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ALTERATIONS OF MICROSOMAL PHOSPHOLIPIDS OCCURRING DURING THE ENZYMIC OXIDATION OF TPNH

A DISSERTATION

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in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

HUBERT EUGENE MAY

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ALTERATIONS OF MICROSOMAL PHOSPHOLIPIDS OCCURRING

DURING THE ENZYMIC OXIDATION OF TPNH

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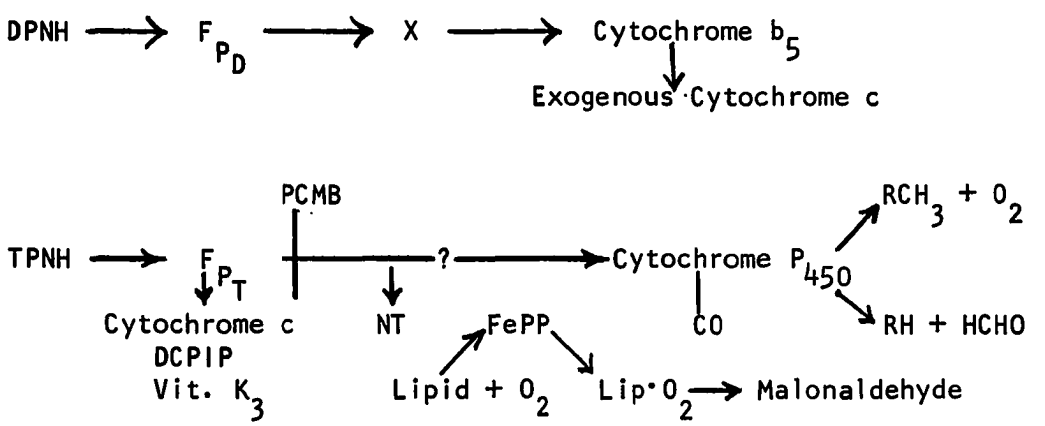
ALTERATIONS OF MICROSOMAL PHOSPHOLIPIDS OCCURRING DURING THE ENZYMIC OXIDATION OF TPNH

CHAPTER I

INTRODUCTION

Microsomes from various tissues contain a variety of mixed function oxidases (1,2) which are concerned with the metabolism of xenobiotics (substances foreign to the metabolic network) and also normal metabolic components such as steroids. These oxidases appear to involve an electron transport chain found in the membrane of the endoplasmic reticulum (3-5). Some if not all, of the pigmented components of the microsomal electron transport chain have been isolated and characterized (6-9). Two cytochromes unique to the microsome have been isolated and it has been shown that their properties are quite distinct from those found in the mitochondrion (6,7). Flavoproteins that oxidize pyridine nucleotides have also been isolated and purified from microsomes and extensively studied (6,10-13). The rate of electron flow, measured by various electron acceptors, appears to be approximately of equal magnitude in mitochondria and microsomes (3). The relationships between the components of the microsomal electron transport system have been established by: a) study of the appearance of various enzymic activities in the developing liver (3-5); b) study of the rate of induction of the components by drugs (14-18);

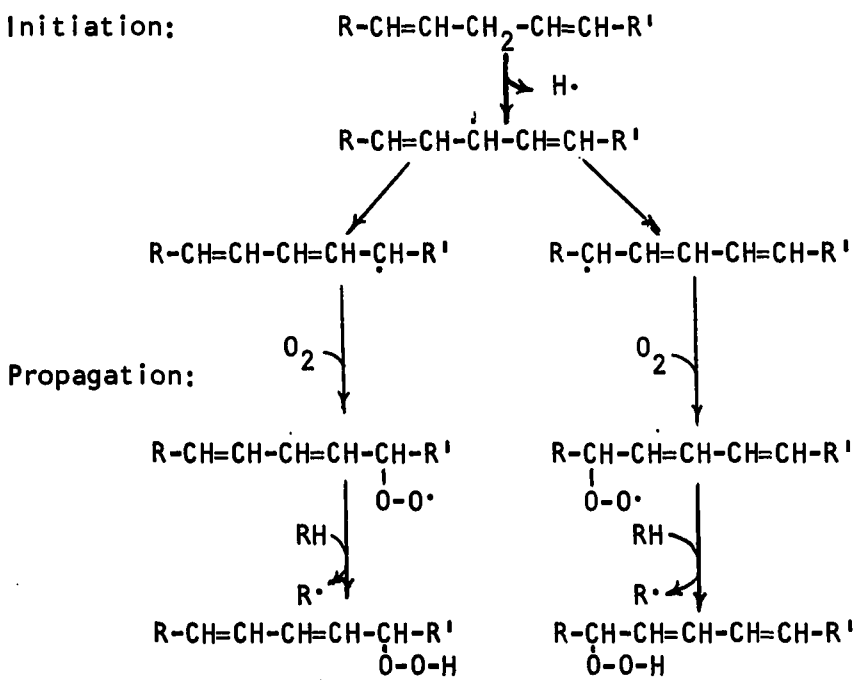
c) studies on the effects of various electron acceptors and inhibitors (19); d) studies of isolated components (1,13,20,21); and e) reconstruction of the enzymic activity of steroid 11 β-hydroxylase of adrenal mitochondria (20). These studies have led to the following scheme which is a combination of that given by Siekevitz (3) and Ernster (19):

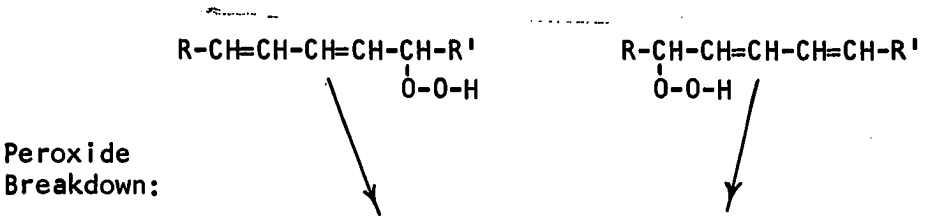


where DPNH and TPNH are di- and triphosphopyridine nucleotides, respectively; PCMB is p-chloromercuribenzoate; DCPIP is dichlorophenolindophenol; NT is a neotetrazolium salt; CO is carbon monoxide; F_{P_D} and F_{P_T} are flavoproteins; and FePP is a pyrophosphate complex of ionic iron. At the present there appears to be two electron transport chains, one for the oxidation of DPNH and another for the oxidation of TPNH. Beloff-Chain et al. (22) reported in 1963 that the addition of adenosine diphosphate (ADP) to rat liver microsomes stimulated the oxidation of TPNH with a concomitant increase in oxygen uptake. Hochstein and Ernster (23) later found that the molar ratio of oxygen uptake to TPNH oxidized was 20 to 1 which was far too great for the expected ratio for the enzymic oxidation of TPNH by known mechanisms. The authors also found a thiobarbituric acid-reacting chromogen produced concomitantly with the stimulated oxidation of TPNH. The formation of this chromogen, which they

believed to be a result of lipid peroxidation, could be prevented by: a) ethylenediaminetetraacetate (EDTA), diphenylphenylenediamine (DPPD) and the α-tocopherol metabolite of Simon (3-(hydroxy-3-methylcarboxy-pentyl) 3,5,6-trimethyl benzoquinone) which are inhibitors of lipid peroxidation (23); b) p-chloromercuribenzoate (PCMB) and β-diethyl-aminoethyl diphenylpropylacetate (SKF-525A), inhibitors of microsomal electron transport (19); and c) inactivation of microsomes by heat prior to incubation with TPNH and ADP (23).

In vitro lipid peroxidation has been studied in model systems for several years and many, although not all, of the basic properties of such systems are known. Lipid peroxidation is thought to be an auto-catalytic series of reactions involving a free radical mechanism. The process can be conveniently divided into four processes (see reviews by Lundberg (24) and Ingold (25)): chain initiation, chain propagation, hydroperoxide breakdown and chain termination.

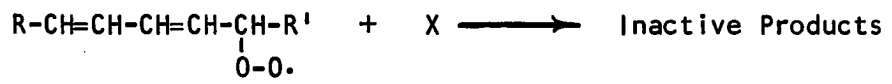




Peroxide Breakdown:

aldehydes, ketones, alcohols, carboxylic acids, polymers, etc.

Chain Termination:



For our purposes RH is an unsaturated fatty acid with a 1,4-pentadiene portion present and X is either another free radical or a compound which reacts with a free radical to form another free radical which is not energetic enough to perpetuate the chain propagation process.

Although many kinds of lipid-derived products are formed by this process (26) carbonyl derivatives are usually formed in relatively larger quantities, among them aldehydes resulting from the cleavage of lipid carbon to carbon bonds are predominant (26). When polyunsaturated fatty acids are used as substrates for lipid peroxidation a thiobarbituric acid-reacting chromogen, presumably malonaldehyde, is formed and a measure of this chromogen constitutes an index, although not a quantitative one, of the extent of lipid peroxidation (27).

Reports have appeared describing <u>in vivo</u> peroxidation of lipids and the subject has been reviewed by Tappel (28). Many of the studies, however, appeared not to have been done with sufficient precaution to prevent lipid peroxidation post mortem in the tissues investigated. Studies in this laboratory have failed to show significant differences in lipid peroxides found in tissues from animals sufficient and deficient in α-tocopherol (29). Negative evidence, however, does not

rule out lipid peroxidation *in vivo* since it may be that the oxidation products are removed from the tissues as fast as they are formed and, therefore, do not accumulate. For example, malonaldehyde when injected into the rat is rapidly metabolized (30). Thus although lipid peroxidation probably occurs *in vivo* the question is still in the author's opinion an open one.

Certain compounds of biological origin have been shown to peroxidize lipids very effectively. For example, hematin compounds are very efficient peroxidative catalysts and the literature up to 1962 was reviewed by Tappel (31,32).

Enzyme-associated lipid peroxidation has been reported (33,34) occurring in association with the activity of the rat liver microsomal enzyme L-gulonolactone oxidase in α-tocopherol-deficient animals. This was the first report of lipid peroxidation associated with the functioning of an enzyme. It has been difficult, however, to demonstrate directly that lipid changes occurred during the function of this enzyme (35).

The microsomal TPNH-oxidizing system described by Beloff-Chain (22) and by Hochstein and Ernster (23) offered another opportunity for investigating directly the formation of lipid peroxidation products by a functioning enzyme system under mild conditions compatible with physiological conditions.

The purpose of this study was:

1) To determine by a variety of analytical techniques whether or not lipid changes occurred in the microsome during the functioning of microsomal TPNH oxidase.

2) To study the effect of various enzymic parameters of TPNH oxidase on any lipid changes that might occur.

3) To study the effect of dietary α-tocopherol levels on the reaction and also to study the effect of drug-stimulated induction of microsomal hydroxylases on the various reactions.

4) To establish the characteristics of these reactions and the nature of the products formed sufficiently: a) to determine whether or not a classical lipid peroxidation mechanism is involved and b) to justify a future search for similar reactions in vivo.

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Adult male albino rats (180-250 g), bred and maintained in this laboratory, were used in these experiments. These rats were originally derived from the Holzman-Sprague Dawley strain and are now highly inbred. Animals were fed either a commercial pellet diet or a synthetic diet described below.

Materials for Diets

Casein, vitamins (except α-tocopheryl acetate), cod liver oil 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-tocopherol and α-tocopheryl acetate 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 (36). The salt mixture and vitamin mixture were prepared according to the method of Hubbell et al. (37).

Composition of Vitamin Mixture

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

Composition of Salt Mixture

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

Composition of Basal Diet

	Percent Composition
Casein, vitamin free	17.0
Sucrose	37.3
Corn Starch	36.0
Lard	3.0

	Percent Composition
Cod Liver Oil	3.0
Salt Mixture	3.0
Vitamin Mixture	0.7

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

α-Tocopherol-supplemented diets. These diets were identical to the α-tocopherol-deficient diet except α-tocopheryl acetate was added. The following diets were used and their nomenclature is given.

Nomenclature	α-Tocopheryl Acetate per 100 g of Diet
- E	0
+ E1	10 mg
+ E2	20 mg
+ E3	30 mg
+ E6	60 mg
+ E9	90 mg
+ E15	150 mg

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, menadione, vitamin B_{12}, 1% calcium carbonate, 0.5% dicalcium phosphate, 1% sodium chloride, and traces of manganese oxide, copper oxide, cobalt carbonate, iron carbonate, zinc oxide, and calcium iodate. The manufacturers guaranteed the following analyses: crude protein, 24%; crude fat, 4%; and crude fiber, 6%.

Reagent Chemicals

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

Triphosphopyridine nucleotides (TPN and TPNH), sodium D-glucose-6-phosphate, D,L sodium isocitrate, and p-hydroxymercuribenzoate were obtained from Sigma Chemical Company, St. Louis, Missouri.

Adenosine 5'-diphosphate was obtained from P L Laboratories, Milwaukee, Wisconsin.

The following chemicals were obtained from Fisher Scientific Company, Philadelphia, Pennsylvania: tris (hydroxymethyl) aminomethane, monobasic potassium phosphate, dibasic potassium phosphate, chloroform, methanol, diethyl ether, benzene and the inorganic salts used in this study.

The following chemicals were obtained from Eastman Organic Chemicals, Rochester, New York: 2,4-dinitrophenylhydrazine, sodium ethylenediaminetetraacetate, 2-thiobarbituric acid, pyrogallol, hydroxylamine-HCl and α,α-dipyridyl.

L-Gulonolactone was obtained from K and K Laboratories, Plainview, New York.

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

Boron trifluoride-methanol reagent, phospholipid standards and some standard methyl ester mixtures were obtained from Applied Science Laboratories, State College, Pennsylvania.

Sodium phenobarbital (40 mg/ml) in isotonic saline for injection was obtained from Connie's Prescription Shop, Oklahoma City, Oklahoma.

Some methyl ester standards and free fatty acids were obtained from the Hormel Institute, Austin, Minnesota.

Solvents

All solvents used were reagent grade except n-hexane which was chromatoquality (Matheson Coleman & Bell Company, East Rutherford, New Jersey). In certain chromatographic procedures, so designated, the solvents required further purification. "Carbonyl free" benzene was prepared by refluxing reagent grade benzene in the presence of dinitrophenylhydrazine (10 g/l) (DPNH) for 8 hours followed by distillation (38). Purified methanol was prepared similarly but required two distillations to remove DPNH impurities. Chloroform was also purified in the same manner.

Chromatographic Materials

Reagent grade silicic acid, 100 mesh, from Mallinckrodt Chemical Works, St. Louis, Missouri, and labeled "suitable for chromatography" was used for column chromatography of lipids after activation at 110° C for 3 hours. Silica gel G (Research Specialties Company, Richmond, California) was used for thin-layer chromatography. A cation exchange resin of the sulfonic acid type (AG50-X4, BioRad Laboratories, Richmond, California) was used in column chromatographic separations of

dinitrophenylhydrazones. This resin (H^+ form) was washed with carbonyl free methanol and then a mixture of carbonyl free methanol and carbonyl free benzene (1:1) prior to its use in column chromatography. Sephadex G10, G15 and G25 (Pharmacia, Uppsala, Sweden) equilibrated with methanol were also used for column fractionation of dinitrophenylhydrazones.

Enzymes

Cobra venom (Naja naja) or rattle snake venom (Crotalus adamanteus) obtained from the Ross Allen Reptile Institute, Silver Springs, Florida, were used as a source of phospholipase A (α-phosphatide-β-acylhydrolase). Purified glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate : triphosphopyridine nucleotide oxidoreductase) was obtained from Sigma Chemical Company, St. Louis, Missouri. Purified D-isocitric dehydrogenase (D-isocitrate : triphosphopyridine nucleotide oxidoreductase) was obtained from Cal Biochem, Los Angeles, California.

Instruments and Equipment

Spectrophotometric measurements were made either using a Beckman DU or Beckman DU-2, from Beckman Instruments Company, South Pasadena, California. Absorption spectra were recorded automatically using a Cary Model 14 Recording Spectrophotometer, Applied Physics Corporation, Pasadena, California. Centrifugations were done using an International Refrigerated Centrifuge, International Equipment Company, Boston, Massachusetts and a Spinco Model L Ultracentrifuge, Beckman Instruments Company, Spinco Division, Palo Alto, California. Enzyme incubations were sometimes carried out in a Dubnoff Shaker equipped with a constant temperature water bath, Precision Scientific Company, Chicago, Illinois.

Organic solvents were removed from lipid samples by use of a Rotating Evaporator obtained from Calab, Berkeley, California. Fatty acid methyl esters were quantitated using a Perkin-Elmer Model 226 Gas Chromatograph, Perkin-Elmer Corporation, Norwalk, Connecticut.

Methods

Preparation of Rat Liver Microsomes

Male rats (180-250 g) were stunned by a blow on the head and exsanguinated by severing the neck vessels. The liver was removed, chilled with cold potassium phosphate buffer (0.15 M, pH 7.5), blotted dry and weighed. Five milliliters of cold phosphate buffer (0° C) was added to each g of liver and then homogenized using a homogenizer of the Potter-Elvehjem type. The homogenate was centrifuged in a refrigerated centrifuge at 8000 x g for 15 minutes to sediment cellular debris, nuclei, and mitochondria. The supernatant fraction so obtained was then centrifuged for 90 minutes in a 30 rotor at 30,000 rpm (105,000 x g) in a Spinco Model L Ultracentrifuge in order to sediment the microsomes. The microsomal pellet was resuspended in the same volume of cold phosphate buffer and recentrifuged for 60 minutes at 30,000 rpm. This washing process was repeated once. The buffer was drained from the final microsomal pellet and the pellet was stored at -20° C. 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 g 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 thiobarbituric acid (TBA) chromogen in the control incubation systems.

Assay of Microsomal TPNH-Oxidizing and TBA Chromogen-Forming Systems

Assay of TPNH oxidation. Although the exact nature of the enzyme reaction is not known the following partial reaction can be written:

$$TPNH + H^{+} \xrightarrow[\text{microsomes}]{O_2,\ ADP\text{-}Fe^{+3}} TPN^{+}$$

TPNH oxidation measurements are based on the fact that TPNH has an absorption maximum at 340 mμ while TPN absorbs very little at this wave length. Thus a decrease in absorption at 340 mμ can be used as a measure of TPNH oxidation (39). The TPNH oxidase system under study functions when microsomes, TPNH, and ADP-Fe^{+3} complex are incubated at pH 7.5. Although any of these parameters can be varied a typical assay mixture contained per ml: 0.1 ml of microsomes, 0.3 μmoles TPNH, 4.0 μmoles ADP-Fe^{+3} (ADP/Fe^{+3} is 333/1) and 0.9 ml of tris-HCl buffer (0.1 M, pH 7.5). One milliliter systems were incubated in 10 ml beakers at 37° C in air. The enzyme reaction was terminated by the addition of 3 ml of ethyl alcohol. After termination of the enzyme reaction the mixture was placed in an ice bath for at least 30 minutes in order to precipitate fully all interfering materials that absorb at 340 mμ. The samples were centrifuged and the optical density of the solution was determined at 340 mμ. Measurement of the absorption of an equivalent amount of TPNH not incubated with the microsomal system permits one to calculate the TPNH oxidized as follows:

$$\mu\text{moles TPNH oxidized} = \frac{(OD_{340} \text{ before incubation} - OD_{340} \text{ after incubation})}{\epsilon}$$

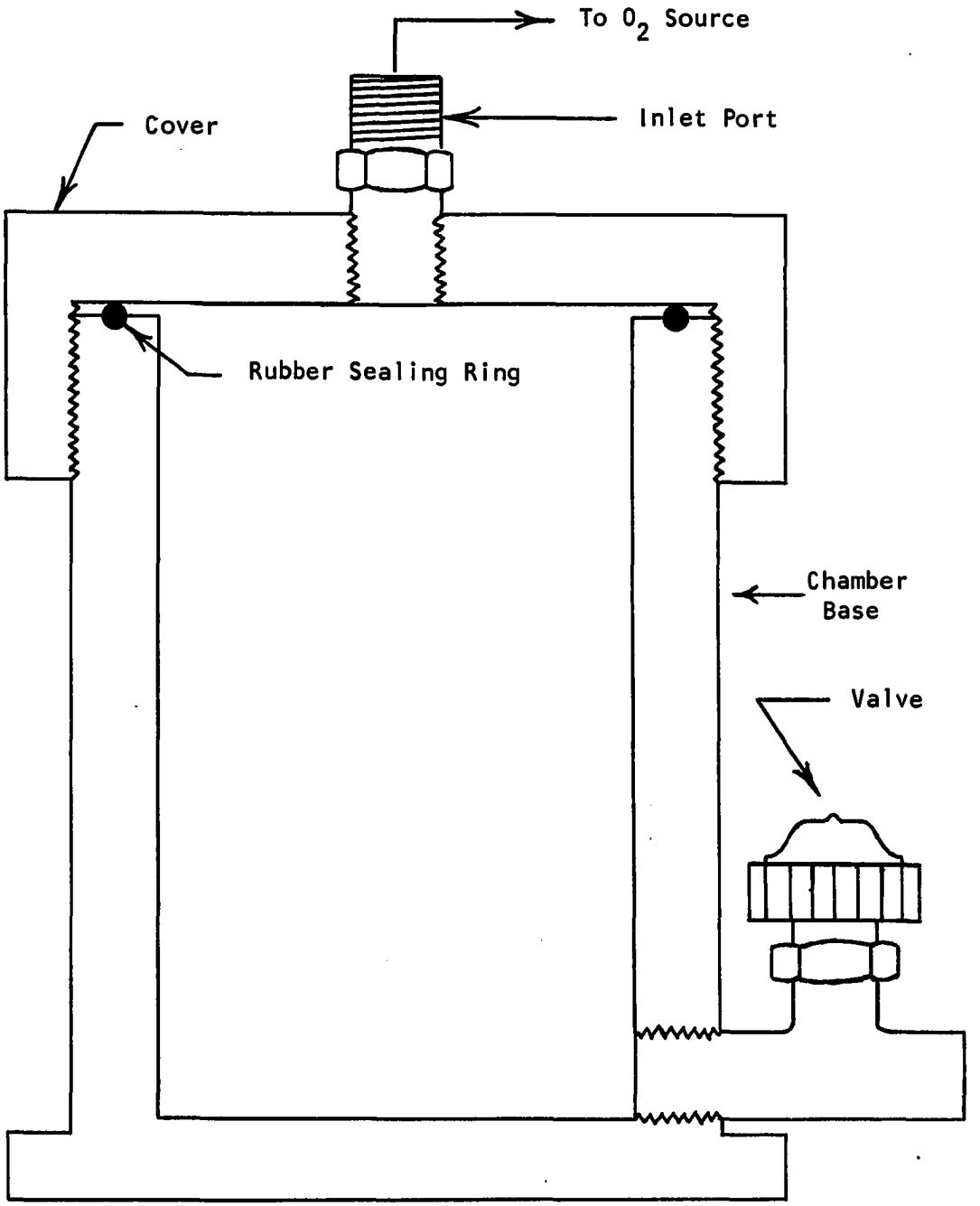
where OD_{340} is the optical density at 340 mμ and ϵ is the OD_{340} if 1 μmole of TPNH is dissolved in 1 ml.

Assay of formation of TBA chromogen. A typical assay mixture contained per ml: 0.1 ml of microsomes, 4.0 μmoles ADP-Fe^{+3} and 0.9 ml of tris-HCl buffer (0.1 M, pH 7.5) were incubated with (Experimental) and without (Control) 0.3 μmoles of TPNH at 37° C in air. One milliliter incubations were carried out in 10 ml beakers. The enzyme reaction is terminated by the addition of 0.5 ml of 35% (weight/volume; W/V) trichloroacetic acid (TCA). One milliliter of 0.75% TBA (W/V) was added and the mixtures were heated in a boiling water bath for 15 minutes according to the method of Ottolenghi (40). After cooling, 1 ml of 70% TCA (W/V) was added to each tube and swirled gently. The samples were then centrifuged and the optical density of the clear pink supernatant was determined at 532 mμ. In many instances sample colors were too dense to be read directly and were first diluted with an acid dilution mixture with the same composition as that of the sample. Samples that were turbid were extracted with chloroform (to remove the lipid turbidity) and the optical density of the clear aqueous layer was determined.

Incubations carried out at hyperbaric oxygen pressures. Incubations carried out at oxygen pressures greater than one atmosphere were performed in the specially constructed stainless steel chamber shown on page 16.

Injection of Animals with Sodium Phenobarbital

In some experiments male rats were given daily injections of sodium phenobarbital intraperitoneally. Doses of 100 mg per kg body weight were given per injection according to that used by Orrenius (15). The number of daily injections varied from experiment to experiment.



Stainless Steel Hyperbaric Pressure Chamber

Large Incubation Systems for Lipid Analyses

Large (10 to 300 ml) control (without TPNH) and experimental (with TPNH) incubation systems were used when lipid analyses were carried out. (Lipid phosphorus in 10 ml incubation systems ranged from 300 to 500 μg). Plastic cups 3 inches in diameter were used when incubations were carried out at 5 atmospheres. Not more than 40 ml was incubated at one time in these vessels in order to insure complete equilibration of the gas phase with the liquid phase. Forty milliliter enzyme systems were incubated separately and pooled for extraction when very large amounts of lipid were required. When air was used as the gas phase Erlenmeyer flasks were used for the larger incubation volumes.

Extraction of Lipids from Microsomal Systems

The method of extraction used was that of Folch, Lees, and Sloane-Stanley (41). After the enzyme incubation was completed, twenty volumes of chloroform-methanol (2:1, V/V) was added to each volume of the microsomal incubation system. The resulting mixture was single-phased with respect to the solvents and was permitted to stand at least 15 minutes with occasional shaking to insure complete lipid extraction. When 0.2 volumes of aqueous sodium chloride solution (0.5% W/V) was added and the mixture was shaken, two liquid phases resulted, one of which was primarily chloroform (referred to hereafter as the chloroform layer) and the other was approximately 60% methanol in water (referred to hereafter as the water-methanol layer). After phase separation the two layers were isolated and treated further according to the design of the experiment. The chloroform layer, which contained the lipids, was usually evaporated to dryness and the lipid residue was redissolved

in a small but known volume of chloroform and stored in the refrigerator under nitrogen until the lipid was analyzed.

Chemical Analyses of Lipids

General considerations. All chemical analyses were done in duplicate. Appropriate standards with known concentrations were assayed with each set of samples.

Determination of lipid phosphorus. The chloroform was removed *in vacuo* from the lipid samples containing 4 to 60 μg of lipid phosphorus. The sample was mineralized in sulfuric (1.2 ml of 5 N) and nitric acids (2 drops). The resulting inorganic phosphate was then determined by the method of Fiske and SubbaRow (42).

Determination of ester groups. Lipid ester groups were determined by the method of Stern and Shapiro (43), as modified by Antonis (44). Chloroform was removed from the sample and the ester (0.3 to 3.0 μmoles) was treated with alkaline hydroxylamine. The resulting hydroxamic acid was determined as a ferric complex which absorbed at 520 mμ. The intensity of the color complex was compared with that developed by standard triolein.

Determination of lipid amino nitrogen. The lipid amino nitrogen content was measured by the method of Lea and Rhodes (45). Ninhydrin (200 mg) was dissolved in 5 ml of ethyleneglycol monomethylether (EGME) and stannous chloride (8 mg) was dissolved in 5 ml of citrate buffer (0.2 M, pH 5.0). Equal volumes of the ninhydrin and stannous chloride solutions were mixed (reagent I). The dry lipid sample (1.0 to 1.5 μg

lipid amino nitrogen) was dissolved in 0.5 ml of EGME and 0.5 ml of reagent I was added. Color was developed by heating the resulting mixture in a boiling water bath for 20 minutes. The mixture was cooled, 3 ml of EGME was added and the optical density at 570 mμ was measured. The quantity of amino nitrogen was determined by comparison with known quantities of standard ethanolamine hydrochloride.

Determination of α-tocopherol. α-Tocopherol was determined by the method of Emmerie and Engel (46) which is based on the reduction of ferric ion by tocopherol to ferrous ion. Ferrous ion is then determined as the red α,α-dipyridyl complex. An aliquot of the total lipid extract (chloroform layer) was evaporated to dryness in vacuo. The residue was taken up in acetone and cooled in a dry ice-alcohol bath to precipitate the phospholipids. The cold acetone was separated from the precipitate by a Pasteur pipette and the precipitate was washed twice with cold acetone (-40° C). The acetone fractions were combined, evaporated to dryness, and taken up in 1.2 ml of absolute ethanol. Addition of 0.05 ml of 0.2% (W/V) ferric chloride solution and 0.05 ml of 0.5% (W/V) α,α-dipyridyl solution was followed by color development in the dark for 10 minutes. The color intensity at 540 mμ was determined immediately and compared with a standard curve prepared with authentic α-tocopherol.

Determination of unsaturation in lipids by bromination. This method is based on the quantitative bromination of lipid carbon to carbon double bonds according to the method of Trappe (47). Samples containing 30 μg of lipid phosphorus were dissolved in 1 ml of

chloroform. Two milliliters of 0.01 N bromine solution (in saturated NaBr) was added and the mixture was placed in the dark 4 to 16 hours. The excess bromine was determined as free iodine liberated upon the addition of 1 ml of 2% (W/V) aqueous potassium iodide. The iodine was titrated with 0.005 N sodium thiosulfate. The difference between the iodine liberated by the bromine reagent in the absence and presence of lipid is a measurement of double bonds in the lipid. Triolein was used as a standard to check the accuracy of each set of measurements.

Determination of unsaturation in lipids by catalytic hydrogenation. Reduced palladium-charcoal catalyst was prepared by incubation of palladium-charcoal with hydrogen gas in a Warburg vessel until no more hydrogen was taken up. A known quantity of lipid (5 to 10 mg) dissolved in methanol was placed in the side arm of a Warburg vessel and 25 mg of the reduced catalyst was suspended in 1.5 ml of methanol and placed in the main compartment of the vessel. The vessel was gassed with hydrogen and equilibrated at 37° C until hydrogen uptake had ceased. The lipid sample was then tipped into the catalyst compartment and the hydrogen uptake was permitted to go to completion. The ratio of μmoles of hydrogen uptake to μmoles lipid phosphorus was used as an index of lipid unsaturation. The same method of hydrogenation was used to prepare saturated methyl esters for gas chromatographic studies of methyl esters derived from microsomal lipids.

Preparation of 2,4-Dinitrophenylhydrazones

The chloroform layer (15 ml) from the Folch extract was treated with 4 mg of 2,4-dinitrophenylhydrazine. After standing 1 hour at 25° C

the chloroform was removed in vacuo and the residue was taken up with 0.5 to 1.0 ml of methanol-benzene (1:1) or methanol and chromatographed on BioRad AG50W-X4 or Sephadex G10 as described previously. The hydrazones of the water-methanol layer from the Folch extract were prepared similarly. The hydrazones were extracted from the water-methanol solution with chloroform (usually four 50 ml portions) until the water-methanol layer was colorless. The chloroform was then evaporated and the hydrazones were dissolved in the appropriate solvent and chromatographed as above.

Treatment of Microsomal Lipids with Snake Venoms

Cobra and rattle snake venom were used as sources of phospholipase A which hydrolyzes the β fatty acid of phosphatidyl ethanolamine and phosphatidyl choline (48) resulting in the formation of lysophosphatides and free fatty acids. One milligram of lyophilized venom was dissolved in 1 ml of 0.005 M calcium chloride solution. Dry lipid containing 60 μg of phosphorus was dissolved in 5 ml of peroxide free ether and 0.01 ml of venom solution was added. The lysophosphatides precipitated at room temperature in 2 to 24 hours depending on the lipid source. The lysophosphatides were separated from the ether soluble free fatty acids either by means of a Pasteur pipette after centrifugation or by fractionation of a chloroform solution of the entire mixture on a small silicic acid column. Completeness of the venom action was monitored by thin layer chromatography.

Column Chromatography

Silicic acid column chromatography. Silicic acid (49) was used to

separate neutral lipids from phospholipids and also to fractionate microsomal phospholipids derived from both control and experimental incubation systems. Silicic acid was dried for 3 hours at 110° C prior to its use. Not more than 25 mg of lipid per g silicic acid was used. Activated silicic acid was slurried in chloroform and packed in a column of appropriate size. (For example, 0.4 X 4.0 cm columns were used to separate neutral lipids from phospholipids containing 0.3 mg of lipid phosphorus and 1.0 X 10.0 cm columns were used to fractionate lipid mixtures containing 7.0 mg of lipid phosphorus). Flow rates of 1 to 2 ml per minute were used. After the lipids were placed on the column in a small volume (1 to 5 ml) of chloroform the neutral lipids were eluted from the column with chloroform (at least 10 column volumes) until no lipid material could be detected in several successive fractions by charring with concentrated sulfuric acid. Phospholipids were eluted with successively increasing concentrations of methanol in chloroform as shown in each individual experiment. Phospholipid elution patterns were followed by determining lipid phosphorus and lipid amino nitrogen in successive fractions eluted from the column. In cases where comparisons of elution patterns of lipids derived from control and experimental incubation systems were concerned, similar chromatographic conditions such as amount of lipid fractionated, column size, elution solvent concentrations and volumes, flow rats etc., were maintained as nearly as possible. Recoveries of lipid phosphorus from columns ranged from 89 to 96%.

Column chromatography of 2,4-dinitrophenylhydrazine derivatives of lipids on BioRad AG50W-X4 cation exchange resin. This cation exchange resin is a Dowex 50W-X4 resin (200-400 mesh) which was treated by BioRad

Laboratories to remove a large part of the color and was used by Schwartz et al. (38) to remove free 2,4-dinitrophenylhydrazine (DNPH) from 2,4-dinitrophenylhydrazones. A small column (0.4 X 6.0 cm) of this resin will retain at least 8 mg of DNPH (freshly recrystallized from carbonyl free benzene) while the 2,4-dinitrophenylhydrazone of stearaldehyde is quantitatively recovered. If the DNPH was not freshly recrystallized, yellow impurities were eluted from the column under the conditions used.

A slurry of the resin was prepared in water and packed into the column. The packed resin was washed with 4 bed volumes of carbonyl free methanol and then with 2 bed volumes of benzene-methanol (1:1, V/V), both carbonyl free. The sample was applied in a minimun volume of the latter solvent mixture and the hydrazones were eluted at a flow rate of up to 3 ml per minute in about 5 bed volumes or until the effluent was colorless (38). Some phospholipid bound hydrazones, however, did not elute under these conditions and required the addition of acid to the eluting solvent mixture. It was found that elution of the column with benzene-methanol (1:1) made 0.6 N with concentrated HCl eluted lipid phosphorus quantitatively. An estimation of the quantity of dinitrophenylhydrazones eluted was obtained by determining the ultraviolet absorption at 360 mμ and assuming a millimolar extinction coefficient of 22.0 (38).

$$\mu\text{moles dinitrophenylhydrazones} = \frac{\text{ml soln X } OD_{360}}{22.0}$$

Sephadex chromatography of 2,4-dinitrophenylhydrazine derivatives.

The basis of separations on Sephadex, a polydextran, is that of molecular

sieving. Sephadex G10 or G15 was slurried in methanol and permitted to swell for 3 hours. The Sephadex was poured into the column and packed under atmospheric pressure in the presence of methanol. We have found that free DNPH separates from the dinitrophenylhydrazones of stearaldehyde and acetone. The sample was applied to the top of the column (1.0 X 15.0 cm) in a minimum volume of methanol (0.5 to 1.0 ml) and fractions were eluted with methanol at a flow rate of 10 ml/hour. The yellow bands were collected and estimations of the quantities of hydrazones were made as described above.

Thin Layer Chromatography (TLC)

Thin layer chromatography on silicic acid was used to compare the characteristics of lipids from control and experimental incubation systems, to follow fractionation procedures and also to detect chemical and enzymic modifications of microsomal lipids. In general, two kinds of separations were effected in this study by TLC, namely, neutral lipid separations and phospholipid separations. Silica gel G was slurried in water (20 g/50 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. Microsomal lipid containing 7 to 10 μg of lipid phosphorus was spotted in a compact spot (3 mm) for phospholipid plates and lipid containing 0.6 to 1.0 μmoles of ester per spot was used for neutral lipid plates. Development of the plates was then carried out in one of the following solvent systems: chloroform-methanol-ammonia (75:25:4) was used for phospholipid analyses (50) and petroleum ether-diethyl ether (90:10) or (70:30) was used for neutral lipids (51). After the solvent front had moved 10 cm the plate was removed, air dried

and sprayed with one or several of the spray reagents listed below, depending on the analysis of interest. Lipid standards were run on each plate for positive identification of sample lipids.

Spray Reagents for Thin Layer Chromatography

Rhodamine-6-G-reagent. Rhodamine-6-G (0.005%; W/V) in 95% ethanol was sprayed on neutral lipid plates and observed under ultraviolet light after air drying (52). Absorbing and fluorescing spots were marked.

Ninhydrin reagent. Five-tenths percent (W/V) ninhydrin in n-butanol was used to detect amino containing phospholipids and was usually the first spray used on phospholipid plates. After spraying, the color was developed in an oven at 110° C for 5 to 10 minutes and the pink amino containing spots were marked.

Modified Zinzadze molybdenum reagent. This reagent (53) was prepared in the following manner: Solution I: 1 liter of 25 N sulfuric acid and 40.1 g of molybdenum oxide were boiled gently until the molybdenum oxide was dissolved. Solution II: 500 ml of solution I and 1.78 g of powdered molybdenum were boiled gently for 15 minutes. Equal volumes of solutions I and II were mixed and then added to two volumes of water. This reagent gives an immediate blue color if phosphorus is present. The blue spots were marked and then charring of most organic compounds was accomplished by heating the plate at 110° C for 10 to 15 minutes. This spray reagent was used for charring neutral lipid and phospholipid TLC plates.

Preparation of Methyl Esters

Methyl esters were prepared by either transmethylation under mild

alkaline conditions (54) or by use of a boron trifluoride-methanol reagent (55). The transmethylation consisted of dissolving the dry lipid (300 μg of lipid P) in 0.5 ml of chloroform, addition of 0.5 ml of 0.1 N methanolic sodium hydroxide and heating 20 minutes at 37° C. Then one drop of ethyl formate was added to destroy excess alkali. After the addition of 0.5 ml of distilled water the esters were extracted from the mixture with three 5 ml portions of ether. Methyl esters were also prepared by addition of 1 ml of boron-trifluoride-methanol reagent to a dry lipid sample (300 μg of lipid P) and heating for 15 minutes in a boiling water bath. One milliliter of water was added and the esters were extracted with two 2 ml portions of n-hexane.

Gas-Liquid Chromatographic Analysis of Methyl Esters

Methyl esters derived from the total lipid or fractionated lipid from microsomes were analyzed qualitatively and quantitatively by gas-liquid chromatography (GLC). Ester separations were effected on packed polyester columns such as butanediol succinate or diethyleneglycol succinate. A standard mixture of esters with known composition was run periodically and under instrument conditions identical to those used for samples. Esters in the sample were tentatively identified by comparison of their retention times with those of the standard ester mixture. Further confirmation of the identity of sample esters was obtained after catalytic hydrogenation of the sample. Peaks of unsaturated esters disappeared after hydrogenation and there were concurrent increases in the corresponding saturated esters with the same chain lengths. Since the composition of fatty acids in microsomal lipids is simple, identification of the fatty acids offered no difficulty. Typical

instrument conditions were as follows:

Column:	Butanediol succinate polyester, 8% liquid phase, 6 ft packed column on Chromsorb W. Diethyleneglycol succinate polyester, 13%, 6 ft packed column on Gas Chrom P.
Initial column temparature	190° C
Flash heater temperature	275° C
Flame ionization detector	200° C
Carrier gas:	Helium at a constant flow rate. eg. 60 ml/minute.
Temperature programming of column:	Program from 190° C to 210° C at 4° per minute. Standards run under same programming conditions.

CHAPTER III

RESULTS

Characteristics of the Enzyme System

The basic parameters of the TBA chromogen-forming system were studied in an effort to obtain maximum enzyme activity and thus enhance the opportunity for finding lipid changes in the microsome. Microsomes (0.1 ml, equivalent to 100 mg liver, wet weight) were incubated in the presence of 4 mM ADP-Fe^{+3} (ratio of ADP to Fe^{+3} is 333 to 1) in air at 37° C in the presence of varying concentrations of TPNH. The reaction was carried out in a Dubnoff apparatus. It is shown in Table 1 that the concentration at which TPNH had its maximum effect was dependent on whether a TPNH-regenerating system (glucose-6-phosphate dehydrogenase, G-6-PDH) was present or not. Much lower levels of TPNH (12 μM) were required for the maximum rate of TBA chromogen production when the TPNH-generating system was added. This effect is not surprising since TPNH was rapidly oxidized by microsomes under these incubation conditions. Within 10 minutes (Figure 1) all TPNH was oxidized in the absence of the TPNH-generating system and the chromogen formation stopped abruptly. In the presence of the G-6-PDH system the TPN remained virtually fully reduced throughout the time course of the reaction and the chromogen formation continued until it finally reached a plateau even though TPNH was still present. A maximum value of chromogen formed was reached

TABLE I

EFFECT OF TPNH CONCENTRATION ON THE RATE OF TBA CHROMOGEN PRODUCTION

TPNH (μM)	OD_{532}/5 minutes	
	With TPNH-gen. syst.	Without TPNH-gen. syst.
0.6	0.54	----
1.2	0.60	----
3.0	0.78	----
6.0	1.07	----
12.0	1.58	----
30.0	1.60	----
60.0	1.60	0.65
150.0	1.80	1.04
300.0	----	1.64
600.0	----	1.66

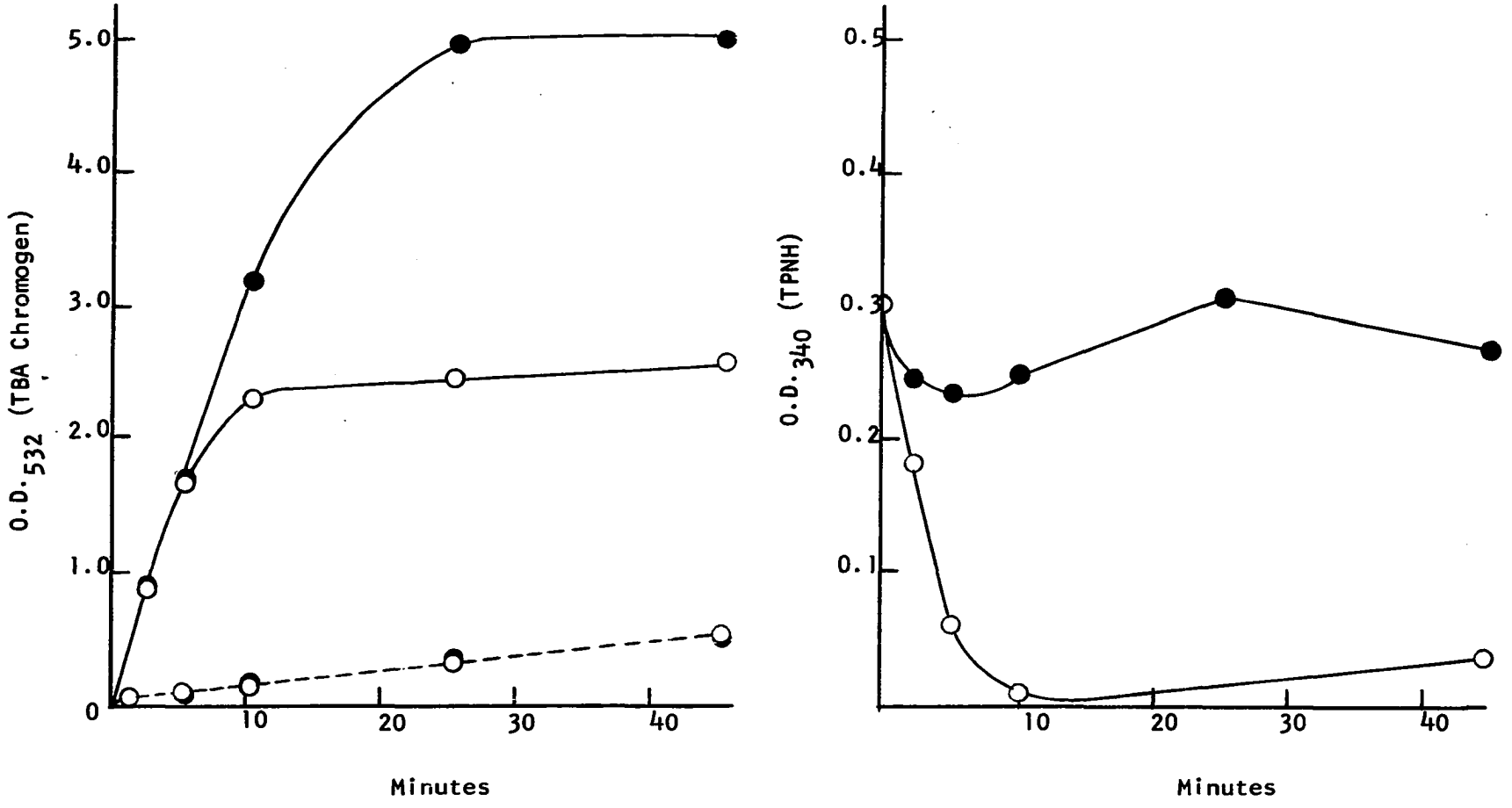


Figure 1. The effect of a TPNH-generating system on the production of TBA-chromogen and on the TPNH levels of microsomal systems incubated in the presence of ADP-Fe^{+3}. O—O 0.1 ml microsomes incubated in the presence of 0.3 mM TPNH, 4 mM ADP-Fe^{+3} in tris buffer (0.1 M, pH 7.5) to a total incubation volume of 1 ml. Incubated in air at 37° C; ●—● represents the same incubation system but with 6 mM glucose-6-phosphate and 0.5 units of purified glucose-6-phosphate dehydrogenase; dashed line represents systems in which TPNH was omitted.

under each set of experimental conditions tested.

The effect of the quantity of microsomes on the production of TBA chromogen is shown in Figure 2. The rate of chromogen synthesis and the maximum quantity of chromogen that can be synthesized are proportional to the quantity of microsomes used (1 to 3 mg protein).

An increase in the oxygen pressure applied to the enzyme system increased the rate and maximum quantity of chromogen formed (Table 2).

TABLE 2

THE EFFECT OF OXYGEN PRESSURE ON THE RATE OF TBA CHROMOGEN FORMATION

Oxygen Pressure (Atm)	Incub. Time (Min)	OD_{532}/ml Incub.	
		Experimental	Control
0.2	7	0.94	----
0.2	15	1.69	0.06
0.2	30	2.22	0.08
5.0	7	2.16	----
5.0	15	3.92	0.16
5.0	30	5.53	0.53

Application of 5 atomospheres oxygen pressure (in a specially constructed stainless steel chamber pictured on page 16) doubled the total amount of chromogen formed by the complete system. This higher oxygen pressure also increased the amount of chromogen formed in the control system (without TPNH). However, there was still more than a 10 fold difference between the control and experimental incubation systems.

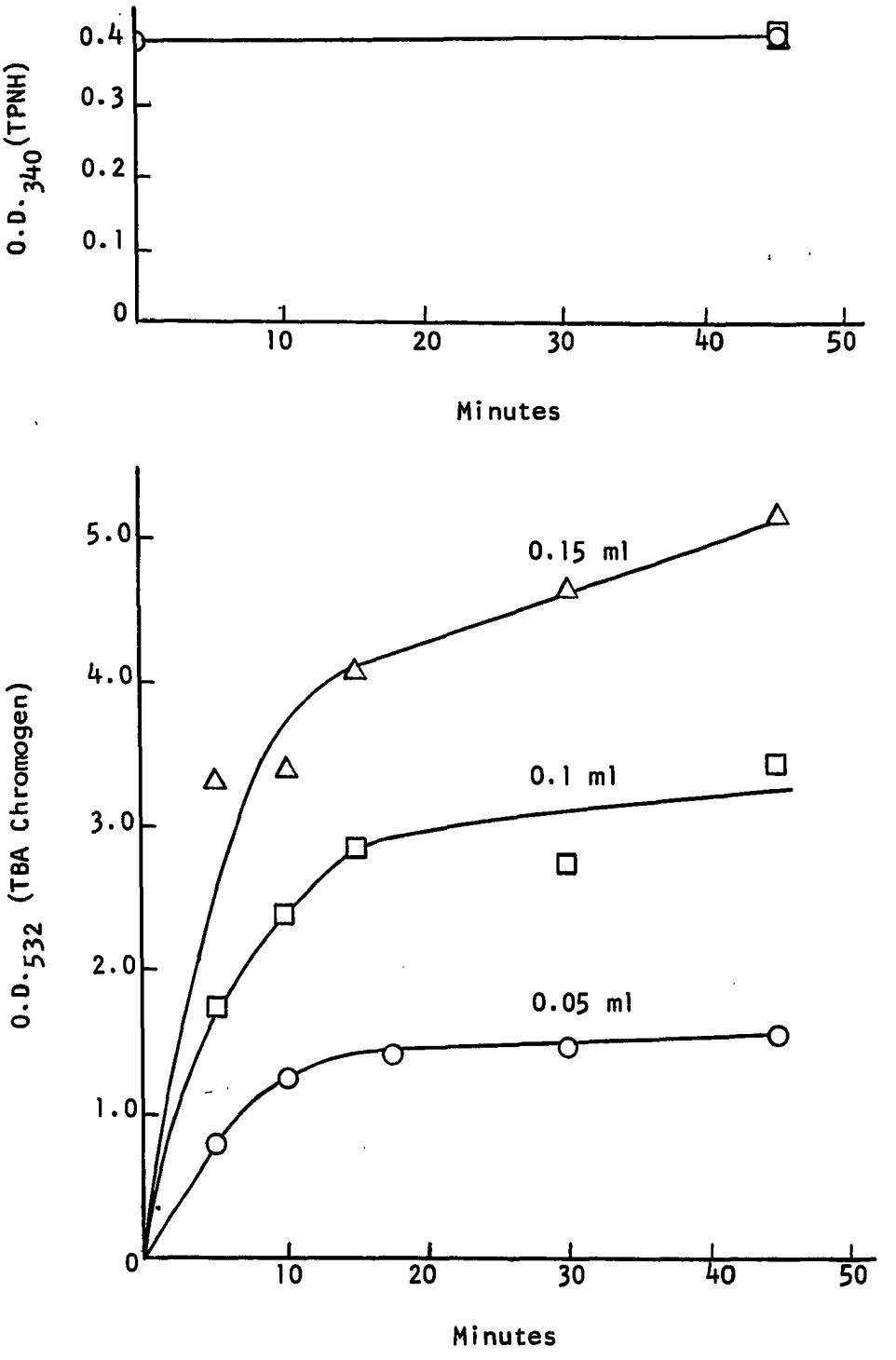


Figure 2. Effect of the quantity of microsomes on the rate and the maximum amount of TBA chromogen produced. Incubation systems contained microsomes, 4 mM ADP-Fe^{+3}, 0.3 mM TPNH, 6 mM G-6-P, 0.5 units G-6-PDH in a 1 ml incubation volume. Incubation in air at 37° C. ○, 0.05 ml microsomes; □, 0.1 ml microsomes; △, 0.15 ml microsomes.

The enzyme system was found to be very easily inactivated by heat. When microsomes were heated for 30 seconds at 65° C, the chromogen-synthesizing and the TPNH oxidase activities were completely lost (Table 3). Significant decreases of these activities occurred with only 15 seconds heating of the microsomes at 65° C.

TABLE 3

HEAT INACTIVATION OF TPNH OXIDASE-LINKED TBA CHROMOGEN FORMATION

Microsome Pretreatment	Incubation Time	TBA Chromogen (OD_{532})		TPNH Oxid. Δ OD_{340}
		Control	Experimental	
None	0	0.03	----	----
None	5	0.04	1.62	0.26
None	10	0.05	2.35	0.31
Heat 15 sec. 65°	0	0.07	----	----
"	5	0.09	0.43	0.21
"	10	0.06	1.97	0.12
Heat 30 sec. 65°	0	0.05	----	----
"	5	0.07	0.12	0.03
"	10	0.11	0.11	0.04
Heat 60 sec. 65°	0	0.04	----	----
"	5	0.12	0.13	0.01
"	10	0.15	0.12	0.03

The effect of varying the incubation temperature on TBA chromogen synthesis is shown in Figure 3. Even at 0° C the chromogen was formed

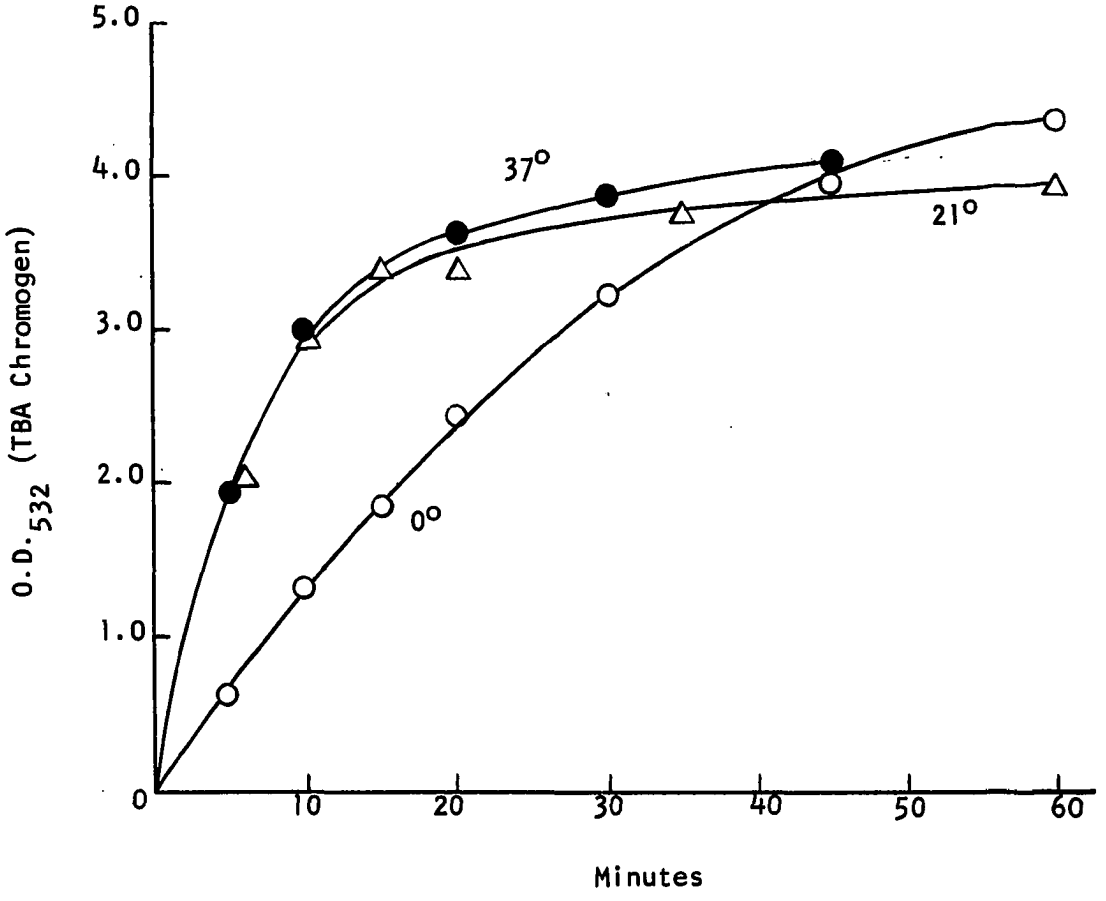


Figure 3. Effect of incubation temperature on TBA chromogen synthesis. Incubation medium same as Figure 1 with TPNH-generating system present.

at about one-half the rate at 21° or 37° C. The maximum quantity of chromogen formed was the same for all three temperatures.

Microsomal Lipid Changes During the Enzymic Oxidation of TPNH

Conditions that gave the maximum quantity of TBA chromogen were selected in order that lipid changes, if they occurred, could be detected. The following incubation systems were used: Control: 0.1 ml microsomes per ml incubation system, 4 mM ADP-Fe^{+3} in tris-HCl buffer (0.1 M, pH 7.5); Experimental: same as control but with 0.3 mM TPNH present. Incubations were carried out at 5 atmospheres oxygen pressure (except where specified otherwise) for 45 minutes at 25° C. Incubation systems ranged from 10 to 300 ml depending on the amount of lipid needed for the analyses. Ten milliliter incubation systems usually contained about 300 μgrams of lipid phosphorus. After incubation the lipid was isolated by the Folch (41) method. All analyses reported in this section were done on the lipid-containing chloroform layer of the Folch extract. Hereafter materials originally derived from control or experimental incubation systems will be referred to as such, e.g. control lipid, control phosphatidyl choline, experimental lipid, etc.

Thin Layer Chromatographic Analysis

Equivalent quantities of lipid phosphorus from the sample lipids as well as the lipid standards were applied on the TLC plates. After development of the phospholipid plates in $CHCl_3$:MeOH:NH_4OH (75:25:4) the amino-containing lipid spots were located by spraying with ninhydrin reagent and then all phosphorus-containing spots were located by the Zinzadze reagent. A typical analysis is given in Figure 4. Marked

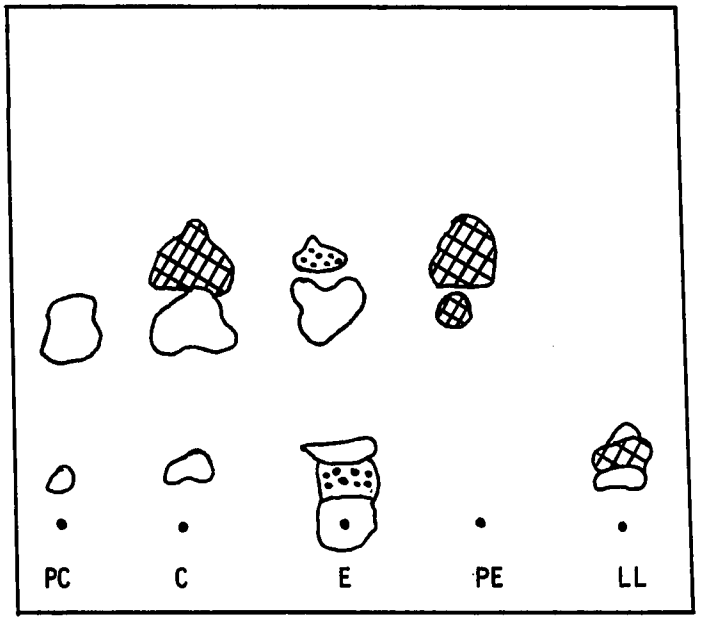


Figure 4. Thin layer chromatographic behavior of lipid extracted from control and experimental incubation systems. Control (C): 1.0 ml microsomes, 4 mM ADP-Fe^{+3} and tris buffer (0.1 M, pH 7.5) to a total of 10 ml. Incubation 5 atmospheres oxygen pressure 45 minutes at 25° C. Experimental (E): same as control but with 0.3 mM TPNH. Solvent system: $CHCl_3$:MeOH:NH_4OH(75:25:4). (PC) Phosphatidyl choline, (PE) Phosphatidyl ethanolamine, (LL) mixture of lysophospholipids. Shaded spots were ninhydrin positive, stippled spots were faintly ninhydrin positive. Only phosphorus positive spots are shown.

losses of phosphatidyl ethanolamine occurred in the experimental lipid. There was also a slow-moving, presumably polar, material present in the lipid from experimental systems which was absent in the control lipid. Some of the slow-moving material was faintly ninhydrin positive indicating the presence of altered amino-containing phospholipid.

Functional Group Analysis

Lipid from control and experimental incubation systems was analyzed for various functional groups. Differences between experimental and control lipid in the ester/P, double bond/P, and amino N/P ratios were used as indexes of the change in the chemical nature of experimental lipid. Although TLC showed gross changes in the chromatographic behavior of the lipid from experimental incubation systems as compared to the control, there was no significant loss of extractable lipid phosphorus (Table 4). No losses in ester groups were observed although

TABLE 4

ANALYSES OF LIPIDS FROM CONTROL AND EXPERIMENTAL SYSTEMS

Lipid Analyzed	μg Lipid P / ml microsomes	Ester[a] / P	Double Bonds/P[a]		Amino N[a] / P
			Bromination	H_2	
Control	332	2.12	3.66[b]	3.44[c]	0.18
Experimental	315	2.02	2.58[b]	2.48[c]	0.12

a Ratios of total microequivalents in lipid extracts of incubation systems.
b Determined by bromination.
c Determined by catalytic hydrogenation.

about 30% of the double bonds (determined by both bromination and catalytic hydrogenation) and about 30% of the lipid amino nitrogen was lost from the experimental lipid (Table 4).

Fatty Acid Distribution

Methyl esters were prepared from the total microsomal lipid of control and experimental incubation systems and analyzed by gas-liquid chromatography. Table 5 shows that losses of the highly unsaturated fatty acids, arachidonic and docosahexenoic acids, amounted to 55 and 75% respectively. Catalytic hydrogenation of the methyl esters confirmed the identity of the arachidonic and docosahexenoic acids since the corresponding saturated esters with the same percent composition appeared in chromatograms of the hydrogenated samples (Table 5).

TABLE 5

FATTY ACID COMPOSITION OF TOTAL LIPIDS

Lipid Analyzed	Wt. Percent of Total Fatty Acids							
	16:0	18:0	18:1	18:2	20:0	20:4	22:0	22:6
Control	19.7	27.9	11.4	16.6	----	21.1	----	3.3
Experimental	25.4	34.5	13.6	16.4	----	9.3	----	0.9
Hydrogenated Control	23.0	48.1	----	----	24.1	----	4.4	----
Hydrogenated Experimental	36.6	54.9	----	----	8.9	----	Trace	----

Ester to phosphorus ratios indicated little or no hydrolytic action by microsomes under these conditions. However, a small amount of selective ester hydrolysis could account for the fatty acid losses observed.

The soaps that would result from the hydrolytic cleavage of phosphatide ester bonds at pH 7.5 might not extract into chloroform. Acidic conditions would convert the soap to the free acid rendering it chloroform extractable. Thus duplicate control and experimental incubation systems were extracted under neutral (normally used) conditions and acidic conditions (pH 0.8). Table 6 compares the functional group analyses and the fatty acid distribution of lipids extracted under these conditions.

TABLE 6

LIPID ANALYSES COMPARING ACIDIC AND NEUTRAL EXTRACTIONS OF CONTROL AND EXPERIMENTAL SYSTEMS

	Neutral Extraction		Acidic Extraction	
Analyses	Control	Experimental	Control	Experimental
Total μg Lipid P	392	422	354	356
Amino N/P	0.28	0.19	0.28	0.27
C=C/P	3.96	2.94	3.94	1.94
Ester/P	2.05	2.27	2.01	1.90
Fatty Acid		Weight Percent		
16:0		25.6	13.9	24.2
18:0		32.8	25.7	36.7
18:1		14.7	10.0	15.0
18:2		16.1	16.2	15.7
20:4		9.4	24.2	7.5
22:6		2.1	9.9	1.0

The gross lipid analyses showed similar results between the two extraction pH's with the exception that under acidic extraction conditions the control and experimental amino N/P ratios were the same and there was a greater loss of double bonds from experimental lipid. The fatty acid composition of the experimental lipid was about the same for both extraction conditions. The control neutral extract was lost by accident, however, it was apparent that C20:4 and C22:6 were not destroyed under acidic extraction conditions. Arachidonic acid was a rather constant fraction (approximately 20%) of the total fatty acids distributed in liver microsomes from animals fed the commercial pellet diet. On the other hand, docosahexenoic acid was not as constant and varied from 3 to 9%.

Silicic Acid Column Chromatography

Large incubation systems (300 ml) were extracted in order to obtain enough lipid to fractionate by column chromatography. This experiment was designed to determine whether certain classes of phospholipids were selectively altered in this enzyme system. Phospholipids were eluted from the columns with successively increasing concentrations of methanol in chloroform (see Methods). The fractionation pattern of control lipid (Figure 5) and experimental lipid (Figure 6) showed some differences. In the experimental lipid, there was a loss of PE (about 50%, Table 7) and the appearance of larger quantities of materials which eluted with higher concentrations of methanol. These results agreed with the TLC data. Yellow-colored material remained at the top of the experimental column and could not be eluted with methanol, hot methanol, methanol-acetic acid, glacial acetic acid, tetrahydrofuran or

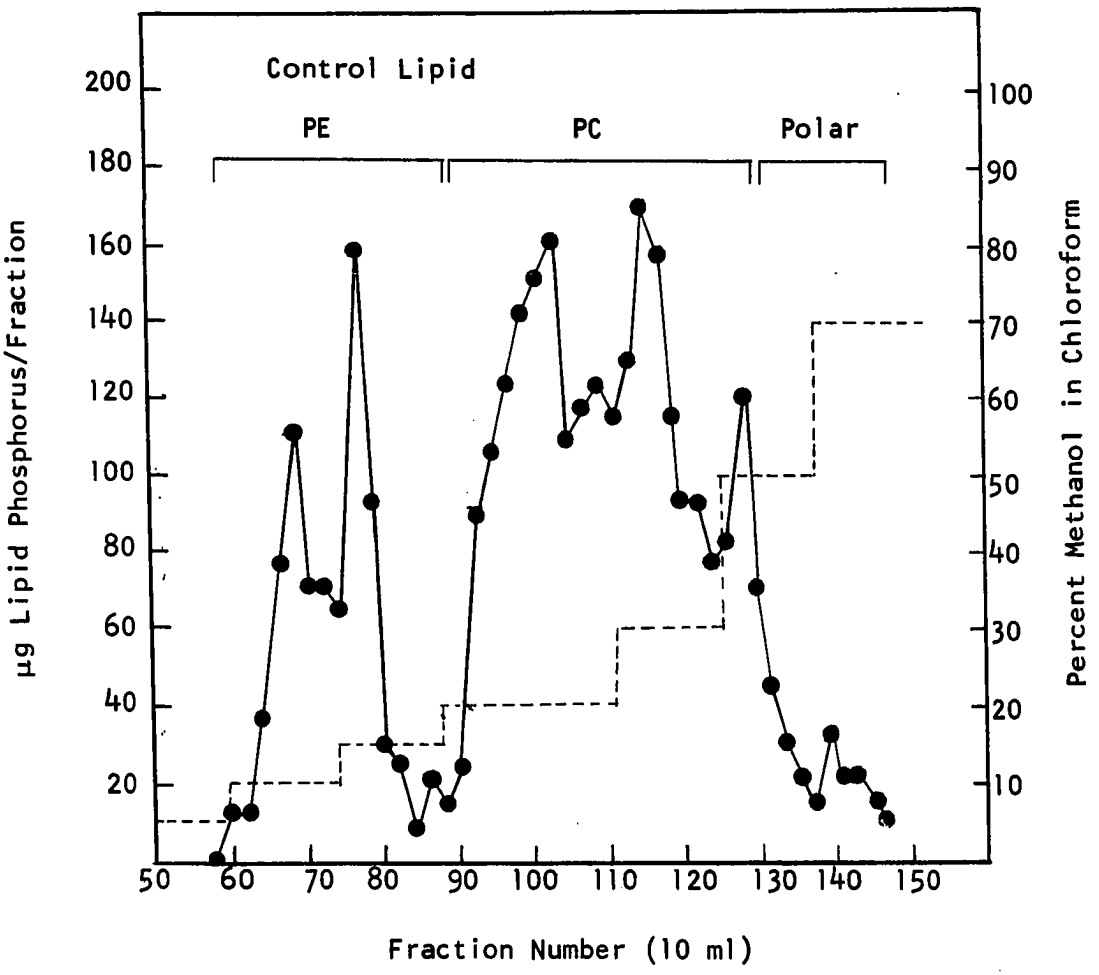


Figure 5. Column chromatography on silicic acid of lipid from control incubation systems. Solid line represents the lipid phosphorus profile and dashed line represents percent methanol in chloroform in elution solvent. Incubation system: 30 ml microsomes, 4 mM ADP-Fe^{+3} in tris buffer (0.1 M, pH 7.5) in a total of 300 ml. Incubated at 5 atmospheres oxygen pressure, 45 minutes at 25° C.

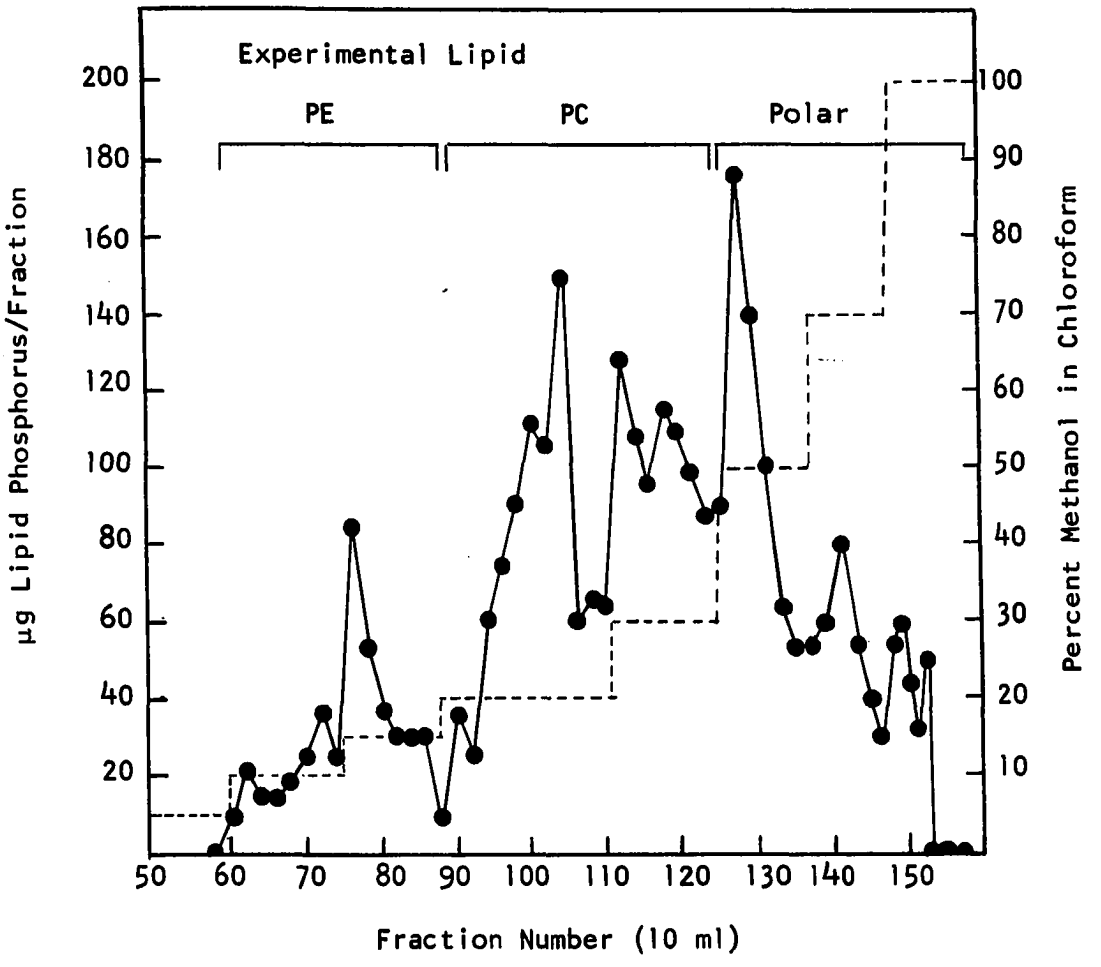


Figure 6. Column chromatography on silicic acid of lipid from experimental incubation system. Solid line represents the lipid phosphorus profile and dashed line represents percent methanol in chloroform in elution solvent. Incubation system and conditions same as given in Figure 5 except 0.3 mM TPNH was added.

TABLE 7

ANALYSES OF LIPIDS FROM CONTROL AND EXPERIMENTAL SYSTEMS AFTER FRACTIONATION ON SILICIC ACID

	Original Lipid		P. E.		P. C.		Highly Polar Materials	
	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Total Lipid P (μg)	226	226	53.8	27.4	149.2	104.6	15.3	64.5
% P Recovered	---	---	23.8	12.1	66.2	46.4	6.7	28.5
% of Total P Recovered	96.7	87.0	----	----	-----	-----	----	----
C=C/P	3.7	2.5	3.1	2.9	3.0	2.3	3.3	2.1
Fatty Acid				Weight Percent				
16:0	20.1	25.0	20.8	21.1	20.9	25.0	36.4	30.6
18:0	24.9	30.9	36.9	42.4	27.6	32.9	37.1	41.8
18:1	10.5	14.5	12.4	12.5	11.5	13.8	12.8	13.0
18:2	20.3	19.5	13.9	15.1	21.6	21.0	12.2	11.5
20:4	20.2	9.4	13.2	7.9	16.4	6.9	1.4	2.7
22:6	3.9	0.8	2.6	1.0	1.9	0.5	Trace	0.3

dimethyl formamide. The loss of this material was reflected by a lower recovery of lipid phosphorus from the experimental column (Table 7). Fractions from the columns were pooled taking into account the lipid phosphorus profile and the TLC analyses of the individual fractions. Table 7 shows the analyses of the pools, the sum of which represents all lipid phosphorus eluted from the columns. Phospholipid components present in relatively small amounts were pooled with the appropriate major component adjacent to them as shown in Figures 5 and 6. The distribution of lipid phosphorus confirmed quantitatively that which TLC had shown qualitatively (compare Table 7 with Figure 4). Analyses of methyl esters derived from the phosphatide classes, however, showed that losses of highly-unsaturated fatty acids occurred to about the same extent in the PE and PC fractions. Thus the selectivity of the enzyme-catalyzed alteration of microsomal lipid was toward the unsaturated fatty acids and not toward a particular phosphatide class.

Effect of Variation of Incubation Parameters on Lipid Changes

It was of interest to determine if marked lipid changes occurred at oxygen pressures in the physiological range. Control and experimental systems were incubated in air at 37° C and at 5 atmospheres oxygen pressure at 25° C. The lipid from each of these systems was isolated, analyzed and compared (Table 8). The total changes in experimental lipid from systems incubated in air at 37° C were at least as great as and sometimes greater than those occurring at 5 atmospheres.

It can be seen from Table 9 that the losses of arachidonic and docosahexenoic acids occurring at 0° C were similar to those occurring

TABLE 8

COMPARISON OF LIPID CHANGES OCCURRING IN SYSTEMS INCUBATED UNDER AIR AND UNDER 5 ATMOSPHERES OXYGEN PRESSURE

Analyses	Air		5 Atmospheres Oxygen	
	Control	Experimental	Control	Experimental
Total P (μg)	449	405	447	440
Amino N/P	0.29	0.15	0.28	0.18
C=C/P	4.65	2.91	4.50	3.20
Ester/P	2.10	1.89	2.08	2.12
OD_{532}/ml inc.	0.15	2.66	1.13	3.81
Fatty Acid			Weight Percent	
20:4	20.8	10.0	21.3	9.8
22:6	3.3	0.9	3.4	1.3

TABLE 9

VARIATIONS OF INCUBATION PARAMETERS AND THEIR EFFECTS ON FATTY ACID ALTERATIONS

Exp.	Incubation System	Temp. (° C)	Weight Percent					
			16:0	18:0	18:1	18:2	20:4	22:6
1	Basal*	0	20.0	24.5	8.9	16.8	23.0	6.8
	Basal + 300 μM TPNH + GS**	0	26.8	33.9	13.2	15.4	9.9	0.7
	Basal	37	18.4	25.9	9.4	17.2	22.3	6.8
	Basal + 6 μM TPNH + GS	37	25.7	33.9	13.3	16.7	9.2	1.1
2	Basal	37	20.8	23.4	11.7	16.9	19.8	7.5
	Basal - ADP-Fe^{+3} + 300 μM TPN + GS	37	23.1	26.0	14.4	18.4	14.8	3.3
	Basal + 300 μM TPNH + GS	37	29.1	30.4	18.5	17.0	4.8	0.2

* Basal: Incubation system per ml: 0.1 ml microsomes, 4 μmoles ADP-Fe^{+3}, 10 μmoles nicotinamide and 0.9 ml tris buffer (0.1 M, pH 7.5).

** GS: TPNH-generating system: 0.5 units/ml incubation of glucose-6-phosphate dehydrogenase and 6 mM glucose-6-phosphate.

at 37° C. The fatty acid losses also occurred at low TPNH levels (6 μM) in the presence of a TPNH-generating system. In experiment 2 it appeared that ADP-Fe^{+3} was not required for the fatty acid losses. This result is regarded as preliminary, however, and should be confirmed.

Lipid Analyses after Treatment with Snake Venom

It was of interest to study the location of the lipid change in the phospholipid molecule. Snake venom treatment offered a good method for positional study with respect to the lipid changes observed (see Methods). Table 10 compares the original lipid from control and experimental incubation systems and their hydrolytic products after venom treatment. Both experiments showed an ester/P ratio greater than 1 for the lysophosphatides of the experimental lipid. The slightly greater double bond/P ratio of the experimental lysophosphatides may or may not be significant. Unsaturated compounds in the ether soluble fraction (fatty acids from the β carbon of the phospholipid which were hydrolyzed by venom) reflected the unsaturation found in the original samples. The total unsaturation of both the original control and experimental lipid samples was accounted for quantitatively by summing the unsaturation in their lysophosphatide fraction and ether soluble fraction (97% recovery for control and 89% for experimental). Table 11 compares the fatty acid distribution of the α' position (from lysophosphatide analysis) and the β position (fatty acids from ether soluble fractions) of phosphatides from untreated microsomes, control and experimental incubations. Comparison of lipid from untreated microsomes and the control system showed that incubation of the microsomes did not significantly alter the fatty acid composition of either α' or β positions of

TABLE 10

LIPID ANALYSES OF CONTROL AND EXPERIMENTAL INCUBATION SYSTEMS BEFORE AND AFTER VENOM TREATMENT

Expt. No.	Analyses	Original Lipid		Lysophosphatides		Ether Soluble	
		Control	Experimental	Control	Experimental	Control	Experimental
1	Ester/P	2.12	2.02	0.94	1.28	----	----
	C=C/P	3.66	2.58	0.47	1.28	3.28[a]	2.01[a]
	Amino N/P	0.18	0.12	0.24	0.17	----	----
2	Ester/P	----	----	1.05	1.25	----	----
	C=C/P	4.55	2.36	0.83	0.90	----	----
	C=C/Total[b]	35.8	23.0	8.0	7.8	26.8	12.7
	Percent[c] Recovery C=C	----	----	----	----	----	----

a Values were calculated based on the original phosphorus and represent the total μequivalents C=C in the ether layer per μmole P in the total venom treated sample. TLC showed the venom hydrolyses were complete.

b Represents μequivalents of C=C in the total sample. All values are directly comparable.

c Percent recovery C=C = $\frac{\text{(Lyso C=C + Ether soluble C=C) 100}}{\text{Total C=C in Original Lipid}}$

Recovery: Control = 97.2%; Experimental = 89.1%

the phosphatides. Almost all of the fatty acid in the β position was unsaturated. Oleic acid appeared to be present in both the α' and β positions. Almost all of the linoleic and more highly unsaturated fatty acids were in the β position of the phosphatides. Fatty acid composition changes in experimental lipids were almost entirely in the β position of the phosphatides. The percentage losses of C20:4 and C22:6 were similar to those observed for the total lipid (compare Table 11 with Table 5).

TABLE 11

FATTY ACID DISTRIBUTION ON α' AND β POSITIONS OF PHOSPHOLIPIDS OF UNTREATED MICROSOMES AND CONTROL AND EXPERIMENTAL INCUBATION SYSTEMS

Lipid Source	Phospholipid Position	Weight Percent					
		16:0	18:0	18:1	18:2	20:4	22:6
Untreated Mic.	α'	36.5	42.8	12.3	3.0	5.3	----
Control Incub.	α'	30.6	50.5	10.0	3.7	4.4	0.7
Experimental Inc.	α'	33.7	53.6	8.8	2.7	1.2	----
Untreated Micr.	β	7.8	5.3	11.4	25.2	41.7	8.5
Control Incub.	β	8.2	6.2	10.0	25.2	41.7	9.4
Experimental Inc.	β	15.0	14.5	15.2	30.0	22.3	3.1

Difference Spectrum of Control and Experimental Lipids

The ultraviolet specta of the Folch chloroform fractions from control and experimental incubation systems were recorded using a Cary Model 14 Recording Spectrophotometer. Both samples showed absorption maxima at 227 mμ and 270 mμ, however, both peaks were higher in the

experimental lipid. The control sample was placed in the reference beam of the spectrophotometer and the experimental sample was placed in the sample beam and the difference spectrum was recorded as shown in Figure 7. The difference spectrum also showed absorption maxima at 227 mμ and 270 mμ.

Solvent Distribution of Lipid Decomposition Products

The possibility that lipid breakdown products would distribute differently between the chloroform and the water-methanol layers was investigated. The TBA chromogen, believed by some investigators to be malonaldehyde, was found to distribute equally between these two solvent phases after incubations were carried out at 5 atmospheres oxygen pressure for 45 minutes (Table 12). (This distribution can be altered by varying the incubation temperature). More chromogen could not be

TABLE 12

DISTRIBUTION OF TBA CHROMOGEN BETWEEN CHLOROFORM AND WATER-METHANOL

Fraction Analyzed*	OD_{532}/ml incub.
Total Incubation System	6.12
Chloroform	1.90
Chloroform, Re-extracted	2.05
Water-Methanol	3.62
Water-Methanol, Re-extracted	3.34

*All fractions from experimental incubation systems.

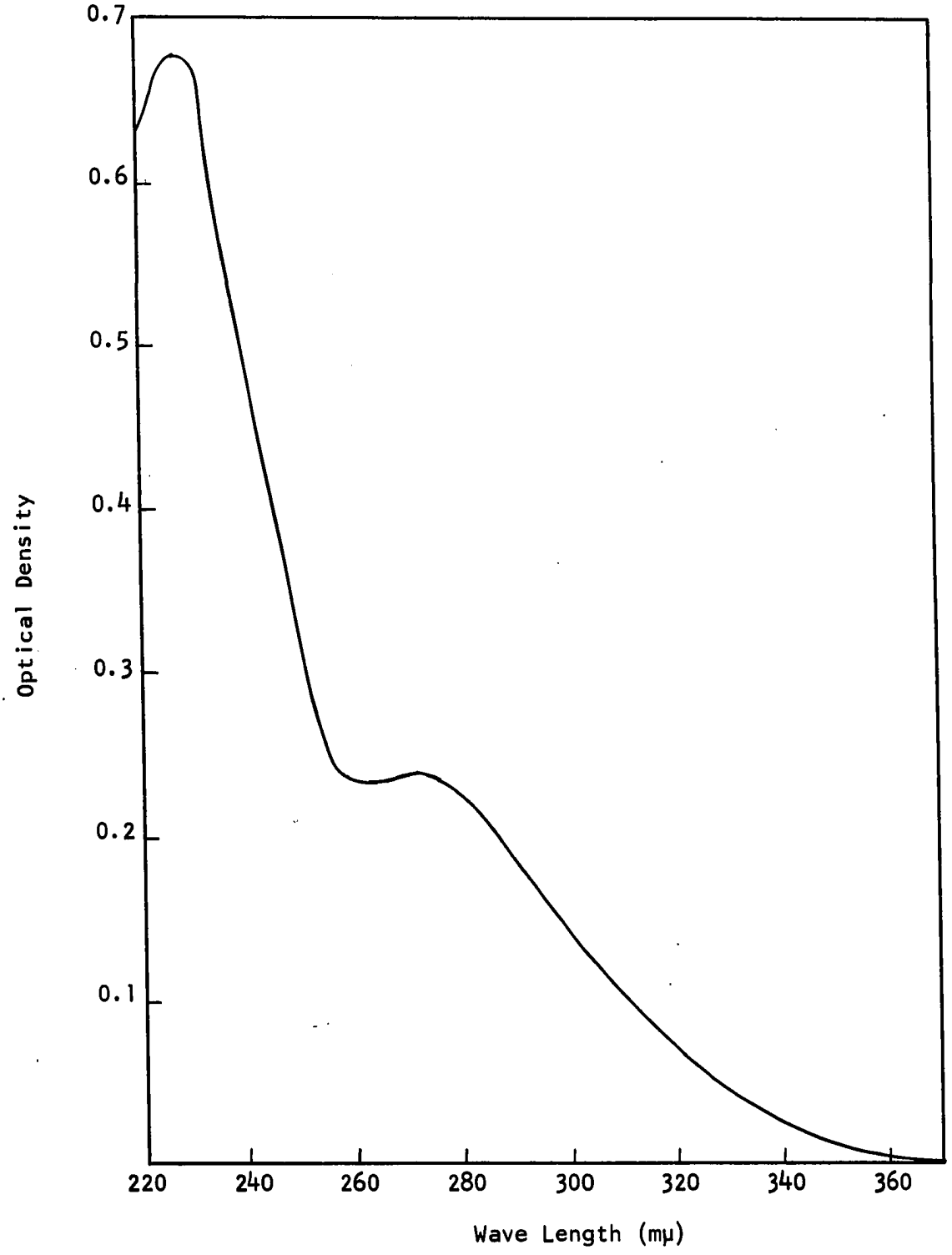


Figure 7. Difference absorption spectrum of lipid from experimental system. Absorption of lipid from control system was subtracted from that of the experimental system in Cary Spectrophotometer.

extracted from either phase by re-extraction with a fresh solvent with the composition of the opposite phase. From time to time it was noted that the amino N/P ratio of the experimental lipid approached that of the control value. Thus the effect that aging of lipid from the chloroform layer had on the amino N/P ratio and on the TBA chromogen was studied. Figure 8 shows that if the amino N/P ratio was determined immediately after isolation of the chloroform layer, the value obtained was the same as the control value. However, after 24 hours aging, the amino N/P ratio decreased by about 50% in the experimental lipid but remained constant in the control lipid. It will be noted that the chromogen present in the experimental chloroform layer also decreased by about 60% after 24 hours aging while the water-soluble chromogen was stable for at least 120 hours.

This distribution of the TBA chromogen could be explained by chain scission of the unsaturated fatty acids in the β position of the microsomal phospholipids resulting in the formation of products which would be dominated by their phospholipid character (chloroform soluble) and those which would extract into the water-methanol layer (60% methanol). It was of interest to see if the chromogen distribution was constant throughout the time course of the enzyme reaction or not. One milliliter experimental incubations were carried out at 5 atmospheres oxygen pressure and 25° C for varying lengths of time. The total TBA chromogen and the solvent distribution of chromogen from aliquots of the same sample were determined (Figure 9). The distribution of the chromogen was 50% in each solvent layer throughout the time course of the enzyme reaction. At each time point the sum of the values of chromogen in the

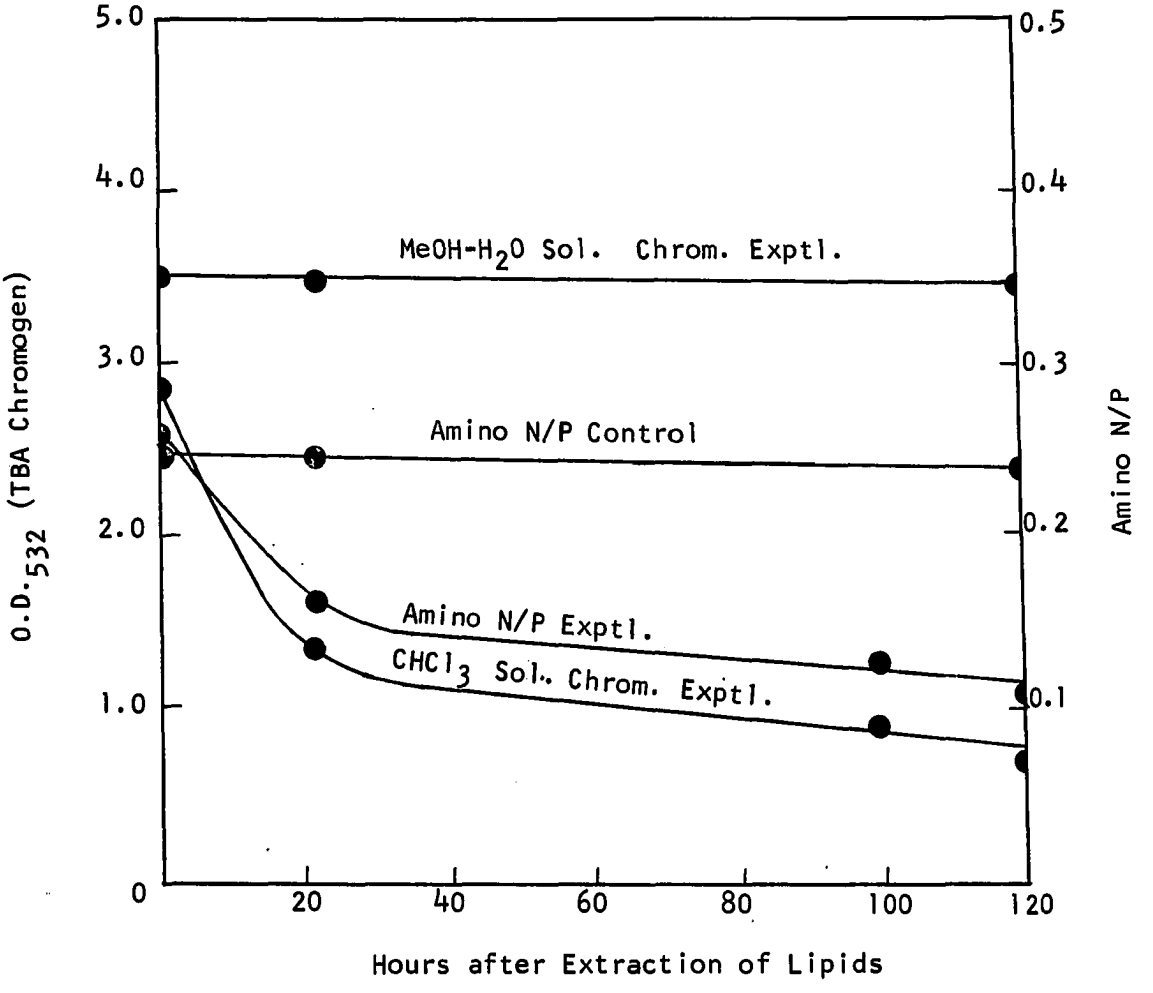


Figure 8. Effect of aging lipid from control and experimental incubation systems on amino N/P ratio and TBA chromogens (Chrom.).

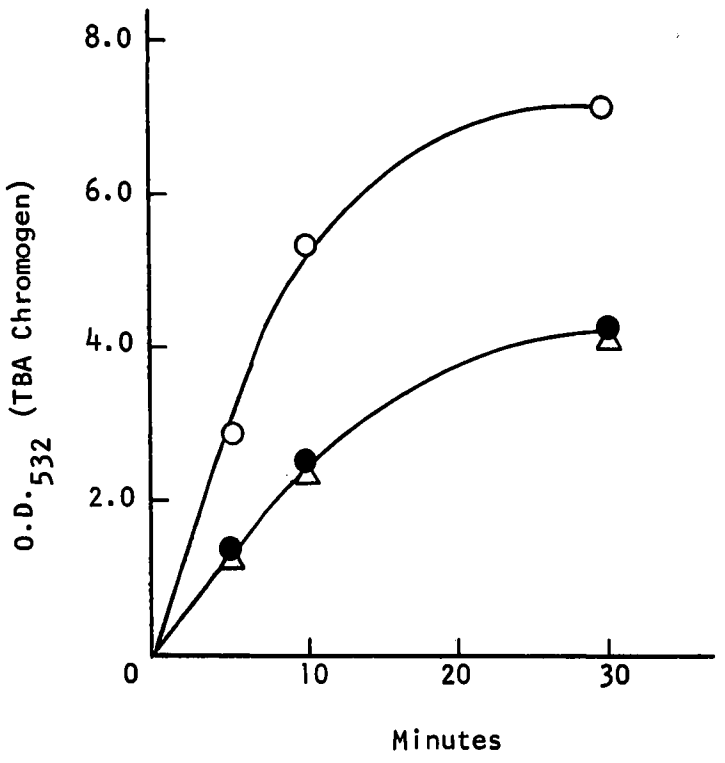


Figure 9. Distribution of TBA chromogen between chloroform and water-methanol as a function of incubation time. ○, total TBA chromogen; ●, TBA chromogen in water-methanol layer; △, TBA chromogen in chloroform layer.

chloroform and water-methanol fractions was equal to that of the total.

Carbonyl compounds were detected in both solvent fractions of the experimental lipid as 2,4-dinitrophenylhydrazones. The carbonyl nature of the TBA chromogen was further supported by the fact that when experimental systems were treated with semicarbazide, dimethylhydrazine or 2,4-dinitrophenylhydrazine (DNPH) they failed to give a TBA test.

The chloroform and water-methanol layers of untreated microsomes, control and experimental systems (10 ml) were immediately treated with 4 mg of recrystallized DNPH (see Methods for details). After the hydrazones were isolated the free DNPH was removed by BioRad AG50W-X4 cation exchange resin chromatography (see Methods). The absorption of the resulting hydrazones was determined at 360 mμ. Blank absorption due to untreated microsomes and reagents was subtracted from all values reported (Table 13). There was a larger amount of carbonyl compounds in the experimental chloroform layer than in the water-methanol layer in both experiments. However, this method would not detect malonaldehyde since its derivative has different absorption characteristics. The carbonyl values of the control systems were lower than those of the experimental systems. The ratio of control hydrazones to experimental hydrazones was about the same as control chromogen to experimental chromogen.

Studies Utilizing Microsomes from α-Tocopherol Deficient and Supplemented Rats

Effect of Variation of Dietary α-Tocopherol on TBA Chromogen Production

Male rats (200 g) were placed on diets containing 0, 10, 20, and

TABLE 13

SOLVENT DISTRIBUTION OF CARBONYL COMPOUNDS FORMED IN MICROSOMES DURING TPNH OXIDATION BY MICROSOMES

Experiment No.	Incubation System[a]	Solvent Layer	TBA Chromogen[b]	DNPH[c]
1	Control	Water-Methanol	0.65	0.27
1	Control	Chloroform		0.10
1	Experimental	Water-Methanol	7.10	0.65
1	Experimental	Chloroform		1.85
2	Control	Water-Methanol	0.96	0.07
2	Control	Chloroform		0.12
2	Experimental	Water-Methanol	6.20	0.18
2	Experimental	Chloroform		1.63

a Incubation system: Control: 2.0 ml microsomes, 4 mM ADP-Fe^{+3}; Experimental same as control but with 0.3 mM TPNH in total incubation volume of 10 ml. Systems incubated at 5 atm. oxygen pressure 45 min. at 25° C.

b TBA reaction, OD_{532} per ml of incubation system.

c Total μmoles of 2,4-dinitrophenylhydrazones assuming a mM extinction of 22.0 at 360 mμ (See Methods). Experiment 1: hydrazones formed during 1 hour incubation at 25° C. Experiment 2: hydrazones formed during 1 hour incubation at 65° C. Hydrazone values from untreated microsomes were subtracted from all values reported.

30 mg of α-tocopherol per 100 g of diet (-E, +E1, +E2 and +E3, respectively) for 5 days. They were sacrificed the sixth day and microsomes were isolated as described in Methods. The rate of chromogen production was studied at 5 atmospheres oxygen pressure at 25° C as described above. Microsomes from animals on the α-tocopherol-deficient (-E) diet showed no lag period in the chromogen production curve (Figure 10). However, microsomes from animals fed diets +E1 and +E2 exhibited a lag period in the chromogen formation curve while those from animals fed diet +E3 failed to produce chromogen in significant quantities. It was found later that microsomes from animals fed diet +E3 failed to produce TBA chromogen because the TPNH was fully oxidized before the chromogen production was initiated.

Time Course of Lipid Changes in Microsomes from Animals Fed Diet +E2

It was of interest to determine whether or not the same lipid changes occurred in microsomes from animals supplemented with α-tocopherol and, if so, to determine the quantitative relationship between enzyme incubation time and total lipid changes. Microsomes from animals fed diet +E2 for 5 days were incubated in 20 ml control and experimental incubation systems. Three experimental systems were incubated 5, 20, and 60 minutes respectively and one control was incubated 60 minutes at 5 atmospheres oxygen pressure at 25° C. Lipid from the chloroform fraction was analyzed as shown in Table 14. The data were plotted in Figure 11 as percent maximum lipid change versus time. There was a lag period in the formation of TBA chromogen but there appeared to be no lag period in the loss of double bonds or the

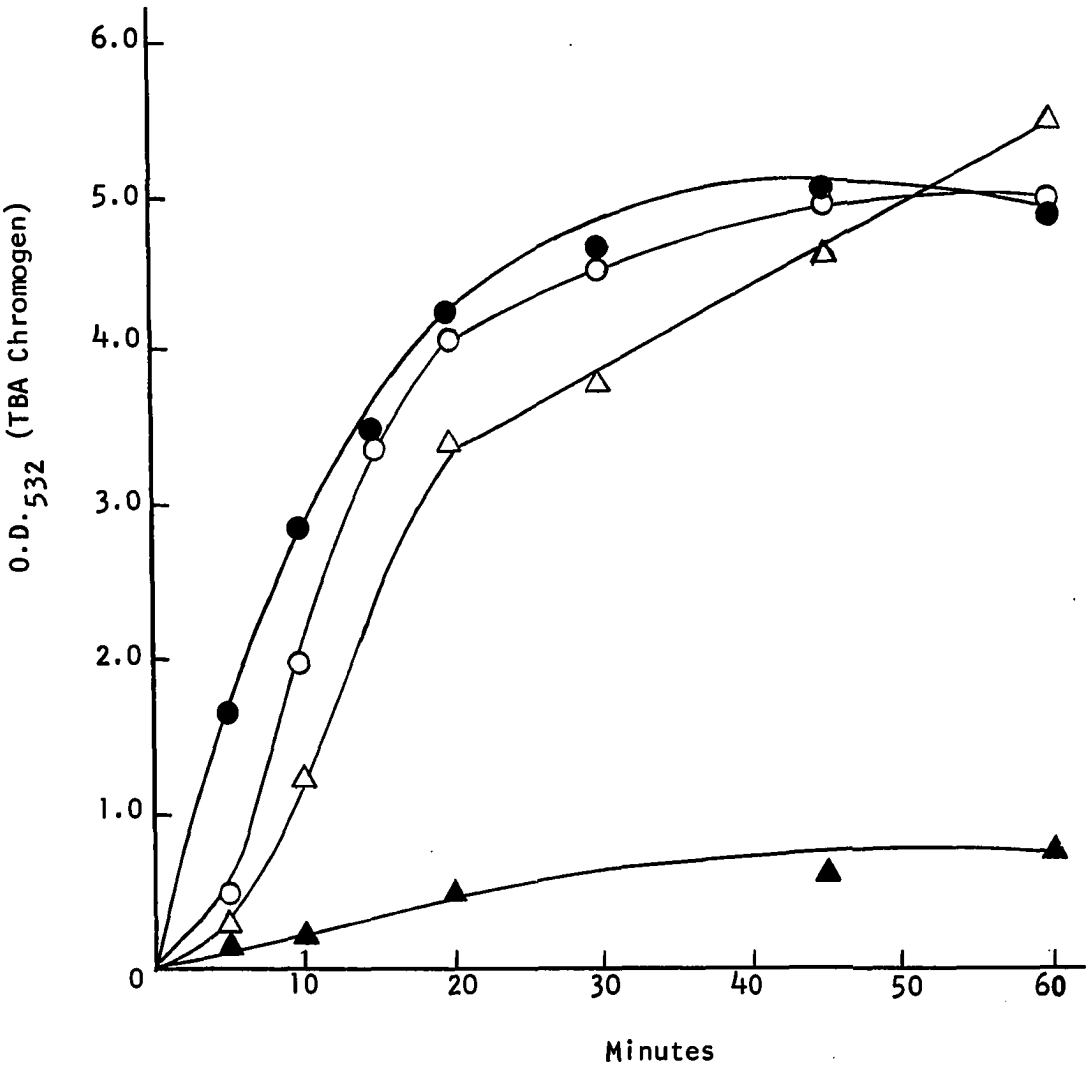


Figure 10. Effect of increasing dietary levels of α-tocopherol on the rate of TBA chromogen formed by microsomes at 5 atmospheres. System: 0.1 ml microsomes, 4 mM ADP-Fe^{+3}, 0.3 mM TPNH, in tris buffer (0.1 M, pH 7.5) to a total incubation volume of 1 ml. Incubated at 25° C. Microsomes: ●, -E; ○, +E1; △, +E2; ▲, +E3.

TABLE 14

TIME COURSE OF LIPID CHANGES OCCURRING IN MICROSOMES FROM ANIMALS SUPPLEMENTED WITH α-TOCOPHEROL (DIET +E2)

	Incubation Time (Min.)			
	Control	Experimental		
Lipid Analyses	60	5	20	60
OD_{532}/ml incub.	0.10	0.29	2.73	5.82
Total Lipid P (μg)	278	300	303	274
Amino N/P	0.37	0.32	0.30	0.25
Ester/P	2.00	1.91	1.88	1.90
C=C/P	4.36	4.18	3.64	3.01
Fatty Acid		Relative Percent		
16:0	100	100	100	100
16:1	21.6	21.9	23.8	31.1
18:0	82.5	80.8	80.4	79.5
18:1	64.2	63.4	64.8	65.6
18:2	27.2	26.2	26.1	23.8
20:4	57.0	52.7	45.2	35.7
20:5	24.6	21.7	17.5	13.6
22:6	44.0	38.5	28.1	21.3

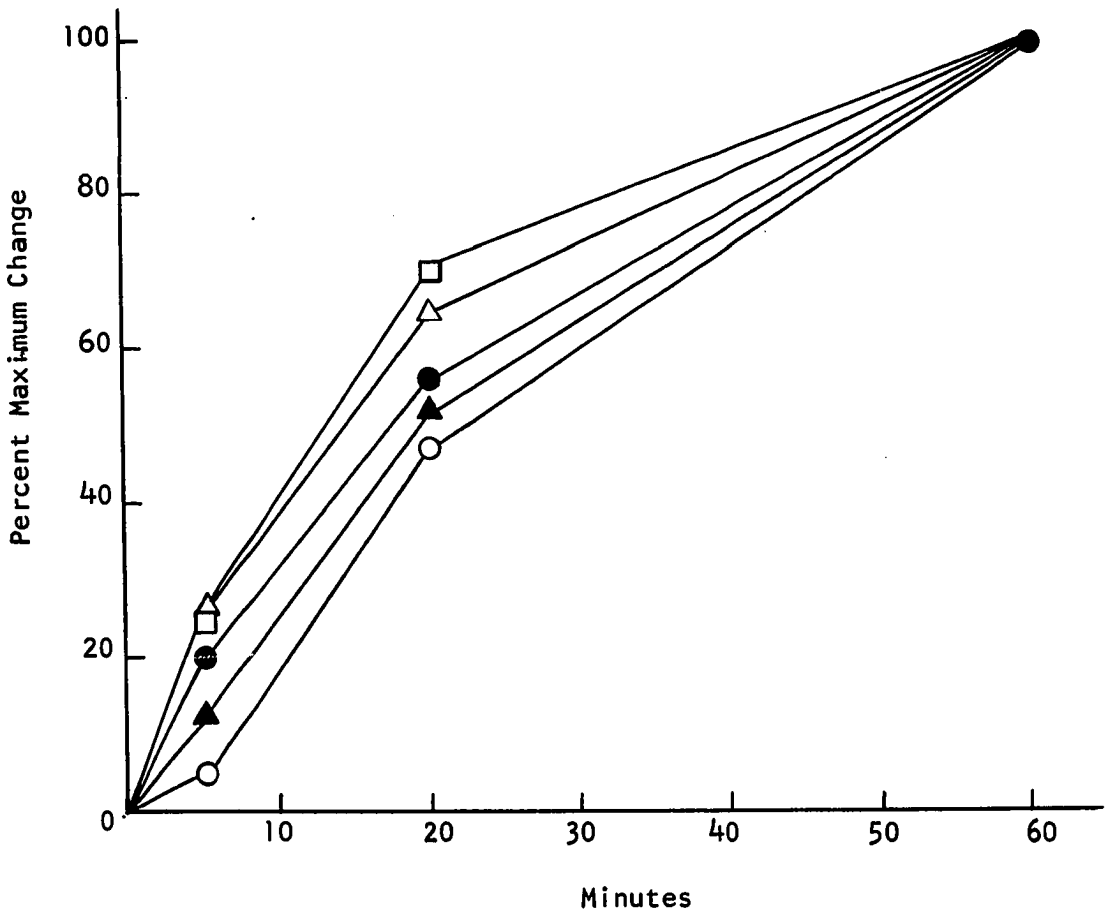


Figure 11. Comparison of the extent of various lipid changes with incubation times of +E2 microsomes. System: 10 ml incubations for each time point. 1 ml microsomes, 4 mM ADP-Fe^{+3} and 0.3 mM TPNH in tris buffer (0.1 M, pH 7.5). Incubated at 5 atmospheres oxygen pressure at 25° C. Analyses on $CHCl_3$ fraction of Folch extract. Ordinate: Percent of maximum change of: ○, TBA chromogen; ▲, double bonds/P; ●, C20:4; △, C20:5; □, C22:6. Abscissa: enzyme incubation time.

unsaturated fatty acids (C20:4, C20:5 and C22:6). The rate of loss of the unsaturated fatty acid was higher for the more highly unsaturated fatty acids and followed the order C22:6 > C20:5 > C20:4. Caution should be exercised in using information from the 5 minute incubations since the changes are relatively small and the data reported was obtained from a difference between two relatively large numbers.

Effect of Concentration of ADP-Fe^{+3} on TBA Chromogen Production

The lag period noted above could possibly be explained by the destruction of α-tocopherol. Since Fe^{+3} oxidizes α-tocopherol if present as the free ion, the possibility that the concentration of ADP-Fe^{+3} might have an effect on the lag periods observed was investigated. Figure 12 shows the effect of 1, 2, and 4 mM ADP-Fe^{+3} on microsomes from animals fed diet +E2. The total chromogen formed was approximately proportional to the amount of ADP-Fe^{+3} present (lower graph). When the data were expressed as percent maximum chromogen so that the shape of the curves could be compared, it could be seen that the concentration of ADP-Fe^{+3} (in this concentration range) had no effect on the lag period.

Variations in the shape of the curves were considered to be within experimental error. Figure 13 shows similar information for microsomes from animals on diets -E and +E1. Again the shapes of the curves were virtually the same in the presence of 2 and 4 mM ADP-Fe^{+3} when the data were plotted as percent of maximum chromogen formed versus time. No lag period in the production of the chromogen was observed with microsomes from animals fed -E diet.

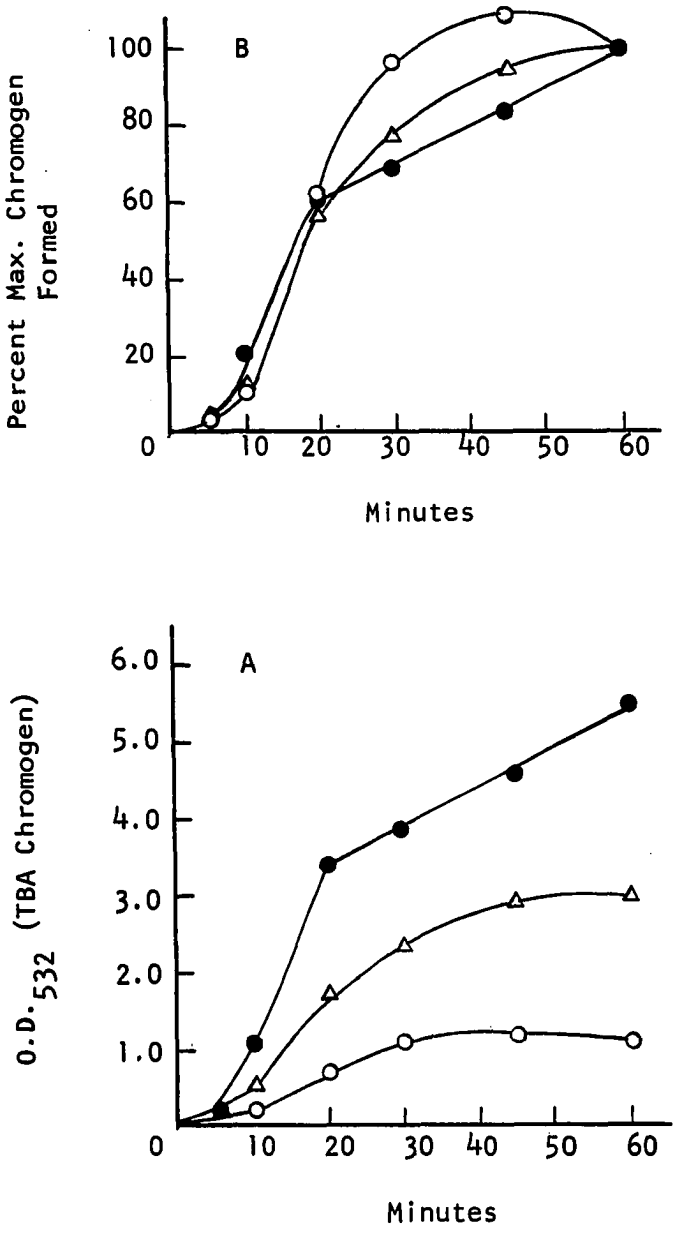


Figure 12. Effect of the concentration of ADP-Fe^{+3} on the lag period in TBA chromogen production by +E2 microsomes. System: 0.1 ml microsomes, 0.3 mM TPNH, ○, 1 mM or △, 2 mM or ●, 4 mM ADP-Fe^{+3}. Incubation: 1 ml systems at 5 atmospheres oxygen pressure, 25° C. A (lower): Total chromogen per ml incubation system. B (upper): Same data expressed as percent total chromogen formed.

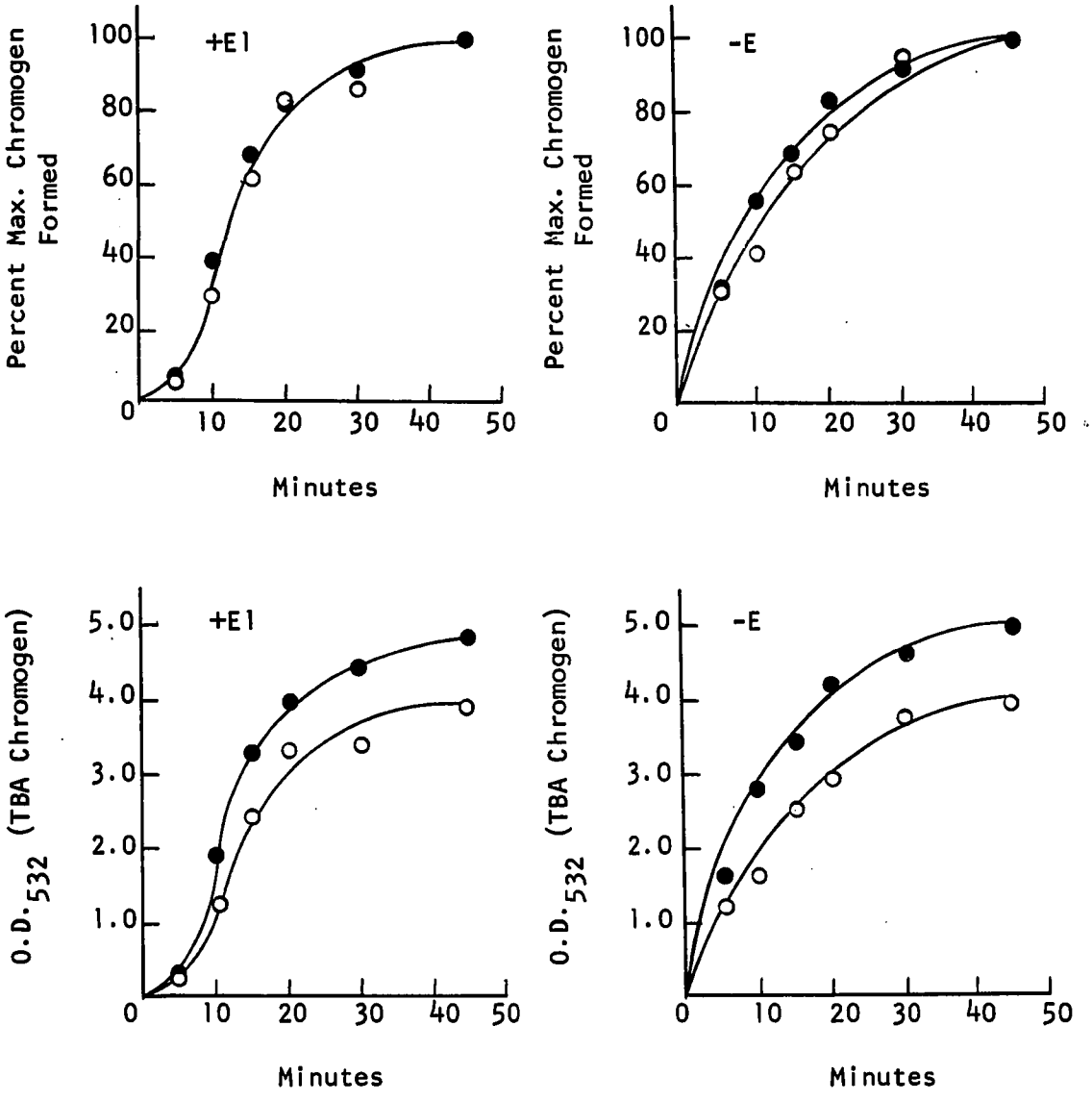


Figure 13. Effect of the concentration of ADP-Fe^{+3} on the rate of chromogen formed by +E1 and -E microsomes. Incubation system and conditions same as Figure 12. ●, 4 mM and ○, 2 mM ADP-Fe.

Relationship Between TPNH Concentration and TBA Chromogen Production

Animals placed on α-tocopherol-supplemented diets but with 5 daily injections of phenobarbital were also found to produce liver microsomes which exhibited a lag period in TBA chromogen formation. A somewhat shorter lag period was observed when these microsomes were used than when those obtained from animals on the same diet but without injections were used.

Figure 14 shows the effect of the TPNH level on TBA chromogen production by microsomes from animals on diet +E3 without (+E3) and with (+E3PB) phenobarbital injections. In the absence of a TPNH-generating system, +E3 microsomes rapidly oxidized the TPNH and produced little or no chromogen. The same effect was observed with +E3PB microsomes. In the presence of the TPNH-generating system, however, both sets of microsomes produced chromogen after a lag period and the TPNH was also maintained at a higher level. These results indicated that TPNH was required throughout the time course of chromogen production. This fact was substantiated by studying the effect of adding more TPNH after the initially added TPNH had been oxidized (Figure 15). In the presence of TPNH without a generating system, microsomes rapidly oxidized the TPNH (upper graph) and the TBA chromogen synthesis stopped. Addition of more TPNH at the end of 10 or 25 minutes resulted in the resumed synthesis of chromogen but again it reached a maximum value when the TPNH was completely oxidized. Addition of the TPNH-generating system after 10 minutes initiated rapid synthesis of chromogen to the level obtained by +E3PB microsomes previously (compare Figures 14 and 15).

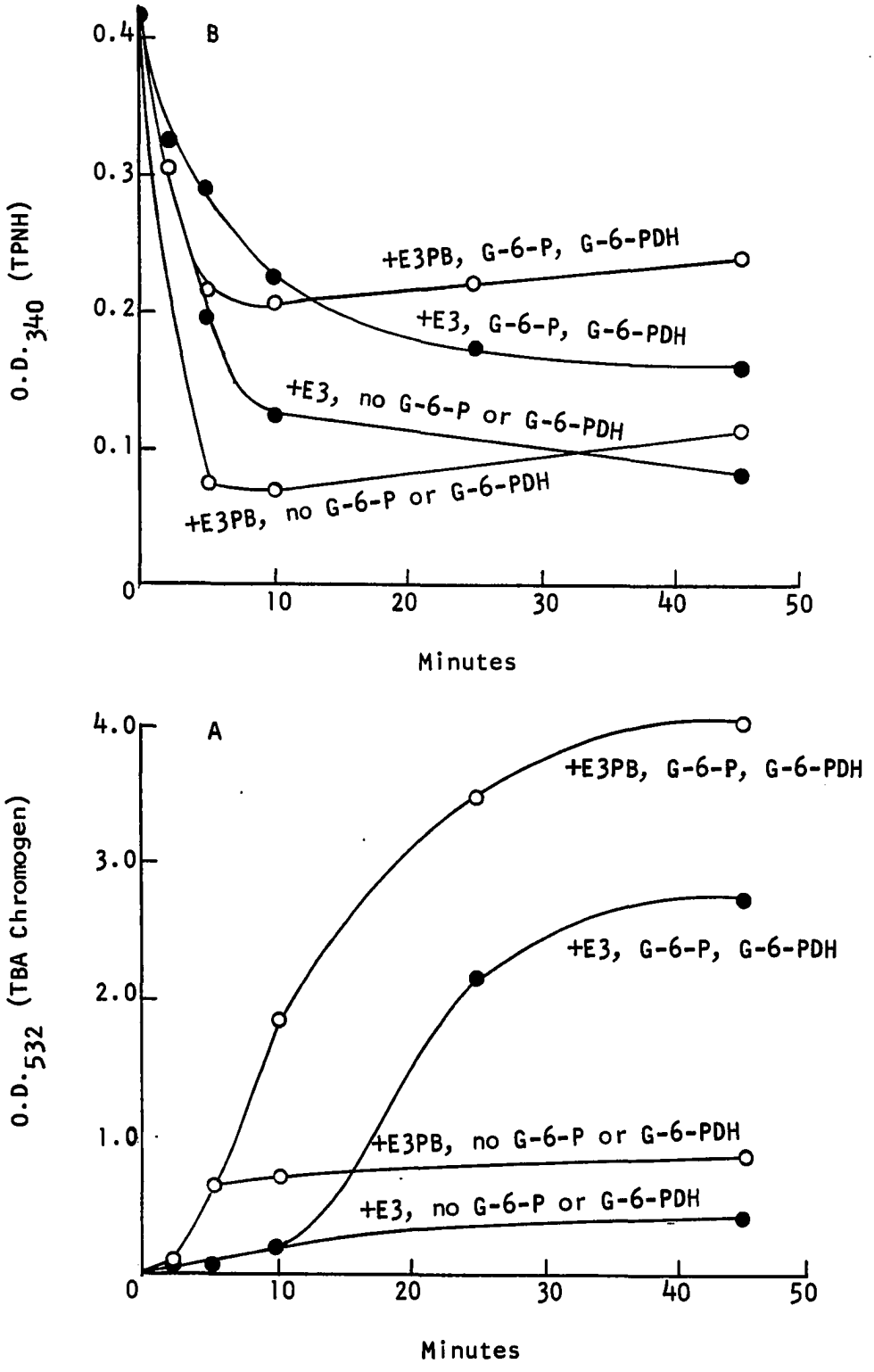


Figure 14. Effect of the presence and absence of a TPNH-generating system on TBA chromogen formed by liver microsomes from α-tocopherol-supplemented rats without (+E3) and with (+E3PB) phenobarbital injections. A (lower): TBA chromogen; B (upper) TPNH levels. See Figure 15 for reaction mixture and incubation conditions.

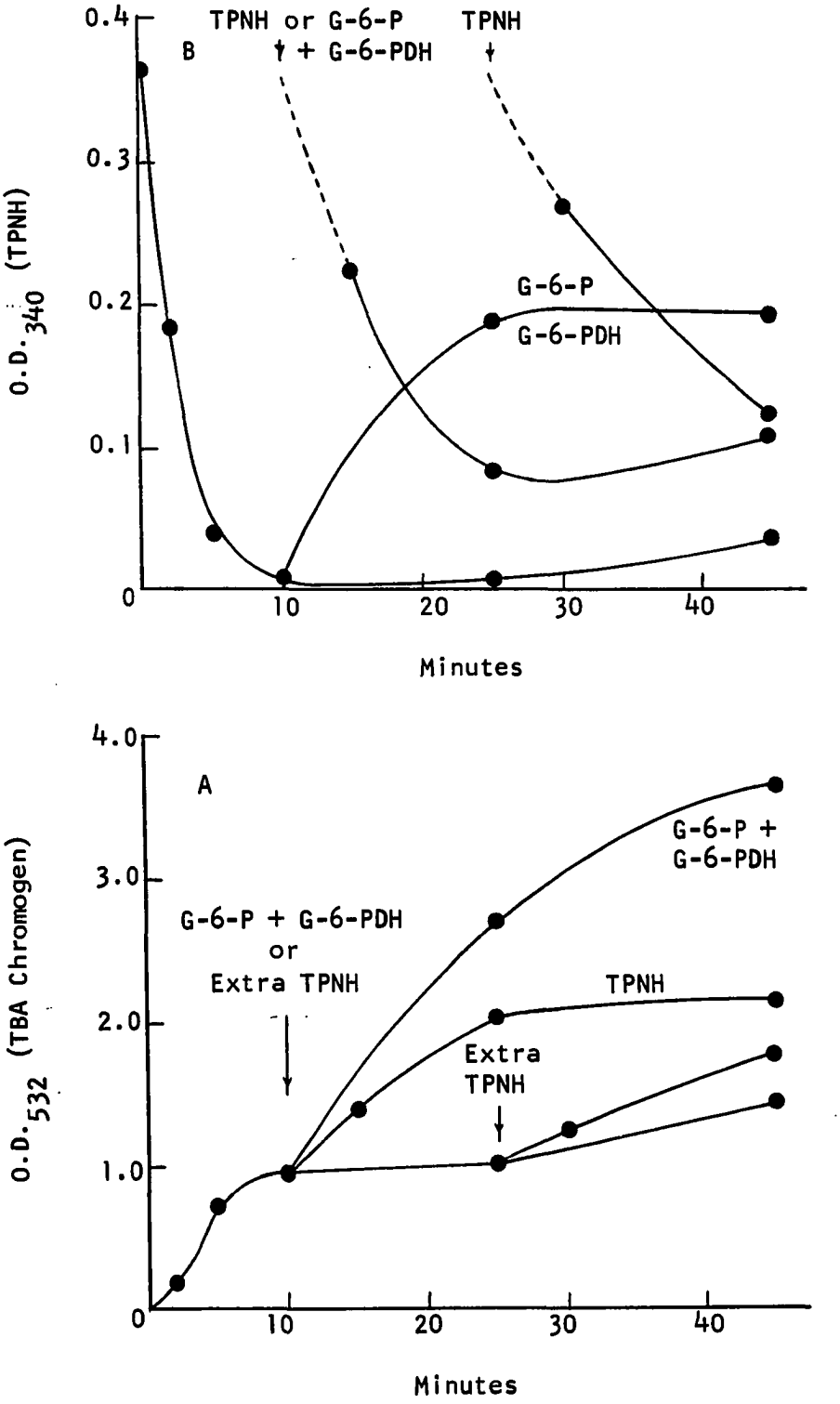


Figure 15. Effect of using limiting amounts TPNH on the formation of TBA chromogen by +E3PB microsomes. System: 0.1 ml microsomes, 4 mM ADP-Fe^{+3} and 0.3 mM TPNH. One ml systems incubated in air at 37° C. Additional TPNH (0.3 mM) added at 10 or 25 minutes, or TPNH-generating system at 10 minutes. A (lower): TBA chromogen formed. B (upper): Corresponding TPNH concentrations.

p-Hydroxymercuribenzoate (PHMB) Inhibition of TBA Chromogen Production

The changes observed in the experimental lipids appear to be consistent with a classical peroxidative mechanism. Therefore, the relationship between the enzyme reaction and the lipid changes is an important problem. Thus the effect of the addition of PHMB to a system that was rapidly producing TBA chromogen was studied. This experiment was an attempt to further confirm that the synthesis of the chromogen required active enzymic oxidation of TPNH at all times. Figure 16 shows that PHMB added at time zero prevented the formation of chromogen and the TPN was predominantly in the reduced form. Addition of PHMB after 11 minutes incubation abruptly stopped further chromogen synthesis.

Comparison of the Effect of Increasing Dietary α-Tocopherol Levels on Microsomes from Animals with and without Phenobarbital Injections

Effect on TBA chromogen formation. Animals were placed on diets containing 10, 30, 60, 90 and 150 mg of α-tocopherol per 100 g of diet for 5 days. Some received daily injections of phenobarbital (100 mg/kg body weight) (animal designations: +E1PB, +E3PB, +E6PB, +E9PB and +E15 PB) and others received no injections (animal designations: +E1, +E3, +E6, +E9 and +E15). Figure 17 shows the chromogen production curve in the presence of +E1, +E3, +E6 and +E9 microsomes. Figure 18 shows the same data for +E1PB, +E3PB, +E6PB, +E9PB and +E15PB microsomes. Increasing levels of α-tocopherol in the diet lengthened the lag period for TBA chromogen production by microsomes from the non-injected animals and chromogen synthesis was almost completely eliminated in +E9 microsomes. Attempts to maintain the TPNH levels in the presence of these

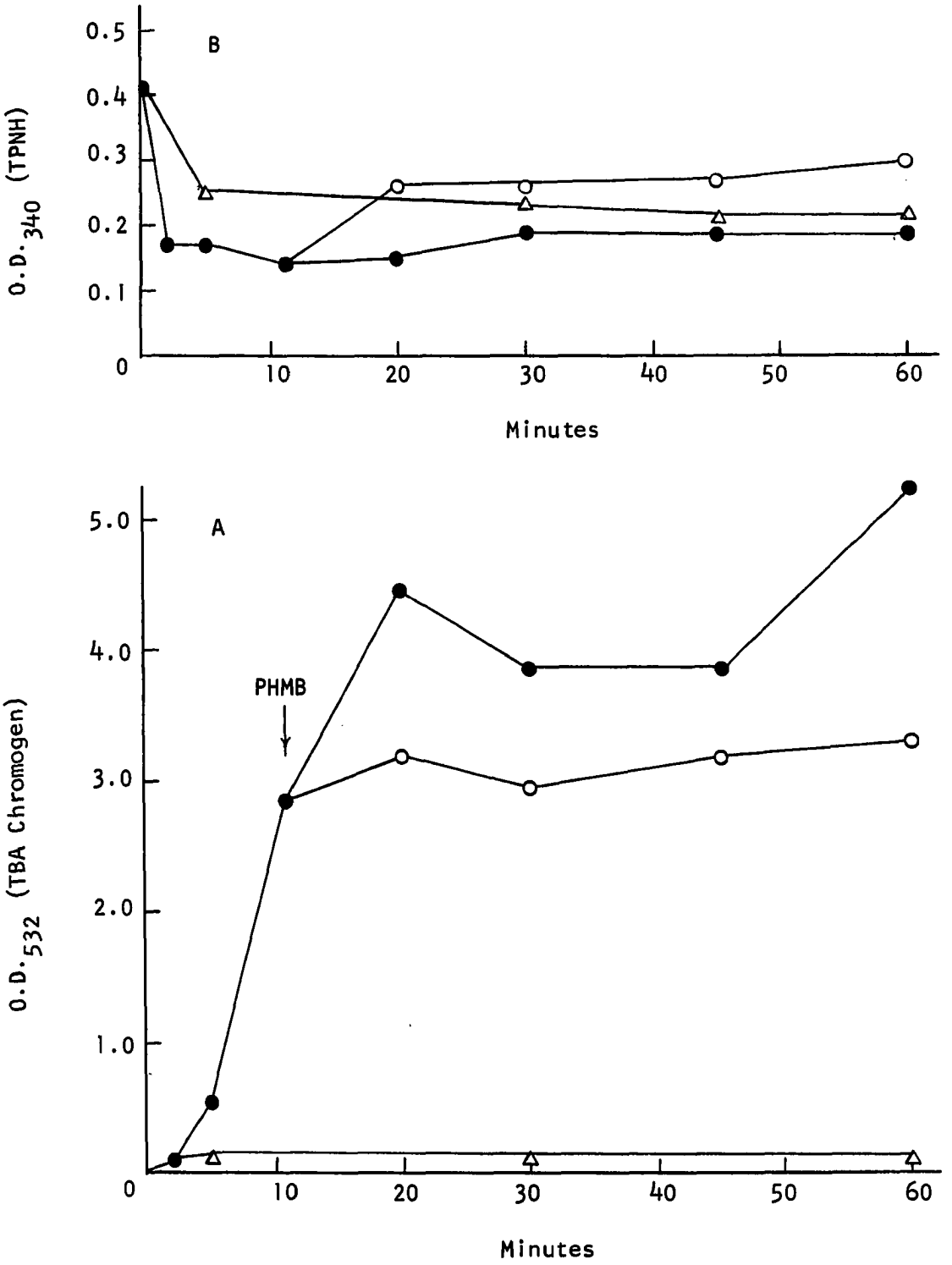


Figure 16. Effect of addition of PHMB to experimental systems rapidly forming TBA chromogen. Incubation: 0.1 ml +E3PB microsomes, 4 mM ADP-Fe^{+3}, 0.3 mM TPNH, 6 mM G-6-P and 0.5 units G-6-PDH in 1 ml incubation volume. ●, no PHMB added; O, PHMB added at 11 minutes; Δ, PHMB added at zero time. A (lower): TBA chromogen formed; B (upper): TPNH oxidation.

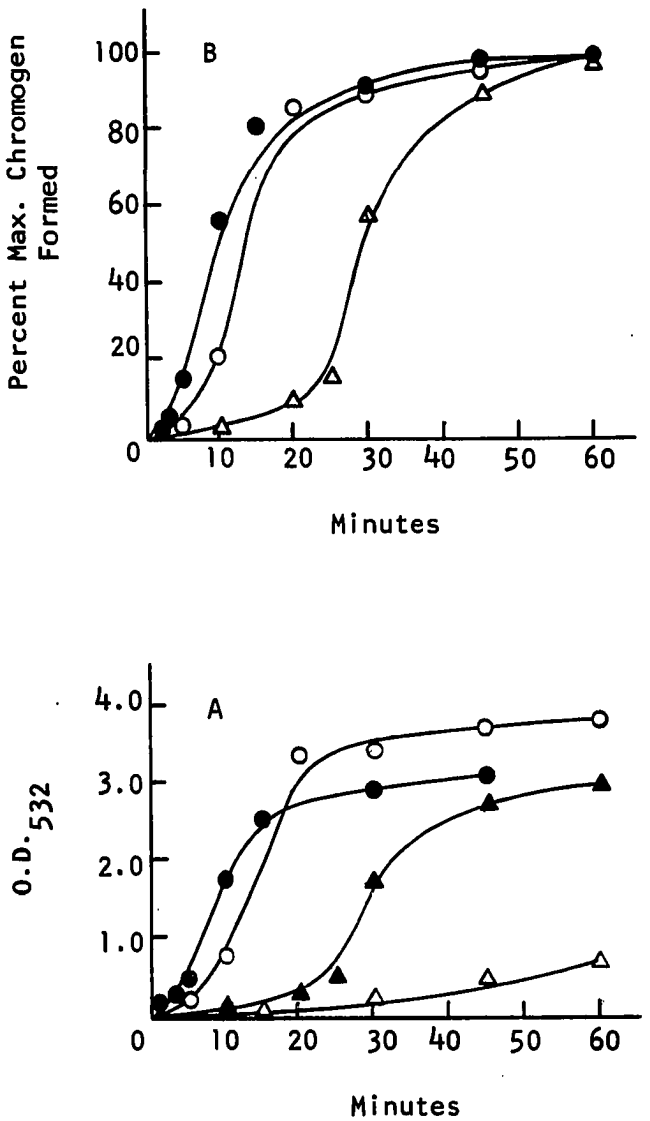


Figure 17. Effect of increased dietary levels of α-tocopherol on the ability of microsomes to form TBA chromogen in the presence of a TPNH-generating system. System: 0.1 ml microsomes, 4 mM ADP-Fe^{+3}, 0.3 mM TPNH, 6 mM G-6-P and 0.5 units G-6-PDH in 1 ml incubation volume. Incubated in air at 37° C. Microsomes: ●, +E1; ○, +E3; ▲, +E6; △, +E9. A (lower): Total chromogen formed. B (upper): Same data expressed as percent of maximum chromogen formed.

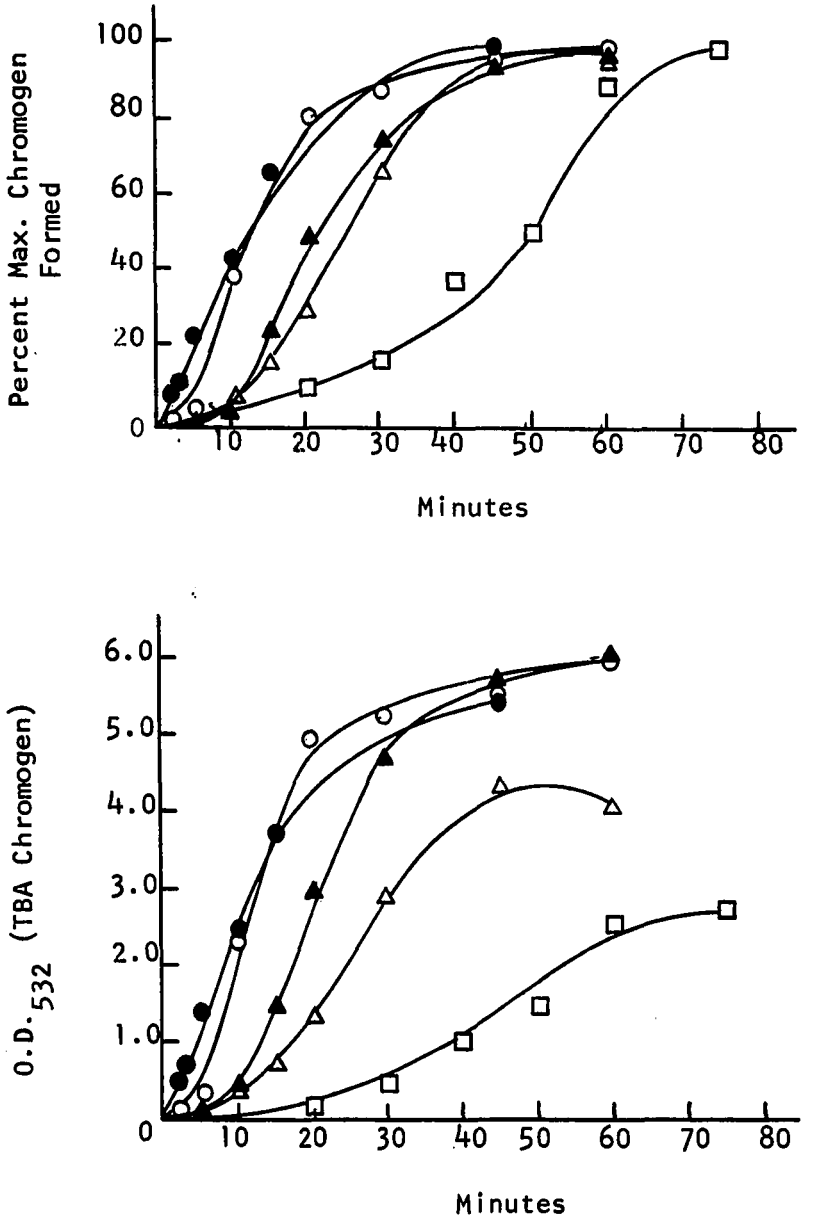


Figure 18. Effect of increased dietary levels of α-tocopherol and of phenobarbital injections on the ability of microsomes to form TBA chromogen in the presence of a TPNH-generating system. Incubation conditions same as Figure 17. Microsomes: ●, +E1PB; ○, +E3PB; ▲, +E6PB; △, +E9PB; □, +E15PB. Phenobarbital injections: 5 daily injections: 100 mg/kg body weight.

microsomes have failed thus far, however, and this may account for the lack of chromogen production. Somewhat shorter lag periods were observed with microsomes from phenobarbital-injected animals (Figure 18), and microsomes from injected animals on diet with α-tocopherol content as high as diet +E15 still produced chromogen while microsomes from the control animals (without injections) produced no chromogen. The TPNH levels could be maintained for long periods of time in systems utilizing microsomes from phenobarbital-injected animals. The maximum chromogen level was depressed in +E15PB and this depression was not related to the quantity of microsomes present (see protein analyses in Table 15). The effect of phenobarbital injection on the lag period of chromogen production is shown in Figure 19. In each case the lag period was shorter in the presence of microsomes from phenobarbital-injected animals. The total chromogen formed was also higher but this was because the microsomes from phenobarbital-injected animals contained more protein and lipid per g of liver (see Table 15).

Effect of α-Tocopherol and of Phenobarbital Injection on the Composition of Liver Microsomes

The microsomes from animals maintained under the dietary and injection conditions stated above were analyzed for protein, lipid phosphorus, α-tocopherol, and fatty acid composition. Figure 20 shows the effect of the dietary level of α-tocopherol on the microsomal protein and lipid content per g of liver wet weight. Microsomes from the control set (not injected) showed no effect on the protein/g liver or lipid P/g liver, however, these ratios appeared to reach a maximum value in microsomes from animals receiving phenobarbital injections. The protein and lipid

TABLE 15

ANALYSES OF RAT LIVER MICROSOMES FROM ANIMALS SUPPLEMENTED WITH VARYING AMOUNTS OF VITAMIN E WITH AND WITHOUT PHENOBARBITAL INJECTIONS

Microsome[a] Source	Liver[b] Weight	µg Vit E g liver	µg Lipid P g liver	mg Protein g liver	Fatty Acid Distribution (Weight Percent) 16:0	16:1	18:0	18:1	18:2	20:4	20:5	22:6
E3 11/16	----	24.8	207	15.1	27.6	5.7	19.2	14.5	6.4	12.5	6.5	7.6
E3 11/23	4.22	6.0	215	14.7	24.5	6.1	18.9	15.6	7.1	14.1	5.6	8.0
E3PB 11/16	----	4.8	236	20.5	22.4	4.2	22.5	12.3	6.7	15.3	6.2	8.2
E3PB 11/23	4.72	13.6	341	19.6	23.9	3.2	27.7	15.3	7.5	11.6	5.6	5.2
E6 12/2	4.68	40.0	209	14.6	24.7	6.6	20.1	17.5	7.5	12.5	4.8	6.2
E6 12/7	4.85	9.6	222	17.2	32.4	4.0	28.0	12.4	6.4	6.0	8.7	2.1
E6PB 12/2	4.98	5.2	480	28.4	24.6	4.1	25.2	15.9	8.8	11.8	5.8	3.8
E6PB 12/7	5.27	13.2	434	32.1	29.1	4.5	24.6	15.1	8.8	11.5	3.5	2.4
E9PB 12/14	----	21.2	375	29.1	23.9	4.4	28.1	15.7	7.6	11.3	5.0	3.9
E15 12/21	3.93	117.6	231	20.1	27.1	5.5	23.4	15.4	8.2	12.4	3.9	4.2
E15PB 12/21	5.01	77.2	358	26.1	26.0	5.2	27.6	12.8	6.4	13.2	4.3	4.5
Pellet	----	----	332	----	19.7	---	27.9	11.4	16.6	21.1	---	3.3

a Animals on designated diet 5 days with or without phenobarbital injections. Isolation data given.
b Average wet weight of liver per 100 g body weight. Three animals were used in most groups.

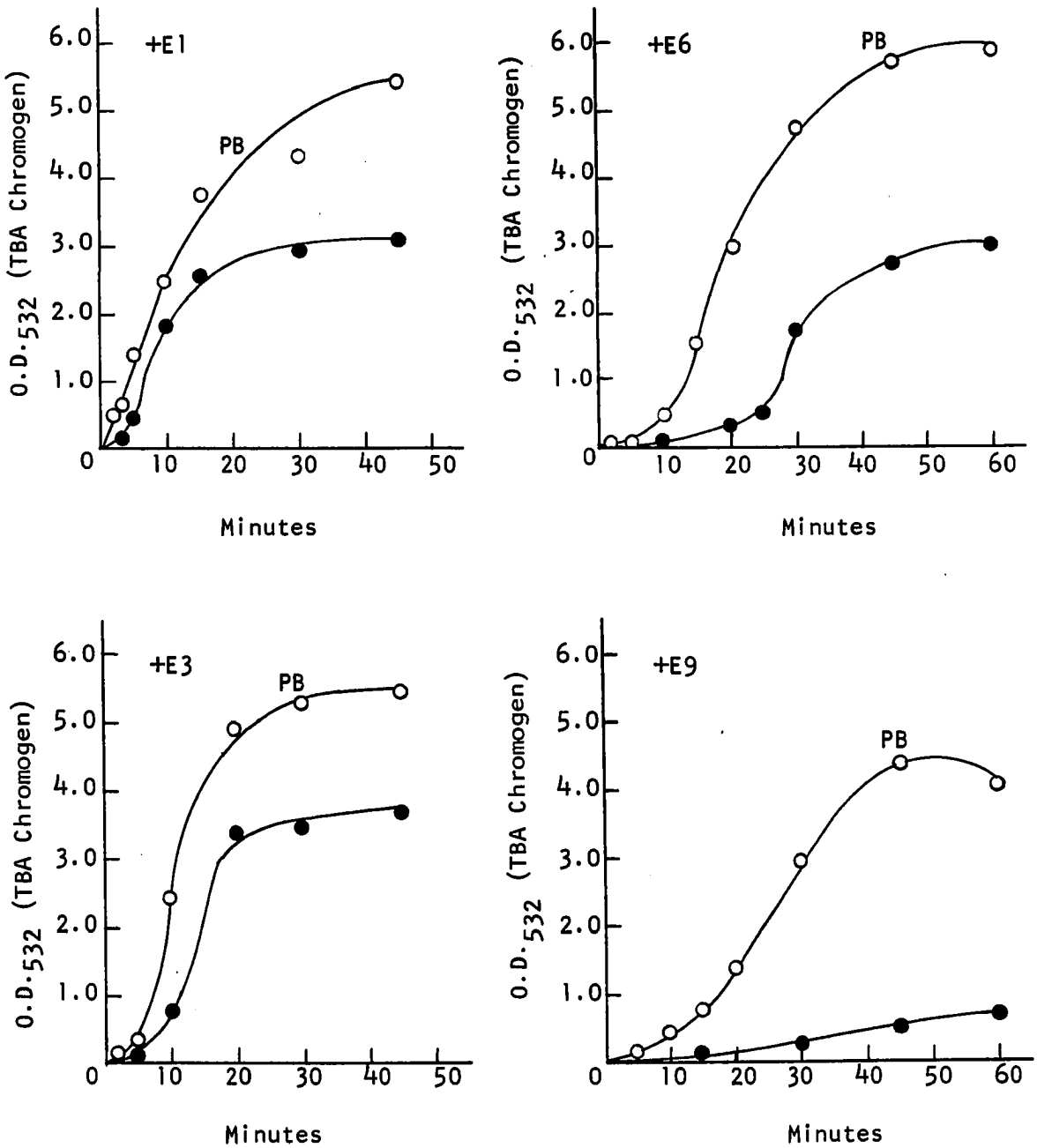


Figure 19. Comparison of lag periods in chromogen formation by microsomes from α-tocopherol-supplemented rats with and without phenobarbital injections. Incubation conditions same as Figure 17. Diet given is shown in each diagram. ●, animals not injected; ○, animals injected with phenobarbital (5 daily injections, 100 mg/kg body weight). Dietary designations given in upper left corner of each graph. PB denotes phenobarbital injections.

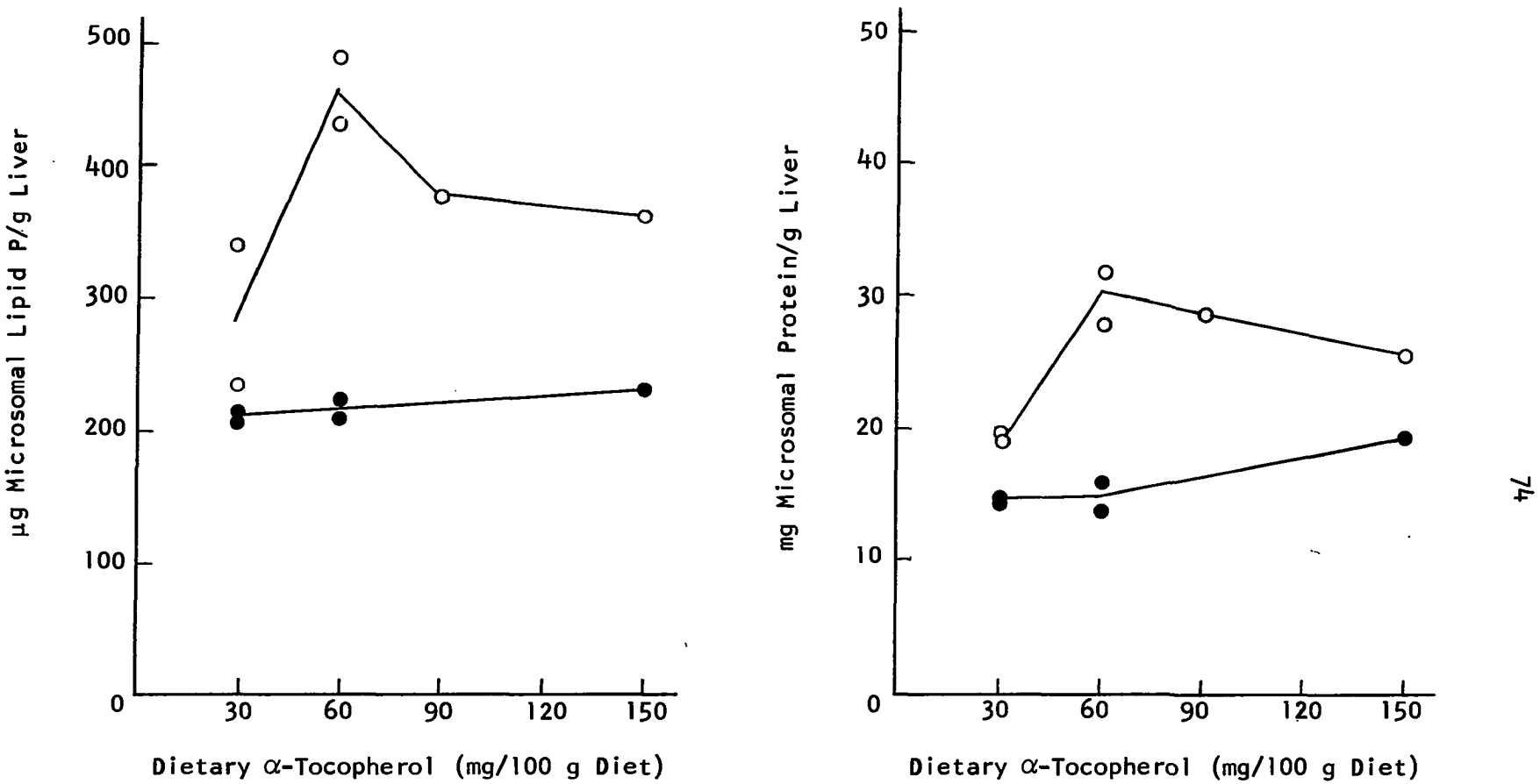


Figure 20. Effect of dietary levels of α-tocopherol on the amount of phospholipid (left) and protein (right) in liver microsomes from rats with and without phenobarbital injections. ○, animals received 5 daily injections of phenobarbital (100 mg/kg body weight); ●, no injections.

phosphorus was approximately doubled in the injected animals. Table 15 shows the fatty acid distribution and effect on liver weight per 100 g body weight and α-tocopherol analyses. Slight but consistently higher liver weights per 100 g body weight were observed in phenobarbital injected animals. The fatty acid composition of microsomes from animals fed the laboratory diets was quite different from that of microsomes from animals fed the commercial pellet ration. Animals maintained on laboratory diets contained much less C18:2 and C20:4 but somewhat more C20:5 and C22:6 than the pellet animals. On the whole the animals on laboratory diets produced microsomes with lesser percentages of unsaturated fatty acids than the pellet animals. Increasing levels of α-tocopherol in the diet appeared to decrease the amount of C22:6 present. Consistent phenobarbital effects on the fatty acid composition of microsomes were not observed.

Effect on TBA Chromogen Production of Placing α-Tocopherol-Supplemented Animals on α-Tocopherol-Deficient Diet

Animals were placed on diet +E6 for 5 days and then placed on an α-tocopherol-deficient diet. As shown in Figure 21 the α-tocopherol effect on chromogen production was lost within 48 hours after α-tocopherol supplementation was terminated indicating that the effect is easily and quickly reversible.

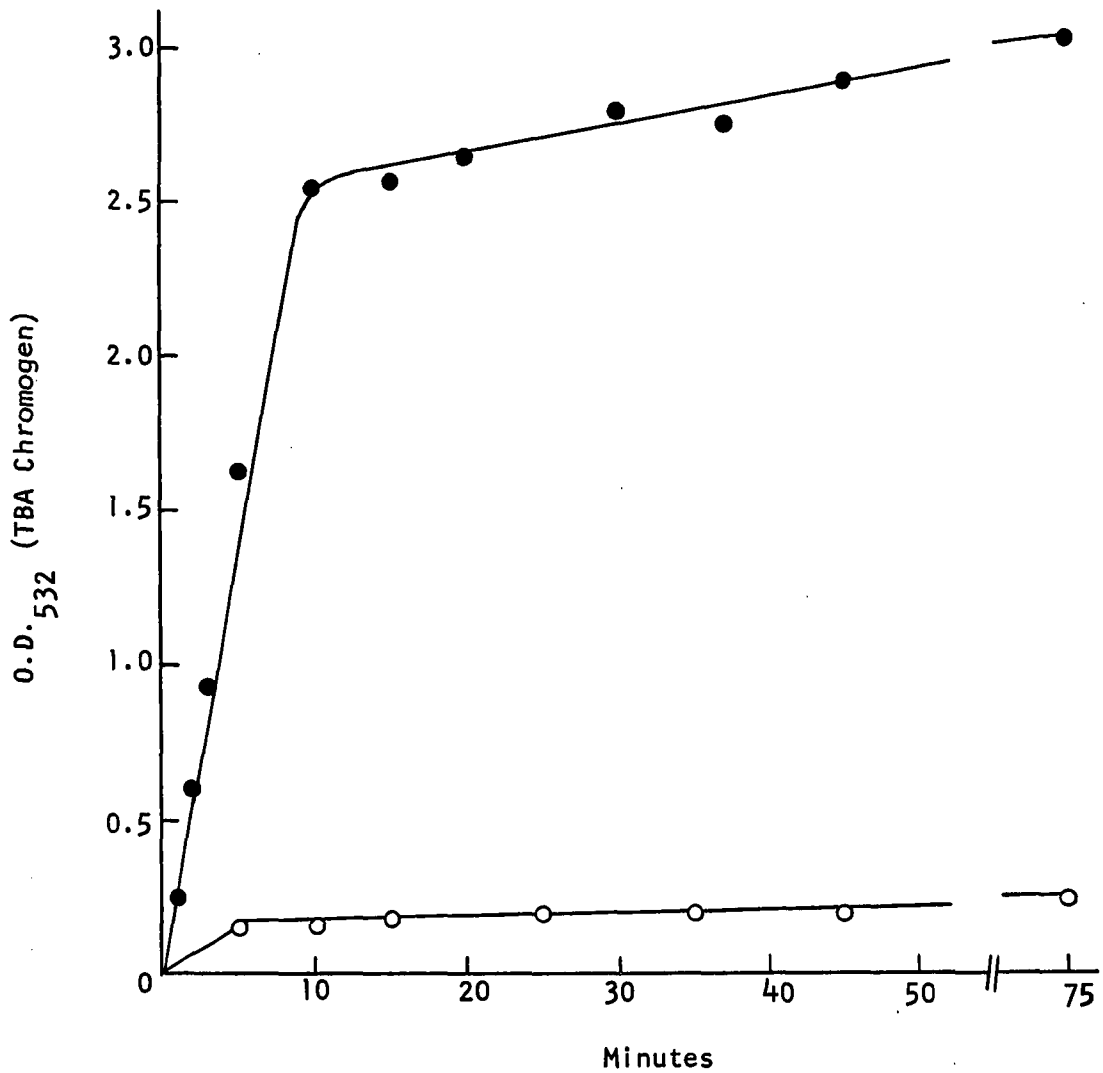


Figure 21. In vivo reversal of α-tocopherol-induced lag period in TBA chromogen production. ○, Animals were supplemented with diet +E6 for 5 days; ●, Animals placed on diet -E after previous supplementation with diet +E6 for 5 days. Incubation system and conditions same as Figure 17.

CHAPTER IV

DISCUSSION

Relationship Between Microsomal Electron Transport and Lipid Alterations

It is probable that there are two electron transport systems present in the microsome (1,3,19,20). Evidence from Ernster's laboratory (14,19) indicates that the chain involved in drug metabolism is involved in lipid peroxidation. However, recently Beloff-Chain et al. (56) presented evidence for the involvement of cytochrome b_5 in this process and cytochrome b_5 is not thought to be a component of drug-metabolizing systems. Orrenius et al. (14) showed that the addition of drugs such as aminopyrine or codeine inhibited lipid peroxidation. They also reported that the drug-metabolizing enzyme(s) lost activity by aging more rapidly than the relatively stable lipid-peroxidizing activity, and that once the drug-hydroxylating activity was lost, the inhibitory action of the drugs on lipid peroxidation was also lost. It was also reported that carbon monoxide, which inhibits drug hydroxylation, had no effect on either lipid peroxidation (14,17) or luminol-dependent luminescence catalyzed by microsomes (57). Ernster and Orrenius (19) have recently presented evidence that the following enzyme activities are competitively inhibited by TPN and that they have about the same inhibitor constant for TPN: a) aminopyrine demethylase; b) lipid

peroxidation; c) TPNH-neotetrazolium reductase; d) TPNH-DCPIP reductase and e) TPNH-cytochrome c reductase. The K_M for TPNH from our data is approximately 3×10^{-6} M for lipid peroxidation in the presence of a TPNH-generating system, and they report a K_M for TPNH of 2.5×10^{-5} M for the drug hydroxylase system. These K_M values are consistent for a mechanism requiring electrons for peroxidation to come from an electron carrier prior to the hydroxylase. (Qualitatively one would expect half-maximal rates to occur at lower TPNH levels at carriers closer to the TPNH dehydrogenase). Thus, all data considered, it would appear that the electron transport chain involved in drug metabolism is operative in lipid peroxidation and that cytochrome b_5 may also be involved.

The TBA chromogen-forming system worked very efficiently even when extremely low TPNH concentrations (6 μM) were used and the fatty acid changes also occurred under these conditions. Somewhat surprising was the finding that these reactions proceeded rapidly even at 0° C. The requirement for enzymic function at all times was established when it was shown that the formation of chromogen abruptly ceased after the TPNH was totally oxidized and further that this chromogen-producing activity could be restored by the addition of either more TPNH or a TPNH-generating system. The very rapid inhibition of chromogen formation by p-hydroxymercuribenzoate (PHMB), (also see, 19) even when the chromogen was being rapidly formed, confirmed the requirement for an enzyme function at all times. This rapid inhibition by PHMB also precludes a role for substrate quantities of enzyme-formed Fe^{+2} having a role in the peroxidation mechanism. It must be said, however, that added Fe^{+3} could be reduced enzymically in catalytic quantities and thus

still be in accord with the proposal of Beloff-Chain et al. (56) that TPNH reduces Fe^{+3} to Fe^{+2} enzymically and that Fe^{+2} is responsible for lipid peroxidation. An alternative should be kept in mind, however, and that is the possibility that added iron is simply restoring iron lost from a microsomal component during isolation. Bodanska et al. (58) have shown marked influences of the ionic constituents of the isolation medium on the rate of oxidation of TPNH by microsomes. These changes were interpreted in terms of structural changes induced in the microsome by ions, especially Mg^{+2} and Ca^{+2}. These results would lead one to wonder if microsomal components may not be lost during isolation. It should also be pointed out that Beloff-Chain et al. (56) used 100 times more Fe^{+2} (1 mM) than we have used of Fe^{+3} in this study to obtain about the same rate of chromogen formation. Also Hochstein (59) using 12 μM Fe^{+2} (the same molar concentration as that of Fe^{+3} used in this study) in the absence of ADP found only small increases in oxygen uptake by TPNH oxidation. It is hoped that more about the role of iron in this mechanism will be learned, but this is a part of another thesis (60). Preliminary data have been obtained which indicate that microsomes in the presence of TPN, nicotinamide and a TPNH-generating system can catalyze lipid changes including chromogen formation in the absence of added ADP-Fe^{+3}. Thus, added iron may not be required at all.

A considerable amount of evidence exists indicating the involvement in lipid peroxidation of a free radical generated by a component of the microsomal electron transport chain. As cited above microsomal peroxidation is prevented by antioxidants (23). In the presence of TPNH, microsomes are known (61) to catalyze the oxidation of sulfite which is

a free radical trapping agent. We have found that sulfite present in low concentrations insufficient to trap free radicals has no effect on chromogen formation, but when present in quantities sufficient for efficient free radical trapping, peroxidation is almost totally inhibited. Nilsson et al. (57) have also shown recently that microsomes in the presence of TPNH catalyze luminescence by luminol and this luminescence is increased by the addition of a pyrophosphate of Fe^{+2} or Fe^{+3}. This reaction was also found to increase in liver microsomes from animals injected with phenobarbital (drug hydroxylating enzymes are induced under these conditions) and the reaction was not affected by CO but was inhibited by aminopyrine. These data make it appear that a free radical mechanism is operative.

There are three logical candidates present in the microsome as a source of free radicals for participation in lipid peroxidation. Masters et al. (12,13) have concluded that a flavin semiquinone (a free radical) is involved in the catalytic mechanism of purified TPNH-cytochrome c reductase from microsomes. It would be of interest to see if this isolated enzyme could catalyze the luminescence of luminol or the oxidation of sulfite or the peroxidation of added lipid. Tam (62) has shown that the lipid-peroxidizing activity of microsomes can be "solubilized" by sodium cholate and that the soluble preparation when fractionated by ammonium sulfate can catalyze changes in added lipid. Ernster's finding (19) that TPNH-cytochrome c reductase in the intact microsome is not inhibited by PCMB while lipid peroxidation is, would make it appear that the flavin semiquinone is not the source of free radicals in question. Studies on the effect of cytochrome c, menadione, DCPIP,

and neotetrazolium (NT) on lipid peroxidation should be done in order to determine the relationship between the microsomal flavoproteins and lipid peroxidation. A consideration of possible antioxidant effects by such electron acceptors would be required, however. A second possibility as a source of free radicals from the electron transport chain is the iron associated with cytochrome P_{450}. This iron component can in the presence of TPNH give rise to a signal detectable by electron paramagnetic resonance characteristic of unpaired electrons (1). This pigment has been shown to be involved in drug hydroxylations (20,21) and has been shown to combine with CO. Carbon monoxide inhibits drug hydroxylation and has no effect on lipid peroxidation and thus it would appear that cytochrome P_{450} is unlikely as a source of free radicals for lipid peroxidation unless as in the case of cytochrome a + a_3 (63) and xanthine oxidase (64) it is a protein with more than one functionally active site. A third possibility as a source of free radicals and perhaps the most likely, by elimination, is a component whose properties are not known but may be a non-heme iron protein (NHI). Omura et al. (20) have succeeded in the reconstitution of adrenal mitochondrial steroid 11β-hydroxylase by the recombination of purified TPNH-DCPIP reductase, NHI and cytochrome P_{450}, respectively. Schemes that have been written for the pathway from TPNH to drug hydroxylation in liver microsomes include a component(s) of unknown nature (3,19). Non-heme iron is also a known component of liver microsomes (1). The identity of the functional site(s) in liver microsomes involved in lipid peroxidation will need to await further work on the nature and identity of all components of the microsomal electron transport chains.

Lipid Changes Associated with Microsomal Oxidation of TPNH

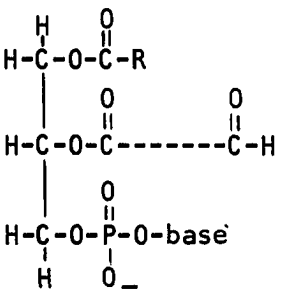
Gross Analyses of Lipid Changes

Lipid peroxidation is associated with the microsomal enzyme L-gulonolactone oxidase of α-tocopherol deficient rats (33,34) but it has been difficult to show significant lipid changes in microsomes during its activity (35). Kitabchi et al. (65) have recently shown that microsomes from beef adrenal cortex incubated in the presence of ascorbic acid show losses of polyunsaturated fatty acids (PUFA).

The lipid changes which occur during the action of microsomal TPNH oxidase from liver in the presence of ADP-Fe^{+3} are striking and represent the only case known to the author in which enzyme catalyzed changes of this type have been demonstrated. Staudinger and Zubrzycki (66) have reported that liver microsomes hydrogenate unsaturated fatty acids in the presence of TPNH.

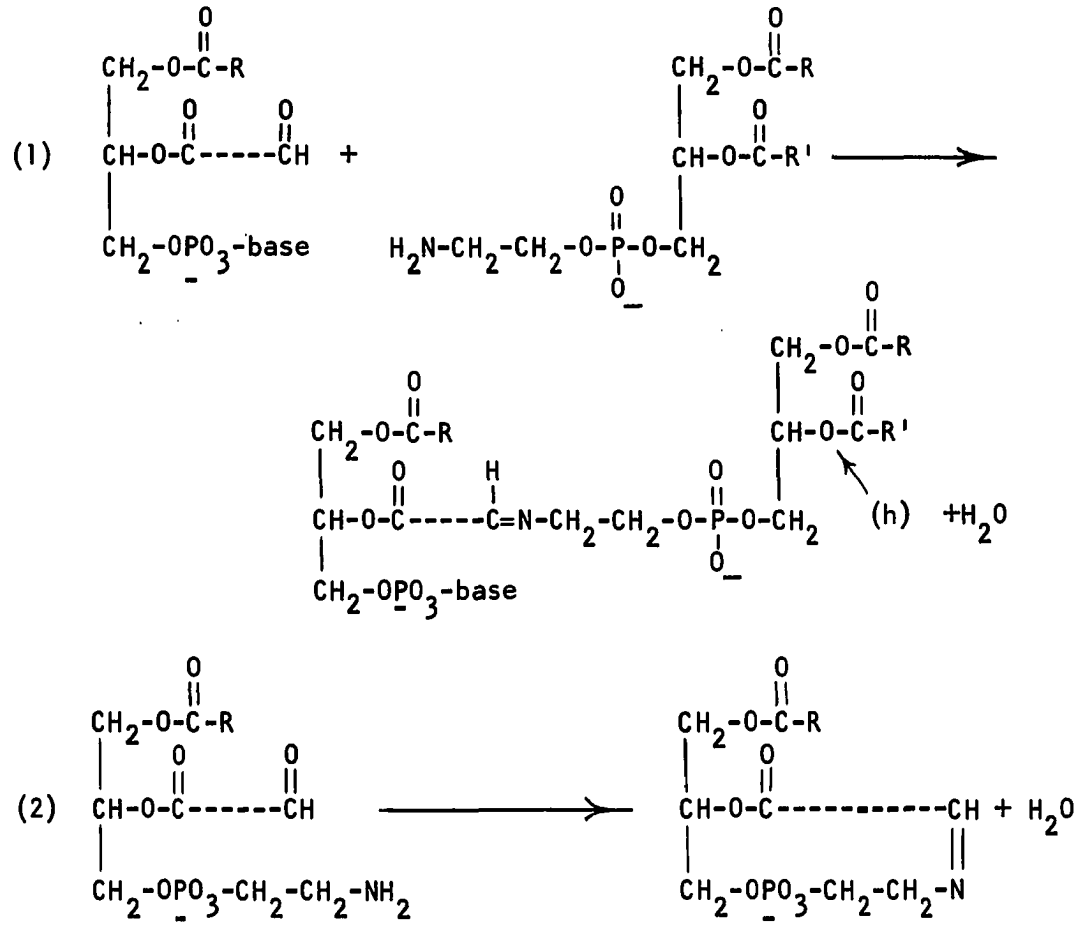
Comparison of the amount of lipid phosphorus extracted from microsomes incubated with ADP-Fe^{+3} in the presence (experimental) and absence (control) of TPNH showed no losses of extractable lipid phosphorus in the experimental lipid. This indicated that extensive lipid-protein polymerization reactions of the type reported by Tappel (67) had not occurred. Thin layer chromatographic analyses showed marked losses (50%) of phosphatidyl ethanolamine and some losses of phosphatidyl choline in the experimental lipid and a concomitant appearance of slow-moving materials, some of which were faintly ninhydrin positive. Losses in double bonds and the PUFA (arachidonic and docosahexenoic acids) without loss of ester groups indicated chain scission of the PUFA present in the phospholipids. The possibility that the loss of arachidonic and

docosahexenoic acids was due to a hydrolytic reaction was eliminated by a lack of loss of ester groups and by the fact that these acids were still lost when the Folch extraction was carried out in an acidic medium. Under these conditions free fatty acids would extract into chloroform and therefore would be detected in the gas-liquid chromatographic analysis of methyl esters derived from sample lipids. The observation that lipid amino groups disappeared upon aging of the isolated experimental lipid and paralleled a decrease in the lipid-bound thiobarbituric acid-reacting chromogen, suggested a possible carbonyl-amino condensation to form a product such as a Schiff base. This type reaction was suggested by Lea (68) for altered phosphatides. Added polarity in phosphatides is known to slow their movement on silicic acid TLC plates. For example, lysophosphatides move more slowly than the corresponding intact phosphatide by the methods used in this study. Carbonyl groups associated with the phosphatides were detected and one would expect a carbonyl compound of the type shown below to cause the phosphatide to move more slowly. Lea and Nendoerffer (69) recently reported, however,



that peroxidized phospholipids, when chromatographed on silicic acid TLC, behaved in a way similar to that of the unaltered compounds. A polymerized product is another possibility and is perhaps more likely to

be the slow-moving material noted on TLC and column chromatography. A condensation product between an aldehyde of one phospholipid molecule and the lipid amino group of another would give a compound which would probably move with difficulty on TLC plates. Other evidence that was



obtained which is consistent with this type reaction is: a) Silicic acid column chromatography of experimental lipid resulted in the retention of a yellow material at the top of the column which could not be eluted except by methylation with BF_3-methanol. b) The lysophosphatides from phospholipase A treated experimental lipids had an ester/P ratio of 1.25 while that of the control lipid was 1.0. Assuming that bond (h) in reaction (1) above was hydrolyzed by the phospholipase, the

liberated hydroxy group may render the dimeric compound insoluble in the phospholipase A reaction medium (ether) and thus remove the β ester linkage of the second phosphoglyceride from contact with the enzyme. Such cleavage would result in an ester/P ratio observed in experimental lysophosphatides. c) Such condensation reactions would also account for the fact that only small amounts of new compounds detected by GLC were found even though losses of PUFA were quite large. The transesterification procedure would methylate all ester linkages, but any fragment attached to the amino group of another phosphatide or, alternatively, an intramolecular condensation product, see reaction (2), would remain attached to the phosphorylglycerol moiety after methylation and would not extract into hexane with the methyl esters. Another reason for loss of oxidation fragments may be that fragments undergo reactions of unknown nature during the methylation procedure since dark colored products are formed during methylation. The use of reagents that would be expected to block a condensation between carbonyl and amino groups have failed thus far to yield useful information.

The loss of such large amounts of arachidonic and docosahexenoic acids (50 to 90%) from the phospholipid fraction is very striking and the possible significance will be discussed later.

Column chromatography on silicic acid showed that the phosphatides, in general, lost PUFA from the experimental lipid. No apparent specificity for a phosphatide class was observed with regard to losses of PUFA. These results confirmed quantitatively what TLC showed qualitatively, namely losses of PE and PC and the appearance of more polar materials which could only be removed from the column by methylating procedures.

Effect of Incubation Parameters on Lipid Changes

Although initially 5 atmospheres oxygen pressure was used for the microsomal incubation system in order to increase the possibility of finding lipid changes, it was later found that the same lipid changes occurred when the enzyme system was incubated in air. Very mild incubation conditions were found to be equally effective in producing lipid alterations. For example, incubations carried out in air at 0^{o} C showed lipid alterations similar to those occurring at 37^{o} C, but the reaction rates were lower at 0^{o} C. The enzyme system also efficiently catalyzed lipid alterations in the presence of 6×10^{-6} M TPNH and a TPNH-generating enzyme system. Preliminary data indicates that the ADP-Fe^{+3} complex may not be required under conditions in which TPN is maintained in the fully reduced form. Oxidized TPN is a competitive inhibitor of lipid peroxidation (19). These data taken as a whole make it encouraging to look for similar changes under <u>in vivo</u> conditions.

The gross lipid changes observed occurred parallel to the time course of the TBA chromogen synthesis curve. Under all experimental conditions tested to date the degree of lipid changes was reflected by the TBA chromogen produced indicating that the two effects are likely a part of the same general process. The more highly-unsaturated fatty acids were oxidized at a faster rate and the degree of unsaturation appears to govern the rate of alteration of the fatty acids. The rates of oxidation were in the order: C22:6 > C20:5 > C20:4 $\gg$ C18:2. Ease of peroxidizability of PUFA follows the same order.

The Site of Lipid Change

This study shows that the glycerophosphatides are major components altered by microsomal TPNH oxidase. The fatty acid distribution of the β position of these phosphatides was such that almost all PUFA was located there. Utilizing phospholipase A to cleave selectively the β fatty acids, it was demonstrated that 40% of the total fatty acid in this position was arachidonic acid and 50% or more of this total was lost from the experimental lipid. Further, dinitrophenylhydrazones prepared from the phospholipids of experimental incubation systems were released from the phosphatides after treatment with phospholipase A, indicating the presence of carbonyl groups in the β fatty acid moiety. These data indicate that the phospholipid is certainly altered under the experimental conditions described and, further, that the primary point of attack is the unsaturated fatty acid in the β position of the glycerophosphatides.

The Nature of the Lipid Products

The spectral properties of the lipid from the experimental chloroform fraction were similar to those given by Saslaw et al. (70). They found that the water extracts of ultraviolet irradiated linolenic acid contained materials with an absorption maximum at 225 mμ and a broad shoulder at about 275 mμ. Unsaturated carbonyl compounds with conjugated dienes give a spectrum similar to this (70). The distribution of carbonyl compounds between the Folch solvent phases is interesting but the data are insufficient to draw more than tentative conclusions. If an average of the molar extinction coefficients for dinitrophenylhydrazones reported by Schwartz (38) is assumed (30,000) there was about

40% as much carbonyl present in the chloroform fraction as there was loss of arachidonic acid. The breakdown of lipid hydroperoxides in model systems is very complex (26) but chain scission can occur in such a way that an aldehyde and a hydroxy compound (26) is produced with each chain scission. If such a fatty acid chain splitting mechanism were operative in the microsomal system and if the process were random, one would expect about half of the aldehydes formed to be phospholipid bound. From this it can be concluded tentatively that carbonyl compounds present in the lipid-containing chloroform fraction constitute a major portion of the products formed from the microsomal PUFA.

The TBA chromogen was also distributed between solvent layers of the Folch extract and this distribution was the same throughout the time course of the enzyme reaction. Recent experiments have shown that the ratio of the chromogen found in the Folch chloroform fraction to that in the water-methanol fraction can be changed by varying the enzyme incubation temperature. At low temperatures (0^{o} C) 50% of the chromogen was in each solvent layer but at 37^{o} C it was mostly in the water-methanol layer. The reason for this is unknown but it would appear that higher temperatures facilitated release of lipid-bound chromogen(s) to a water-methanol soluble form. The identity of the chromogen(s) is the subject of another thesis (60). However, its carbonyl nature was strongly implied when it was found that the TBA test was negative if the reaction mixture was first heated in the presence of semicarbazide or dimethylhydrazine prior to the addition of TBA. Attempts to find other likely products such as hydroxy compounds and hydroperoxides have not been undertaken as yet.

The Effects of α-Tocopherol

The TBA chromogen formation curve exhibited a lag period when microsomes from animals supplemented with α-tocopherol were used. By increasing the amount of α-tocopherol in the diet the lag period could be lengthened to a point that the peroxidation reaction was entirely eliminated. In vitro addition of α-tocopherol also prevented lipid peroxidation. An interpretation of the lag period could be that it is the length of time required to destroy α-tocopherol by free radicals formed by the microsomal electron transport chain. Alternatively the ADP-Fe^{+3} could be destroying the α-tocopherol. The latter appears to be less likely in view of the fact that lower levels of ADP-Fe^{+3} reduced the rate of chromogen formation but had no appreciable effect on the lag period. Animals placed on diets with levels of α-tocopherol high enough to produce long lag periods or completely eliminate chromogen production, rapidly lost this α-tocopherol effect when placed on an α-tocopherol-deficient diet. Within 48 hours after placing these animals on diets deficient in α-tocopherol, the lag period in the chromogen synthesis was virtually lost. This probably represents a rather rapid turnover of α-tocopherol in the liver microsome. Carpenter (71) has found that L-gulonolactone oxidase from liver microsomes does not respond within 48 hours to the removal of dietary α-tocopherol as described above.

The intraperitoneal injection of phenobarbital into rats has been shown to induce synthesis of the microsomal enzyme system associated with drug hydroxylations (15,72,73). Our data are in agreement with these reports in that increased amounts of microsomal lipid and protein per g of liver wet weight were observed. Injection of animals with

phenobarbital did not produce an effect on either the rate of chromogen formation or on the total amount of chromogen formed when the data were expressed per mg protein, however, both rates and totals were 50 to 100% higher when the data were based on liver wet weight. Microsomes from animals fed α-tocopherol supplemented diets and, in addition, injected with phenobarbital showed considerably shorter lag periods in TBA chromogen production than microsomes from corresponding animals (control set) fed the same diet but not injected. α-Tocopherol analyses of microsomes from animals fed the various diets with or without phenobarbital injections showed rather large variation from one set of animals to the next which were subjected to the same conditions. It is not certain whether or not the values reported for α-tocopherol analyses represent only α-tocopherol and therefore conclusions about microsomal α-tocopherol levels are not possible. If it is assumed that injection of phenobarbital has no effect on the absorption of α-tocopherol by the intestinal tract or on the storage of the vitamin in liver microsomes, some interesting speculations can be made in connection with the lag period observed. One case involves the assumption that the α-tocopherol content of microsomes, as isolated, depends on the dietary α-tocopherol level under which the animals were fed and that phenobarbital injections have little or no effect on this level. Thus, the microsomal electron transport chain in phenobarbital treated animals would be induced and the amount of α-tocopherol per unit of enzyme system would be lower than that in the control animals. This lower ratio of α-tocopherol to enzyme would result in a dilution effect on α-tocopherol and would probably result in a diminished lag period. A more attractive case would

be the assumption that absorption and storage of α-tocopherol are unaffected by the phenobarbital injections and that the α-tocopherol content of microsomes is lower in the injected animals. In this case the *in vitro* interpretations would be similar but there may be more *in vivo* implications. Lower α-tocopherol content under these assumptions would indicate a faster turnover of α-tocopherol in the injected animals. This would be the expected result if α-tocopherol were acting as an antioxidant or regulator by some other means *in vivo* and were regulating an increased number of free radicals produced by the induced microsomal electron transport chain associated with drug metabolism. Experiments designed to test this possible interpretation should be devised.

Lipid alterations in microsomes from animals on α-tocopherol-supplemented diets were essentially similar to those occurring in microsomes from animals fed a commercial pellet diet. Lipid changes were reflected by the TBA chromogen produced.

Interpretations and Experimental Approaches

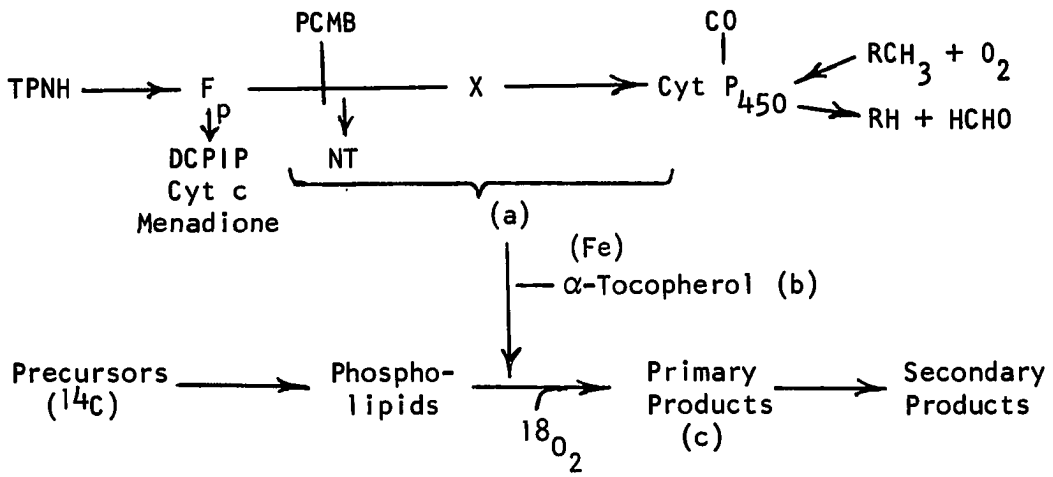
Present data indicate that during the enzymic oxidation of TPNH rat liver microsomes also catalyze lipid alterations that are characteristic of lipid peroxidation. There is a requirement for continuous enzymic activity in order for lipid changes to occur continuously. The nature of the link between the enzymic oxidation of TPNH and the lipid changes is yet unknown. It is possible that a free radical associated with a component such as non-heme iron might be involved. The fact that enzymic activity is required at all times is in itself inconsistent with lipid peroxidation in model systems. In model systems lipid peroxidation is a self-perpetuating reaction once started unless oxygen is

removed. For example, α-tocopherol when added to a model system undergoing rapid lipid peroxidation has little inhibitory effect on the process (74). It would appear that in the microsome either there are large quantities of free radical "chain breakers" or that something peculiar to the structure of the microsomal membrane prevents efficient chain propagation reactions. Still another possibility, which is more attractive, is that there is a fundamental difference between the lipid peroxidation in model systems and the changes catalyzed by microsomes. Experimental conditions have not yet been found under which the TBA chromogen in the lipid fraction was greater than 50%. Poyer (60) has found that the TBA chromogen(s) of air-oxidized methyl arachidonate is predominantly extractable into chloroform. These data imply that the microsomal enzyme system may be involved in the cleavage of the carbon to carbon bond of the β polyunsaturated fatty acid of the glycerophosphatides or, alternatively, a nonenzymic chain scission occurs which is faster than the enzymic reaction.

There is not enough information available to propose a detailed mechanism for these reactions. The following points would need to be considered in any mechanism that was considered seriously: a) free radical participation in the initiation process but some means of keeping the overall reaction under enzyme control; b) reactions that lead ultimately to chain scission of PUFA present in the microsome; c) possible but not certain involvement of added iron and d) involvement of α-tocopherol in the control of the mechanism.

In consideration of the possible experimental approaches to microsomal peroxidation of lipids it is convenient to refer to the inter-

relationships shown in the following diagram:



In Vitro Experiments

The final identity of the link(s), (a), of the microsomal electron transport chain involved in the lipid changes observed will need to await the isolation and characterization of all components of the chain. Present information, however, indicate the site(s) lies between the TPNH-oxidizing flavoprotein and cytochrome P_{450}. Further information could be obtained by a study of the interrelationships of the various electron acceptors such as DCPIP, cytochrome c, menadione, NT and drugs on the one hand, and inhibitors such as PCMB, sulfite and other possible inhibitors on the other and their effects on the peroxidation reactions. The possible inhibitory effect of the free radical, diphenylpicrylhydrazyl, and luminol should also be investigated. The "soluble" system of Tam (62) may make possible the use of competing substrates in the elucidation of the site of the electron transport chain involved in lipid peroxidation. Of particular interest would be the interrelationship between TPNH-NT reductase and lipid peroxidation

both of which are PCMB sensitive (19). The reductase is also sensitive to lipase (12) and we have found that the peroxidation activity is sensitive to snake venom (presumably phospholipase A). It would be of interest to see if NT and lipid compete for the same site. Brush and May (75) have reported the use of competitive substrates in a study of the mechanism of action of L-gulonolactone oxidase in liver microsomes and concluded that oxygen and DCPIP react at different sites. The use of kinetics in such studies could prove fruitful but as pointed out by Webb (76), interpretation of kinetic data of complex enzyme systems is difficult.

Information on the role of α-tocopherol, (b), in the in vitro system could be obtained by an analytical study of the α-tocopherol level in control and experimental lipids utilizing microsomes from animals supplemented with high levels of dietary α-tocopherol. (Experiments of this type are already in progress). The use of ^{14}C or ^{3}H α-tocopherol would facilitate finding α-tocopherol derived products if they are formed by the system. Also careful studies of lipids from microsomes extracted prior to the end of the lag period of TBA chromogen formation should be done. If it should happen that lipid changes, including conjugated diene formation (determined by ultraviolet absorption), do not occur during the lag period one would conclude that the role of α-tocopherol was to prevent the initial reaction of the sequence. If on the other hand, α-tocopherol acted as a lipid free radical trap it would seem almost certain that conjugation of lipid double bonds would occur during the lag period and this could be detected spectrophotometrically. If fatty acids with conjugated double bonds were formed under these

circumstances they would probably not be detected by GLC analyses.

Many of the reactions that occur in the lipid are not known except by implication. Failure to find phospholipid bound fatty acid derivatives that can be methylated and chromatographed by GLC has been disturbing. The use of microsomes labeled with ^{14}C arachidonic acid would perhaps give a great deal of information concerning the distribution of the fragments during isolation of the lipids and ultimately lead to a better understanding of the nature of such products. Useful information in the study of the stoichiometry of the reaction could also be obtained from such experiments. That the carbonyl-forming reactions described constitute a major pathway in the destruction of arachidonic and docosahexenoic acids seems evident, however, the stoichiometry between oxygen uptake, fatty acids lost, carbonyls formed and TPNH oxidized should be determined. The relationship between oxygen uptake and TPNH oxidized may not be necessarily meaningful since this relationship can be altered by the experimental conditions used (77). The use of $^{18}O_2$ as the gas phase would be useful in determining the ratio of carbonyl oxygen formed and oxygen incorporated into other functional groups formed. The amount of ^{18}O released to water by the interaction of carbonyls and DNPH would represent the amount of carbonyl compounds formed. Comparison of this value with the total ^{18}O-containing organic compounds, corrected of course for that which was present in the control incubation system, and the overall oxygen uptake, determined manometrically, would give quantitative information concerning the relative importance of the reactions involved. (This study would be easier or perhaps may require the use of ^{14}C-labeled PUFA-containing microsomes). Once the relative quantities

of the various functional groups were known, intelligent use of various reactions of organic chemistry should lead to a somewhat detailed analysis of the products of the reaction. Knowledge of the products formed *in vitro* would be valuable in attempting to find the same or similar products formed *in vivo*.

A particularly interesting tool is now available and should prove very useful in studies of the type listed above, and that is the use of the mass spectrometry in conjunction with gas-liquid chromatography. Both techniques are extremely sensitive, give complementary information and can be arranged so that most of the sample can be recovered and used for further study. Ryhage (78) has used the mass spectrometer in lipid analysis. The technique has the advantage that molecular weights are given directly from the fragment pattern in many cases (79). Incorporation of $^{18}O_2$ into organic compounds could be studied, for example, by comparing fragment patterns of compounds formed in an $^{16}O_2$ atmosphere with those formed in an atmosphere rich in $^{18}O_2$. Compounds containing the heavier isotope would display a fragment pattern in which fragment weights were shifted by two atomic weight units per oxygen atom in the fragment.

In Vivo Experiments

It cannot be said that the reactions described in this study have any significance *in vivo* since the experimental design was such that *in vivo* information was not obtained. That these reactions appear to be enzyme controlled at all times suggests that the same type reactions may occur *in vivo*. It would be especially encouraging if further work on the iron requirement should prove that its addition is not required.

Even if an iron requirement remains valid it cannot be said that pyrophosphate bound iron does not exist in the cell. The reactions occur under physiologic TPNH concentrations (6 μM) and temperature conditions. Gilliam (80) has preliminary evidence that the ratio of ^{14}C-acetate incorporated into PUFA to that incorporated into saturated fatty acids is higher in the liver of chicks which are deficient in α-tocopherol than in the control animals. These results imply a role for α-tocopherol in the metabolism of PUFA. If the reactions we have described occur in vivo one would predict an increased turnover of PUFA under experimental conditions that would favor the process, such as α-tocopherol deficiency. It would seem justifiable then to devise experiments to test the hypothesis that a process resembling lipid peroxidation catalyzed by components of the endoplasmic reticulum occurs in vivo and that α-tocopherol is involved in its control. In the author's opinion it should not be decided in advance that such a process, if it occurs in vivo, has strictly pathologic consequences. It is known that the components of the endoplasmic reticulum, including lipid, turnover rather rapidly (16,18). One could imagine that controlled reactions similar to lipid peroxidation could be involved in such reactions as those that initiate the breakdown of the endoplasmic reticulum. Other processes that would be regarded as physiologic could also be visualized as involving reactions of this type.

If peroxidation of lipids occurs in vivo several points are evident: a) peroxidation products are formed whether they have been detected or not (item (c) in the scheme above); b) substrates are used; c) experimental conditions which promote acceleration of the process will result

in at least an increased turnover rate of the lipids involved and possibly a decreased content of the lipid substrates (PUFA) undergoing peroxidation in the tissues (depending on the relative rate of lipid synthesis and breakdown). Based on these points, experiments can be suggested to investigate: a) possible products formed; b) factors that will influence the lipid substrate formation and/or breakdown; c) factors that alter the amount of biological catalysts present; d) combinations of these factors.

Efforts to find products derived from lipid peroxidized in vivo under carefully controlled experimental conditions have been largely unsuccessful even when the experimental animal was put in a situation of stress which should result in increased peroxidation of lipid, such as α-tocopherol deficiency (29). As stated before this could be largely a matter of further metabolism of the primary products to unrecognizable secondary products at rates too rapid for accumulation of such recognizable products. Placer (81) has suggested the use of certain inhibitors of the metabolism of functional groups such as aldehydes which are potential products of lipid peroxidation. Experiments of this type are worthy of consideration. Use of radioactive precursors of PUFA that would yield a maximum of specific incorporation into lipids with a minimum amount of incorporation of radioactivity into pathways unrelated to lipid metabolism is desirable and should make the task of finding products of lipid peroxidation much easier.

An experiment that may be difficult to carry out that should yield interesting information is as follows: Inject an animal with ^{14}C-linoleic or arachidonic acid and after the maximum incorporation of ^{14}C into

lipid has occurred place the animal in an atmosphere rich in $^{18}O_2$. After the oxygen exposure is completed all ^{14}C from the organs could be isolated and separated after a Folch extraction into convenient pools, such as lipid, water soluble, protein bound etc. The amount, if any, of ^{18}O incorporated into carbon-containing compounds could be determined and the ratio of ^{14}C to ^{18}O should give information about the possibility that peroxidation had occurred. If the ratio were encouraging, attempts could be made to fractionate and characterize ^{14}C compounds and note changes in ^{14}C to ^{18}O ratios. Only one pathway of fatty acid catabolism has been described that would incorporate molecular oxygen into lipid and that is omega oxidation, in which case dicarboxylic acids are formed. The predominant pathway in lipid breakdown is apparently β-oxidation and this pathway incorporates oxygen from water into lipid products. Thus, the primary pathway of molecular oxygen in the oxidation of lipids would be the mitochondrial electron transport chain in which case water is formed. If lipid peroxidation occurs *in vivo*, experiments of this type would yield ^{18}O-containing compounds that fractionate with the ^{14}C compounds derived from lipid peroxidation. As was mentioned above the corresponding *in vitro* experiment would also be of interest and probably should be done first. Again it would appear that good use of mass spectrometry could be applied to a study of this type.

A second obvious fact if lipid peroxidation occurs *in vivo* is that lipid substrates are used. The highly-unsaturated fatty acids are likely to be the substrates and one would expect a decrease in the amount of PUFA when the animal is subjected to conditions designed to increase

lipid peroxidation. Literature reports on this point are in conflict in that reports have been made that PUFA increase (82), do not change (83) and are decreased (84) in α-tocopherol deficiency. Results by Shires (85) indicate that there is no difference in the fatty acid composition of tissues from chicks that are sufficient or deficient in α-tocopherol. Again this may be explained on the basis that the PUFA are resynthesized sufficiently fast that differences cannot be detected. Efforts to find losses of PUFA due to lipid peroxidation can be directed in the following ways: a) reduction in the rate of synthesis of PUFA by limiting the amount of precursors available or inhibition of enzymes on the lipid biosynthetic pathway in α-tocopherol-sufficient and -deficient animals; b) supplying experimental conditions that should increase the peroxidation rates; c) a combination of these conditions. Perhaps it would be possible to reduce the rate of PUFA synthesis thus making the peroxidation reactions faster than the synthetic reactions by reducing for a relatively short period of time the amount of dietary linoleic acid. Care would need to be taken not to limit linoleic acid long enough to produce essential fatty acid-deficiency complications. This should be no problem since fatty acid turnover is rather rapid. Fasting may produce the same result on PUFA synthesis if it were for a relatively short time. Comparison of lipid from animals on α-tocopherol-sufficient and -deficient diets under these circumstances might show differences in PUFA composition. Another approach, if possible, would be to inhibit chain elongation and/or the desaturation reactions involved in the synthesis of arachidonic and docosahexenoic acids. An added source of stress may be the induction by drug administration of

the microsomal hydroxylases for the purpose of supplying the liver with a proportionally greater source of free radicals to carry out peroxidation. Comparisons of the fatty acid content of microsomes from animals injected with phenobarbital and on α-tocopherol-supplemented and -deficient diets with the corresponding control sets without injection would be interesting. The same experiment using low linoleic acid levels in the diet for short periods of time would also be interesting.

In view of the fact that PUFA distribution appears not to change in α-tocopherol deficiency, a higher turnover of PUFA would still be expected in tissues from α-tocopherol-deficient animals if lipid peroxidation is significant in vivo. As stated above, Gilliam (80) has preliminary evidence that the incorporation of ^{14}C-acetate into PUFA is more rapid in the liver of α-tocopherol-deficient chicks than animals supplemented with α-tocopherol. An extension of these results to include the turnover rates of the various fatty acids should give valuable information in support or against in vivo lipid peroxidation. Since variation of factors that will change the rates of lipid peroxidation should as a minimum requirement give corresponding changes in turnover rates of lipids, turnover studies are the most logical preliminary studies in search for in vivo lipid peroxidation. If turnover rates do not change it would be difficult to leave the question of in vivo lipid peroxidation open. On the other hand, turnover studies or any of the experiments suggested here will only give results that indirectly support or are against lipid peroxidation in vivo except those experiments that show directly the formation of products compatible with lipid peroxidation, derived from lipids, under conditions that

are certain to occur *in vivo*.

In conclusion, it would seem that the results from this study and those of others are suggestive enough to stimulate a search for similar reactions *in vivo*.

CHAPTER V

SUMMARY

Lipid alterations occurring during the microsomal oxidation of TPNH have been studied. This enzyme system catalyzed rather extensive changes in microsomal lipids especially in the glycerophosphatides. The lipid changes consisted of extensive losses (50 to 90%) of arachidonic and docosahexenoic acids, losses in lipid associated double bonds and amino nitrogen, and the formation of carbonyl compounds. Alterations involved predominantly the polyunsaturated fatty acids located in the β position of the glycerophosphatides.

Studies of various enzymic parameters and their relationships to the lipid changes revealed that the extensive lipid alterations occurred under very mild conditions compatible with physiological conditions. Reaction products identified were similar to those expected for lipid peroxidation but the overall reactions appeared to be under enzymic control which suggested that either a mechanism different than lipid peroxidation in model systems was operative or that certain enzyme to lipid structural relationships accounted for the differences.

The effect of dietary levels of α-tocopherol on these in vitro reactions was studied. It was found that α-tocopherol in the diet could produce a lag period in the formation of a thiobarbituric acid-reacting chromogen used as an index of lipid peroxidation in this study. Further,

dietary α-tocopherol levels could be increased sufficiently to prevent entirely the production of the thiobarbituric acid-reacting chromogen. Microsomes from animals fed the same diets but injected with phenobarbital displayed shorter lag periods in the thiobarbituric acid-reacting chromogen production than the control animals. The possible role of α-tocopherol in this system was discussed.

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