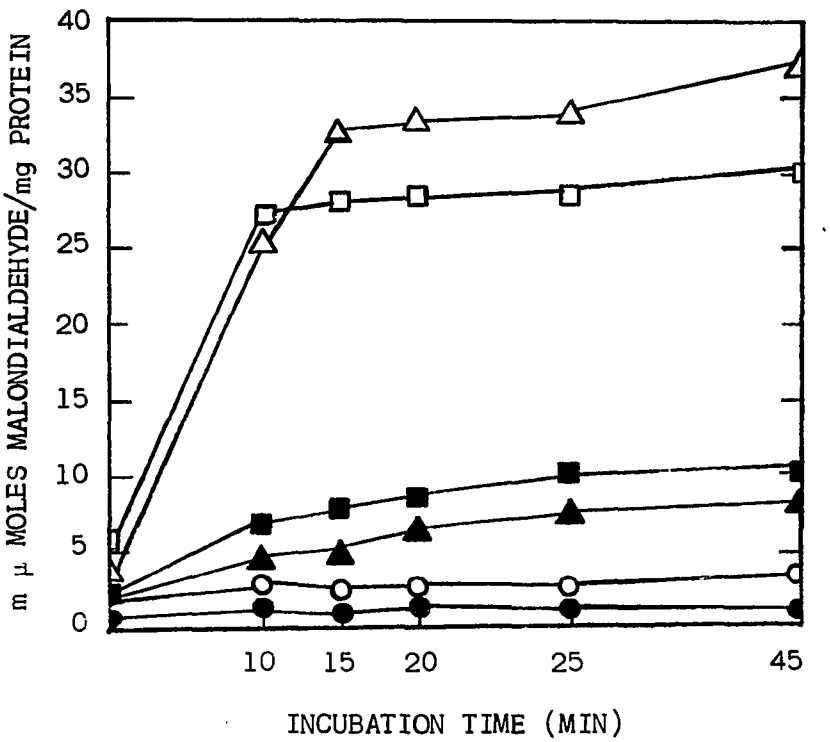


Figure 20. Progress curve for the NADPH-dependent malondialdehyde formation by liver microsomes of α-tocopherol-deficient and α-tocopherol supplemented rats and rats fed a commercial ration.

The systems were as described under "Methods." Incubation volume was 1 ml at 37°C containing 1.24 mg microsomal protein. △, experimental system with microsomes from tocopherol-deficient rats; ▲, control system with microsomes from tocopherol-deficient rats; O, experimental system with microsomes from tocopherol-supplemented rats; ●, control system with microsomes from tocopherol-supplemented rats; □, experimental system with microsomes from rats fed a commercial ration; ■, control system with microsomes from rats fed a commercial ration.

CHAPTER IV

DISCUSSION

The results of these studies described disclose that there is possibly a previously unrecognized enzyme system in liver for metabolizing polyunsaturated fatty acids associated with certain phospholipids. The phospholipids of liver cells as well as in cells of all other tissues constitute a major component of nearly all the structures found in such cells; (a) the external cell membrane; (b) cytoplasmic membranes (Golgi, endoplasmic reticulum, vesicles, etc.); (c) nuclear membrane, and (d) the mitochondrial membranes. The metabolic system which is discussed here is located in the cytoplasmic fraction of membranes of the cell.

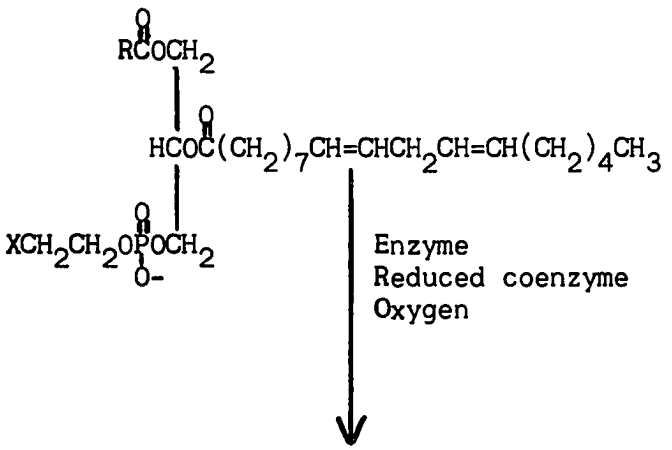
The membrane phospholipids altered by this microsomal enzymic reaction were found to contain carbonyl function on the β-position fatty acids as final products of the reaction <u>in vitro</u>. Thirteen different types of carbonyl-containing fatty acids have been obtained from microsomal phospholipids as dinitrophenylhydrazine derivatives at the end of the reaction. Carbonyl compounds containing no lipid phosphorus were also detected in the supernatant fraction of the incubation system. Phosphatidylcholine and phosphatidylethanolamine molecules which contain either arachidonic, linoleic, or docosahexaenoic acids at the β-position were substrates for the reaction. The data suggested that an oxidative

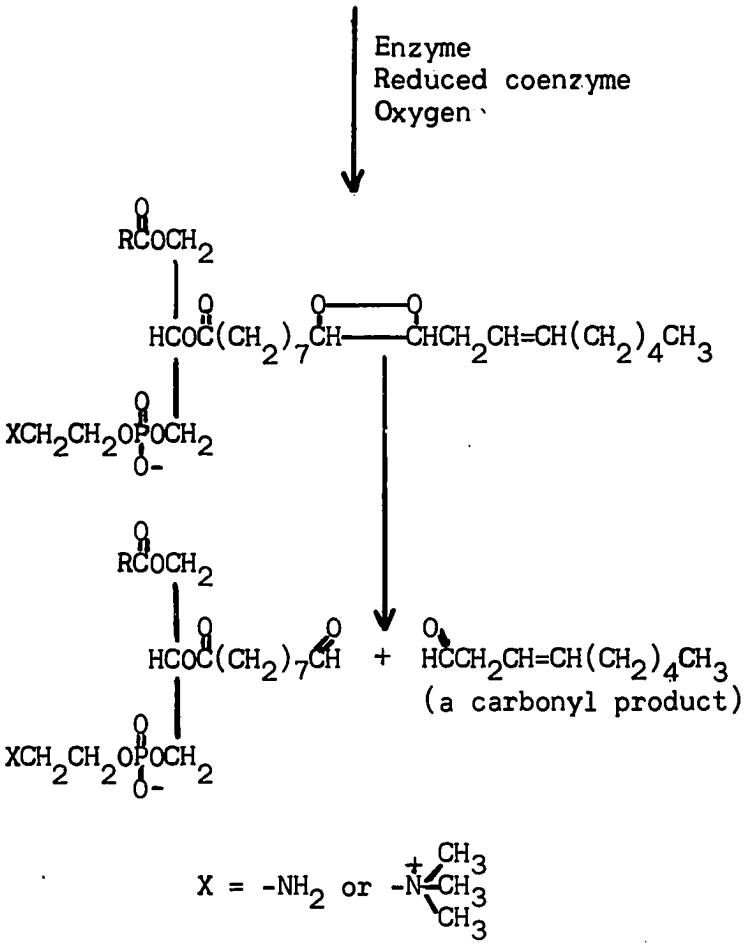
cleavage of these β-position polyunsaturated-fatty acids was taking place as a consequence of NADPH oxidation. The quantity of oxygen consumed, and the fact that one of the water-soluble carbonyl products was proven to be malondialdehyde, suggested that peroxidative cleavage of the unsaturated hydrocarbon chain was occurring.

The present studies give substantial support to this hypothesis by demonstrating that NADPH oxidation in liver microsomes is accompanied by a transitory formation of peroxides on membrane polyunsaturated-fatty acids which then cleave to form aldehydes. Free polyunsaturated-fatty acids are not substrates for this reaction. Only those bound to phosphatidylcholine and phosphatidylethanolamine are known to be utilized.

The number of derivatives obtained indicates that cleavage may occur at various points along the fatty acid carbon chain. This number closely approximates the number of double bond positions in the phospholipid fatty acyl groups which are substrates. For the sake of better illustration and understanding, the structure and the possible oxidative cleavage of cis-9, 12-octadecadienoyl (18:2) is shown below:

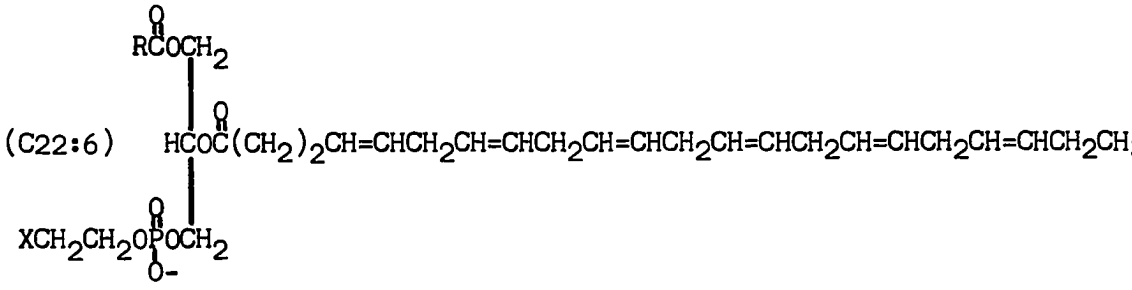
Generalization of the reaction





With regard to the overall reaction, it appears certain that the process occurs in at least two steps. The reaction requires oxygen, the reduced coenzyme, NADPH, and one or more enzymes. The first step involves the addition of oxygen molecules to the polyunsaturated portion of the phospholipid. The result is the formation of a phospholipid peroxide. This substance has been isolated from internal membranes of liver cells in which the reaction was known to be occurring. The phospholipid peroxide in the membrane undergoes a further reaction which cleaves the polyunsaturated fatty acid portion of the phospholipids into several smaller fragments, leaving the phospholipid molecule with its polyunsaturated fatty acid moiety considerably reduced in size and containing additional

oxygen atoms in the form of carbonyl groups. The phospholipids functioning as substrates for the reaction are those containing docosahexaenoic, arachidonic and linoleic acids, with reactivity in that order:



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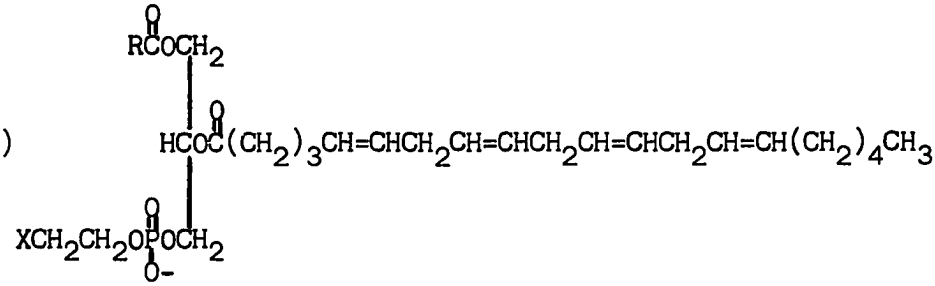

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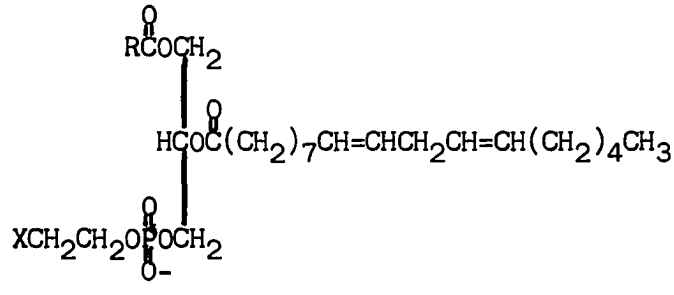
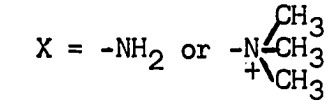
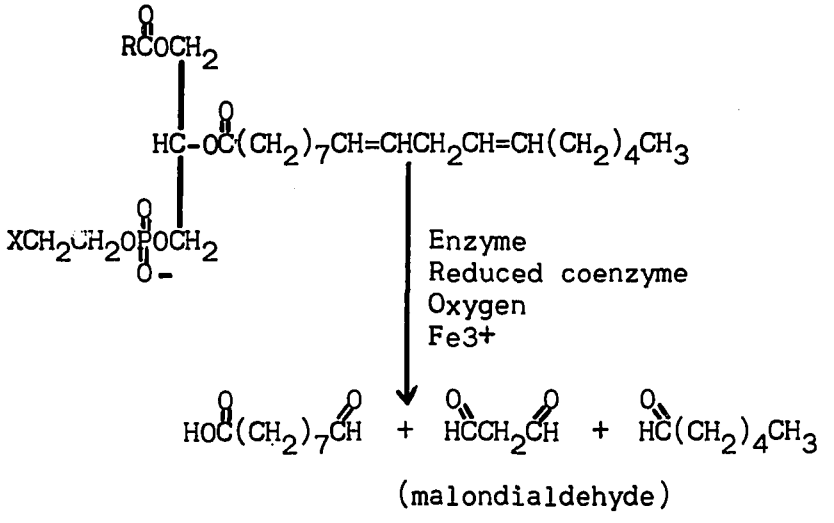


(C18:2)



If peroxidative cleavage of these fatty acid chains were a function of double bond location, it might result in the production of at least 12 different products providing the specificity for an attack at any particular double bond position were low. Production of the small quantity

of malondialdehyde may be the result of cleavage simultaneously at two neighboring double bonds on the same carbon chain. For example:



If this was true, it must be an infrequent event since malondialdehyde formation can only account for about 5% of the oxygen uptake (40). This possible mechanism of formation might also explain why the total malondialdehyde formation constitutes only a small percentage of the total phospholipid equivalents which undergoes peroxide formation, in that for this particular product to form, simultaneous cleavage of both double bonds may have to occur, for otherwise it would seem that conjugation of double bonds would prevent malondialdehyde formation if the attacks were sequential.

A study of turbidity changes during the reaction indicated that the membrane structure must have undergone some significant alteration. Since regulation of permeability is a major function of membranes, it is possible that the alterations may result in changes in the permeability of this internal membrane structure, due to the formation of charged groups on the hydrophobic portion of an appreciable number of the phos-

pholipids. Turbidity changes occurring during the reaction also suggest that partial fragmentation of the microsomes might have occurred.

Investigations by Hill et al., (63) on the incorporation of ^{14}C-glycerol by rat liver slices indicate that the more unsaturated glycerophosphatides may turn over at a faster rate than the less unsaturated ones. Since the polyunsaturated-fatty acids composition of the liver microsomal phospholipids remains essentially constant on a standardized diet, a metabolic reaction utilizing polyunsaturated-fatty acids in microsomal phospholipids would require an equal rate of replacement.

This enzyme system appears to compete for reducing equivalents in common with the drug hydroxylating system (64), presumably because the two systems have one or more components in common. This relationship now bears further investigation since Lin and Chen have shown that tetralin hydroperoxide is an intermediate in the NADPH-linked hydroxylation of tetralin by liver microsomes (65). Whether the enzyme-catalyzed, peroxidative cleavage of the microsomal phospholipids occurs as a manifestation of an explicit function for these phospholipids in the membrane or whether it represents chemical damage to the membrane consequent to electron transport from NADPH to oxygen is an open question.

If the reaction which has been described occurs in vivo to any extent, the consequences of incorporating large amounts of polyunsaturated fatty acids into tissues could be severe if the site of phospholipid peroxide production caused sufficient alteration in membrane structure to disrupt an essential process. One of the most interesting facts about the phospholipid-cleaving reaction is that there is an associated conversion of α-tocopherol to an unidentified compound during the process (41).

The relationship between the dietary intake of polyunsaturated fatty acids and α-tocopherol has been thoroughly documented (66). High levels of polyunsaturated fatty acid intake require appropriately increased levels of α-tocopherol to prevent the development of tissue damage of several types which in most species is fatal. Work done in this laboratory has shown that enzymic phospholipid cleavage is depressed in animals given higher than normal levels of α-tocopherol. On the other hand, animals which are deprived of α-tocopherol have higher activities than animals fed a balanced laboratory ration. All of these aspects discussed above suggest that a function of α-tocopherol in animal tissues, particularly in the endoplasmic reticulum, may be to assure that the formation of phospholipid peroxides does not exceed safe limits. It may well be that the described alteration of phospholipids is an unavoidable consequence of electron transport from NADPH in the endoplasmic reticulum which is kept within manageable limits by the presence of α-tocopherol. We are dealing with a highly-ordered membrane structure in which the polyunsaturated fatty acid moieties of the phospholipids may be positioned in highly-vulnerable loci in the membrane with respect to the potent oxidizing functions associated with NADPH oxidation (cholesterol side-chain cleavage, squalene oxidation, drug detoxification, etc.). It then becomes reasonable to consider that the relationship between the nutritional levels of α-tocopherol and polyunsaturated fatty acids may be a function of the susceptibility of these acids (on being incorporated into phospholipids of the endoplasmic reticulum) to peroxidative cleavage when an insufficient amount of tocopherol is present. This concept fits nearly all of the accumulated data relating to a biochemical function for

α-tocopherol. The most pertinent question to be answered is: Does the enzymic process as manifested in the test tube occur as such in vivo? There is no definitive answer to this question at the moment although studies have been undertaken in an attempt to resolve the problem. However, there are several lines of evidence which seem to indicate that it is reasonable to assume such is the case. First, there are the cases of fat malabsorption in humans in which the uptake of α-tocopherol from the intestine is very low. These patients exhibit a number of symptoms, among them evidence of α-tocopherol deficiency. The cells of the intestinal mucosa in these patients do not possess normal endoplasmic reticulum structure according to electron microscopic studies. There is a lack of positive contrast which has been thought to be due to the presence of the polyunsaturated fatty acids in the membrane. However, after treatment of these patients with α-tocopherol for 4 months, the endoplasmic reticulum had a normal appearance in the mucosal cells (67). These observations suggest that α-tocopherol is required to maintain the endoplasmic reticulum in the intestinal mucosa. Although it is not known if the enzyme system under study is present in human intestinal mucosa, it has been determined that the system is present in other human tissues (68).

In the case of rabbits deprived of α-tocopherol, the organization of the contractile filaments, which are situated in a network of the sarcoplasmic reticulum, is lost, culminating in acute, fatal muscular dystrophy. The phospholipid peroxidizing system is known to be present in skeletal muscle.

Studies with C^{14}-labeled fatty acids in tocopherol-deficient

and -sufficient animals have indicated that polyunsaturated fatty acids turn over more rapidly in the deficient animals (66). Since the reaction described in this report has specificity for these acids and its activity is accelerated by tocopherol deprivation, it would contribute to an increased turnover of polyunsaturated fatty acids in deprived animals in addition to altering the structure of the endoplasmic reticulum.

Intracellular conditions appear to be nearly optimal in liver tissue for the reaction to occur. Nearly all of the NADP in the cytoplasm is in the required reduced form; the concentrations of inorganic iron and of ADP are also more than sufficient to provide maximum activity. It is not certain at present to what extent the reaction may occur in vivo since neither the phospholipid peroxides or aldehydes derived from them appear to be stable in the presence of other cellular components. However, we have obtained 2,4-dinitrophenylhydrazine derivatives of phospholipids from normal rat liver microsomes.

Studies of the reaction show that NADPH can be replaced by H_2O_2. The utilization of phospholipid polyunsaturated fatty acids by the H_2O_2 system follows the same pattern as the NADPH-requiring system and is equally heat labile. The activity of the H_2O_2-dependent system was found to be inhibited by the same agents which were known to inhibit the NADPH-dependent system. One distinguishing exception was the effect of catalase inhibitors which prevented the activity of the H_2O_2-dependent system to a large extent but had little effect on the NADPH-dependent system. This phenomenon may be explained by the fact that the concentration of H_2O_2 used in the experimental system was rather high. At the same moment, the turnover rate for catalase is higher than that of any other known en-

zyme; one molecule of catalase can decompose 44,000 molecules of hydrogen peroxide per second into oxygen and water. Catalase reacts with H_2O_2 to form a relatively stable enzyme-substrate complex of uncertain structure. It is in this form only that catalase may react with the specific inhibitor 3-amino-1, 2, 4-triazole. As a consequence to this inhibitory reaction, large quantities of hydrogen peroxide molecules are then left untouched. Since hydrogen peroxide is a very strong oxidant, inactivation of the H_2O_2-dependent enzyme may result. There is at least one report that free sulphydryl groups of a protein were diminished after H_2O_2 treatment of red cells in the study of hemolysis (69). On the other hand, it may be that catalase produces some form of activated oxygen during the decomposition of H_2O_2 which can attack phospholipids in the manner described. Our studies also show that PHMB and iodoacetic acid did inhibit both the NADPH-dependent and the H_2O_2-dependent enzymic systems. The two inhibitors are known to react with the sulphydryl groups of proteins. Thus, it appears that there could be at least two enzymes involved in each of these systems. For example, in the H_2O_2 system, catalase and another sulphydryl-containing protein may be required.

Both the NADPH-dependent and H_2O_2-dependent systems apparently produce the same component or components which react with phospholipids to form phospholipid peroxides, the latter then undergoing degradation to carbonyls. The component(s) may involve the superoxide anion which has been suggested by Nilsson (70), Yamazaki (71), and Massey (72) to be involved in some biological peroxidative systems.

CHAPTER V

SUMMARY

An intermediate in a new enzyme system catalyzing the NADPH-dependent oxidative cleavage of liver microsomal phospholipids was detected and partially characterized. The intermediate is a phospholipid peroxide which forms transiently during the reaction. The cleavage process is specific for polyunsaturated fatty acids in the β-position of phosphatidylethanolamine and phosphatidylcholine. Free polyunsaturated fatty acids are not substrates for the reaction. The various data support the conclusion that microsomes contain an enzyme system which promotes multiple peroxidative cleavage of endogenous membrane-bound polyunsaturated fatty acids, producing a variety of carbonyl-containing residues. The products of the reaction include phospholipids containing carbonyl groups in the β-position acyl moiety. Concomitant with the alteration of membrane-bound phospholipids, there is evidence for major structural changes in the membrane itself. The reaction may occur as a natural consequence of electron transport from NADPH. On the other hand, the reaction may represent an explicit metabolic function for unsaturated phospholipids during electron transport in the endoplasmic reticulum, or even a possible alternative pathway for polyunsaturated fatty acid oxidation. An additional new finding derived from this work is that NADPH can

be substituted in the reaction by H_2O_2. The utilization of phospholipid polyunsaturated fatty acids by a H_2O_2 system follows the same pattern as the NADPH-requiring system and is equally heat labile. The activity of the H_2O_2-dependent system was found to be inhibited by the same agents which were known to inhibit the NADPH-dependent system. One notable exception was the effect of catalase inhibitors which decreased the activity of the H_2O_2-dependent system to a large extent but had little effect on the NADPH-dependent system. The results suggest that both the NADPH-dependent and H_2O_2-dependent systems produce a component which reacts with phospholipids to form phospholipid peroxides, the latter then undergoing degradation to carbonyls. Dietary supplementation with tocopherol resulted in inhibition of both the H_2O_2-dependent and NADPH-dependent systems, while deficiency of this vitamin in the diet was accompanied by an increased activity of the system.

BIBLIOGRAPHY

1. Hogeboom, G. H., J. Biol. Chem., 177, 847 (1949).

2. Hogeboom, G. H., and Schneider, W. C., Nature, 166, 302 (1950).

3. Palade, G. E., and Siekevitz, P., Fed. Proc., 14, 262 (1955).

4. Palade, G. E., and Siekevitz, P., Biophys. Biochem. Cytol., 2, 171 (1956).

5. King, T. E., H. S. Mason, M. Morrison, Oxidases and related redox systems, John Wiley & Sons, Inc., New York, Vol. 2, 881 (1965).

6. Klingenberg, M., Arch. Biochem. Biophys., 75, 376 (1958).

7. Staudinger, Hj., Page, A. C., Folkers, K., et al., Arch. Biochem. Biophys., 96, 580 (1962).

8. Stritmatter, P., and Velick, S., J. Biol. Chem., 221, 277 (1956).

9. Phillips, A. H., and Langdon, R. G., J. Biol. Chem., 237, 2652 (1962).

10. Garfinkel, D., Arch. Biochem. Biophys., 71, 100 (1957).

11. Omura, T., and Sato, R., Biochim. Biophys. Acta, 71, 224 (1963).

12. Mason, H. S., North, J. C., and Vanneste, M., Fed. Proc., 24, 1172 (1965).

13. Siekevitz, P., Ann. Rev. Physiol., 25, 15 (1963).

14. de Duve, C., Wattiaux, R., and Baudhuin, P., Advances in Enzymol., 24, 291 (1962).

15. Gillette, J. R. Proceedings of the 1st International Pharmacological Meeting, Stockholm, 6, 13 (1962).

16. Dallner, G., Siekevitz, P., and Palade, G. E., Biochem. Biophys. Res. Commun., 20, 142 (1965).

17. Siekevitz, P., Fed. Proc., 24, 1153 (1965).

18. Beloff-Chain, A., Serlupi-Crescenzie, G., Cantanzaro, R., Venettacci, D., and Balliano, M., Biochim. Biophys. Acta, 97, 416 (1965).

19. Orrenius, S., Dallner, G., and Ernester, L., Biochem. Biophys. Res. Commun., 14, 329 (1964).

20. Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O., and Estabrook, R. W., Fed. Proc., 24, 1181 (1965).

21. Gillette, J. R., Brodie, B. B., and La Du, B. N., J. Pharm. Exp. Therap., 119, 532 (1957).

22. Gaudette, L. E., and Brodie, B. B., Biochem. Pharm., 2, 89 (1959).

23. Krisch, K., and Staudinger, H., Biochem. Zeitschr., 334, 312 (1961).

24. Hochstein, P., and Ernster, L., Biochem. Biophys. Res. Commun., 12, 388 (1963).

25. Lundberg, W. D., in H. W. Schultz, E. A. Day and R. O. Sinnhuber (Eds.), Symposium on Foods: Lipids and their Oxidation, AVI Pub. Co., Westport, Conn., pp 31 (1962).

26. Ingold, K. U., ibid, pp 93.

27. Keeney, M., ibid, pp 79.

28. Dahle, L. K., Hill, E. G., and Holman, R. T., Arch. Biochem. Biophys., 98, 253 (1962)

29. Tappel, A. L., in H. W. Schultz, E. A. Day and R. O. Sinnhuber (Eds.), Symposium on Foods: Lipids and their Oxidation, AVI Pub. Co., Westport, Conn., pp 367 (1962).

30. Kitabchi, A. E., McCay, P. B., Carpenter, M. P., Feinberg, R. H., Trucco, R. E., and Caputto, R., Biochem. Biophys. Res. Commun., 1, 216 (1959).

31. Carpenter, M. P., Kitabchi, A. E., McCay, P. B., and Caputto, R., J. Biol. Chem., 234, 2814 (1959).

32. McCay, P. B., J. Biol. Chem., 241, 2333 (1965).

33. Beloff-Chain, A., Cantanzaro, R., and Serlupi-Crescenzi, G., Nature, 198, 351 (1963).

34. Poyer, J. L., May, H. E., and McCay, P. B., Fed. Proc., 25, 301 (1966).

35. Poyer, J. L., Ph. D. Dissertation, University of Oklahoma (1969).

36. Roach, M. K., Reese, Jr., W. N., and Creaven, P. J., Biochem. Biophys. Res. Commun., 36, 596 (1969).

37. Maker, A. B., and Mannering, G. J., Mol. Pharmacol., 4, 484 (1968).

38. Goodman, J. I., and Tephly, T. R., Mol. Pharmacol., 4, 492 (1968).

39. May, H. E., and McCay, P. B., J. Biol. Chem., 243, 2288 (1968).

40. May, H. E., and McCay, P. B., J. Biol. Chem., 243, 2296 (1968).

41. McCay, P. B., Stipe, B., and Shealy, S., Fed. Proc., 28, 757 (1969).

42. Caputto, R., McCay, P. B., and Carpenter, M. P., J. Biol. Chem., 233, 1025 (1958).

43. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., J. Nutrition, 14, 273 (1937).

44. Kokatnur, M. G., Bergan, J. G., and Draper, H. H., Anal. Biochem., 12, 325 (1965).

45. American Oil Chemists' Society, Official and Tentative Methods of the American Oil Chemists' Society, Chicago, Cd-8-53 (1964).

46. Lundberg, W. O., and Chipault, J. R., J. of Am. Chem. Soc., 69, 833 (1947).

47. Kolthoff, I. M., and Sandell, E. B., Textbook of Quantitative Inorganic Analysis, Third Ed., pp 590-600 (1959).

48. Ottolenghi, A., Arch. Biochem. Biophys., 79, 355 (1959).

49. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

50. Folch, J., Lees, M., and Sloane-Stanley, G. H., J. Biol. Chem., 226, 497 (1957).

51. McCay, P. B., Personal communication.

52. Fiske, C. H., and SubbaRow, Y., J. Biol. Chem., 66, 375 (1925).

53. Fioriti, J. A., Sims, J. R., J. Chrom., 32, 761 (1968).

54. Fioriti, J. A., Buide, N., and Sims, J. R., Lipid, 4, 142 (1968).

55. Wagoner, H. L., Horhammer, L., and Wolf, P., Biochem. Z., 334, 175 (1961).

56. Ditmer, J. C., and Lester, R. L., J. Lipid Res., 5, 126 (1964).

57. Morrison, W. R., and Smith, L. M., J. Lipid Res., 4, 600 (1964).

58. Long, C., and Penny, I. F., Biochem. J., 65, 382 (1957).

59. Robinson, J. D., Arch. Biochem. Biophys., 110, 475 (1965).

60. McCord, J. M., and Fridovich, I., Fed. Proc., 28, 346 (1969).

61. McCord, J. M., and Fridovich, I., J. Biol. Chem., 244, 6049 (1969).

62. May, H. E., Ph. D. Dissertation, University of Oklahoma (1966).

63. Hill, E. E., Husbands, D. R., and Lands, W. E. M., J. Biol. Chem., 243, 4440 (1968).

64. Gram, T. E., and Fouts, J. R., Arch. Biochem. Biophys., 114, 331 (1966).

65. Lin, C. C., and Chen, C., Biochim. Biophys. Acta, 192, 133 (1969).

66. Gilliam, J. M., and McCay, P. B., Fed. Proc., 25, 241 (1966).

67. Molenaar, I., Hommes, F. A., Braams, W. G., and Polman, H. A., Proc. of the Natl. Acad. Sci., 61, 982 (1968).

68. Heston, H. H., and McCay, P. B., Unpublished observation.

69. Jacob, H. S., and Lux, S. E., Blood, J. Hematol., 32, 549 (1968).

70. Nilsson, R., Pick, F. M., and Bray, R. C., Biochim. Biophys. Acta, 192, 145 (1969).

71. Yamazaki, I., and Piette, L. N., Biochim. Biophys. Acta, 77, 47 (1963).

72. Massey, V., and Gibson, Q. H., Fed. Proc., 23, 18 (1964).