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RELATIONSHIP BETWEEN MEMBRANE-BOUND OXIDATION SYSTEMS AND LIPID IN NORMAL AND REGENERATING LIVER

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CHAPTER I

INTRODUCTION

An understanding of the cellular function of tocopherols (also called Vitamin E) has been sought since these were first discovered to be required nutritional factors for mammals. A large literature has developed around this subject and many investigators have contributed to the area but no comprehensive explanation for the function of tocopherols has yet evolved.

Deprivation of tocopherol definitely has an effect on the metabolism of lipid in experimental animals. As noted by Menshick (1) the most "striking and common feature" of tocopherol deficiency in mice was the complete absence of subcutaneous and subperitoneal adipose tissue. Studies (2-4) have been made which indicate that tocopherols may function in animals by their lipid antioxidant activity, that is the ability to prevent autoxidation of lipids by molecular oxygen (O_2).

Autocatalytic oxidation of lipids has been extensively

studied in model systems and the general mechanism has been reviewed by Lundberg (5) and Ingold (6). The mechanism appears to be that of a free radical chain reaction which can be described as occurring in four phases:

1) initiation--formation of a free radical on the lipid
2) propagation--formation of a hydroperoxide of the lipid with O_2
3) hydroperoxide breakdown--formation of more free radicals which can initiate additional reaction chains
4) chain termination--formed radicals react with each other or other substances to form stable products

Keeney (7) has reported that the hydroperoxide breakdown results in the formation of many different products, including aldehydes, ketones, carboxylic acids, and alcohols.

Autoxidation can be accelerated by heat, light, and various prooxidant substances which include some metals and their salts, hematin compounds, oxidative enzymes, and photochemical pigments (5). The rate of autoxidation also is proportional to the degree of unsaturation in the lipids being studied. Many substances, called antioxidants, exist which can prevent or slow this oxidation. One of these substances which is active biologically and naturally-occurring is alpha-tocopherol.

Many studies have been made on the effects of feeding

autoxidized lipids to experimental animals (8). Such feeding results in many varied symptoms, ending in death if feeding is extended. The products of oxidized lipids may be generally said to be toxic to mammals when fed in very large quantities, although they do not lead to symptoms developed by animals which are deprived of tocopherol.

The above evidence coupled with studies which have been reviewed by Tappel (3,9) are the basis for suggesting that tocopherols function in mammals by preventing the peroxidation of tissue polyunsaturated fatty acids. This argument rests mostly on the fundamental observation that lipid oxidation products are found in tissues separated from animals deficient in tocopherol and are not found when the animals receive tocopherol before removal of the tissue. Another part of the argument is that the symptoms of tocopherol deprivation are accentuated by feeding autoxidized lipids to animals (8). A second observation is that some tocopherol deprivation symptoms are relieved by the feeding of antioxidants which are structurally dissimilar to tocopherol (10).

An enzymatic system which oxidizes reduced nicotine adenine dinucleotide phosphate (NADPH) in the presence of O_2 and produces a chromogen reacting with thiobarbituric acid (TBA) has been reported by Hochstein and Ernster (11) in rat liver microsomes. The chromogen has been reported as a product of lipid peroxidation. Concurrently Beloff-Chain et al. (12) reported finding the same enzymatic activity and later

(13) confirmed the presence of the chromogen, indicating lipid peroxidation. In a subsequent paper (14) Hochstein et al. reported that chromogen formation associated with this enzymatic activity required iron in either ferric or ferrous form and either pyrophosphate or a pyridine nucleoside di- or triphosphate. Orrenius et al. (15) and later Gotto et al. (16) found that several drugs metabolized by the microsomal NADPH-linked hydroxylating system would decrease the amount of chromogen formation relative to the rate of NADPH oxidation when these drugs were included in the assay mixture. Their results suggested that the drug is competing with lipid for reducing equivalents from the system.

In a recent review of the lipid peroxidation associated with NADPH oxidation, Ernster and Nordenbrand (17) describe the similarities between this system and the non-enzymatic ascorbate-linked lipid peroxidation. Both types of lipid peroxidation cause a striking lysis of microsomes as revealed by light scattering and release of protein (18,19). This lysis is accompanied by characteristic changes in the activity of various microsomal enzymes similar to changes observed when microsomes are exposed to detergents such as deoxycholate.

The changes occurring in microsomal membrane phospholipids as a result of the in vitro activity of the NADPH-linked lipid peroxidation have recently been reported in detail by May and McCay (20,21). Their studies show that this

system requires NADPH and O_2, and oxidizes the polyunsaturated fatty acids, primarily arachidonic acid, which are located in the beta position of the membrane phospholipids. Studies by May (22) show that the enzymic NADPH-linked lipid peroxidation is inhibited in microsomes prepared from animals fed large quantities of tocopherol acetate. Peroxidation activity is highest in animals fed diets restricted in tocopherol content.

Studies by Tappel and Zalkin (23,24) indicate that a similar system may exist in rat liver mitochondria. This system can utilize NADH while consuming O_2 resulting in lipid peroxidation as measured by chromogen formation. Tocopherol fed to the animals from which the mitochondria are prepared inhibits the production of lipid peroxidation products. No study is known to have been made on the effects mitochondrial peroxidation have on the mitochondrial fatty acids. The studies with both the microsomal and mitochondrial systems indicate that parts of the electron transport systems in each of these organelles participate in the production of a chromogen and the oxidation of fatty acids.

The similarities between the <u>in vitro</u> enzymatic destruction of microsomal polyunsaturated fatty acids and the peroxidation of lipids presumed to occur in intact animals or reported in lipids exposed to air prompted us to ask the question: Could the NADPH-requiring phospholipid oxidation activity in the absence of tocopherol be responsible for the

deficiency disease observed in animals deprived of tocopherol?

The purposes of this study and dissertation are:

1) to test the hypothesis that tocopherol functions *in vivo* to prevent NADPH-requiring phospholipid oxidation and as a consequence the abnormal catabolism of the polyunsaturated fatty acids in membrane phospholipids.
2) to search for other factors which affect the *in vitro* measurement of NADPH-requiring phospholipid oxidation.
3) to present an alternative proposal for *in vivo* tocopherol function as a guide to future experimental studies.

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Chickens. One day old white Leghorn cockerels were obtained from the Capitol Hill Hatchery, Oklahoma City, Oklahoma.

Rats. Adult male albino rats (150-300 g), bred and maintained in this laboratory, were used in some of these experiments. These rats were originally derived from the Holtzman-Sprague Dawley strain and are now highly inbred.

All animals were watered with distilled water and fed either a commercially prepared diet or a synthetic diet described below.

Materials for Diets

Casein (vitamin free), vitamins (except alpha-tocopheryl acetate), cod liver oil, and Alphacel (a pure, powdered, non-nutritive cellulose added for bulk) were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. ADM assay protein C-1 (soybean) was obtained from Archer

Daniels Midland Company, Minneapolis, Minnesota. Stripped lard, stripped corn oil (alpha-tocopherol and other volatile materials removed by molecular distillation) and alpha-tocopheryl acetate were obtained from Distillation Products Industries, Rochester, New York. Linoleic acid (purified), sucrose, and inorganic salts were obtained from Fisher Scientific Company, Philadelphia, Pennsylvania. Corn starch (Argo brand) was obtained from Best Foods Division, Corn Products Company, Englewood Cliffs, New Jersey.

Experimental Diets

The experimental diets used in these studies were mixed in the following proportion:

Basal Diet (see Table 1)	100 g
Alphacel	10 g
alpha-Tocopheryl acetate	10 mg

The basal diet for chickens is a modification of the diet used by Kokatnur *et al.* (25) as modified by Caputto *et al.* (26).

For the rats the basal diet used was that of Young and Dinning (27) as modified by Caputto *et al.* (28). Vitamin and salt mixtures (see Table 2) for the rat basal diet were prepared according to the method of Hubbell *et al.* (28,29). For the fat-restricted diet, cod liver oil and lard were left out and 1.3 percent (w/w) linoleic acid was substituted.

TABLE 1

BASAL DIET CONSTITUENTS

Ingredients	Chicken	Rat, Normal	Rat, Fat-Restricted
	g	g	g
ADM Protein	35.30	-----	-----
Casein	-----	17.0	17.83
Sucrose	48.18	37.3	39.12
Corn Starch	-----	36.0	37.76
Corn Oil	10.00	-----	-----
Lard	-----	3.0	-----
Cod Liver Oil	-----	3.0	-----
D, L-Methionine	0.75	-----	-----
Glycine	0.30	-----	-----
Choline Chloride	0.20	-----	-----
Salt Mixture	5.27	3.0	3.14
Vitamin Mixture	27.3×10^{-3}	0.7	0.74
Vitamin A acetate	-----	-----	80.0×10^{-3}
Vitamin D_3	-----	-----	0.33×10^{-3}
Linoleic Acid	-----	-----	1.3

TABLE 2

VITAMIN MIXTURE AND SALT MIXTURE

Vitamin Mixture			Salt Mixture		
Vitamins	Chick	Rat	Salt	Chick	Rat
	mg	mg		g	g
Vitamin A acetate	2.0	---	$CaCO_3$	2.166	1.629
Vitamin D_3	0.15	---	KH_2PO_4	1.050	0.636
Vitamin B_{12}	2.0×10^{-3}	4.6×10^{-3}	$CaHPO_4 \cdot 2H_2O$	0.940	-----
Biotin	60.0×10^{-3}	5.1×10^{-3}	NaCl	0.800	0.207
Thiamine	10.0	0.52	$MgSO_4$	0.250	48.0×10^{-3}
Nicotinamide	10.0	21.0	$FeSO_4 \cdot 7H_2O$	0.030	-----
Riboflavin	1.6	0.52	$MnSO_4 \cdot H_2O$	0.020	1.05×10^{-3}
Calcium Pantothenate	2.0	1.05	$ZnCO_3$	0.010	-----
Pyridoxine HCl	0.6	0.52	$CuSO_4 \cdot 5H_2O$	0.002	1.62×10^{-3}
Folic Acid	0.4	0.52	KI	0.001	0.24×10^{-3}
Menadione	0.5	---	$NaMoO_4 \cdot 2H_2O$	0.001	-----
Inositol	---	104.8	KCl	-----	0.336
Choline Chloride	---	104.8	$MgCO_3$	-----	75.0×10^{-3}
2-methylnapthoquinone	---	26.1×10^{-3}	$AlK(SO_4)_2 \cdot 12H_2O$	-----	0.51×10^{-3}
Dextrose,above triturated in	---	466.1	NaF	-----	3.0×10^{-3}
			$FePO_4$	-----	61.5×10^{-3}

Stock Diets

Chickens. Chickens from the hatchery were maintained for one day on a commercial chick starter diet (All-in-One Krumbies) from Superior Feed Mills, Oklahoma City, Oklahoma. This diet had the following ingredients: ground yellow corn, ground grain sorghums, wheat middlings, dehydrated alfalfa meal (prepared with ethoxyquin), soybean meal, fish meal, meat and bone meal, calcium carbonate, defluorinated phosphate, salt, dried extracted streptomyces meal and fermentation solubles, dried penicillin fermentation solubles, condensed corn fermentation solubles, corn distillers solubles, condensed whey fermentation solubles, corn distillers dried grain, Vitamin B_{12} supplement, Vitamin A palmitate, D activated animal sterol, riboflavin, calcium pantothenate, choline chloride, alpha-tocopheryl acetate, niacin, folic acid, menadione sodium bisulfite complex, ethoxyquin, zinc oxide, manganese oxide, iron carbonate, copper oxide, cobalt carbonate, ethylene diamine dihydroiodide (total added minerals less than five percent). The manufacturer guaranteed the following analysis: crude protein not less than seventeen percent, crude fat not less than two and one-half percent, crude fiber not more than seven percent.

Rats. Rats that were not maintained on an experimental diet were fed a commercial pellet diet from Rockland Laboratories, Tekland Incorporated, Monmouth, Illinois. This diet had the following ingredients: Soybean meal, ground

yellow corn, fish meal, pulverized barley, wheat middlings, ground wheat, dehydrated alfalfa meal, pulverized oats, feeding oat meal, dried skim milk, one percent animal fat, Vitamin A palmitate, irradiated dry yeast, niacin, calcium pantothenate, riboflavin supplement, menadione, Vitamin B_{12}, one percent calcium carbonate, one-half percent dicalcium phosphate, one percent sodium chloride, and traces of manganese oxide, copper oxide, cobalt carbonate, iron carbonate, zinc oxide, and calcium iodate. The manufacturer guaranteed the following analysis: crude protein not less than twenty-four percent, crude fat not less than four percent, crude fiber not more than six percent.

Reagent Chemicals

All chemicals and solvents were reagent grade except n-hexane and toluene which were chromatoquality (Matheson, Coleman, and Bell Company, East Rutherford, New Jersey) and were used as obtained.

Adenosine-5-diphosphate was obtained from PL Laboratories, Milwaukee, Wisconsin.

The following chemicals were obtained from Fisher Scientific Company, Philadelphia, Pennsylvania: tris (hydroxymethyl) amino-methane, trichloroacetic acid, monobasic potassium phosphate, dibasic potassium phosphate, Rhodamine-6-G, sodium potassium tartrate, Phenol Reagent (2N), chloroform, petroleum ether (B.P. 30-60° C) acetone, methanol, diethyl ether (anhydrous), benzene, and the inorganic salts, acids,

and bases used in this study.

The following chemicals were obtained from Eastman Organic Chemicals, Rochester, New York: ascorbic acid, thiourea, 2,4-dinitrophenylhydrazine, 2-thiobarbituric acid, and hydroxylamine-HCl.

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

Boron trifluoride-methanol reagent, phospholipid standards, neutral lipid standards, and fatty acid methyl ester standards were obtained from Applied Science Laboratories, State College, Pennsylvania.

Absolute ethanol and ninety-five percent ethanol were obtained from U. S. Industrial Chemical Company, New York, New York.

The following chemicals for scintillation spectrometry were obtained from the Packard Instrument Company, La Grange, Illinois: 2,5-diphenyloxazole (PPO), 1,4-bis-2-(4-methyl-5-phenyloxazoyl-) benzene (POPOP) and toluene-C^{14}.

Radioactive isotope labelled materials were obtained from Nuclear Chicago Company, Des Plaines, Illinois.

Chromatographic Materials

Reagent grade silicic acid, 100 mesh, obtained from Mallinckrodt Chemical Works, St. Louis, Missouri, and labelled "suitable for chromatography" was used for column chromatography of lipids.

Silica Gel G from Research Specialties Company, Richmond, California, was used for thin-layer chromatography.

Pretested ethylene glycol-succinate polyester on Gas Chrom P (60/80 mesh) was the packing employed for gas-liquid chromatography of fatty acid methyl esters and was obtained from Applied Sciences Laboratory, State College, Pennsylvania.

Helium, hydrogen, and air (purified) for gas-liquid chromatography were obtained from Oklahoma Oxygen Company, Oklahoma City, Oklahoma.

Nitrogen (dry, high purity) for gas-liquid chromatography was obtained from Red Ball Supply Company, Oklahoma City, Oklahoma.

Instruments and Equipment

Centrifugations were done using an International Refrigerated Centrifuge, International Equipment Company, Boston, Massachusetts, and a Spinco Model L2-65 Ultracentrifuge, Beckman Instruments Company, Sprinco Division, Palo Alto, California.

Spectrophotometric measurements were made using either a Beckman DU or a Beckman DU-2 spectrophotometer from Beckman Instruments Company, South Pasadena, California.

Enzyme incubations were 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 <u>in vacuo</u> using rotating evaporators from Rinco Instruments Com-

pany, Greenville, Illinois.

Gas-liquid chromatography was performed on either an Aerograph Autoprep 700 equipped with a thermal conductivity detector from Varian Aerograph Company, Walnut Creek, California, or a Perkin-Elmer Model 881 gas chromatograph equipped with a flame ionization detector from Perkin-Elmer Corporation, Norwalk, Connecticut.

Tissues were homogenized for lipid extraction in a stainless steel homogenizer from Waring Blendor Company, New York, New York. Homogenization for microsome preparation was performed with a teflon and glass tissue grinder from Arthur Thomas Company, Philadelphia, Pennsylvania.

A Packard Tri-Carb Scintillation Spectrometer Model 3375 equipped with an automatic external standard from Packard Instrument Company, Downers Grove, Illinois, was used to determine radioactivity in lipid samples.

Methods

Extraction of Lipids from Animal Tissues

The method of extraction used was that of Folch, Lees, and Sloane-Stanley (39). After removal from an animal the tissue to be extracted was homogenized in a Waring blender with 15 volumes (15 ml for each g of tissue) of a chloroform: methanol mixture (2:1 v/v) for 90 seconds. If the resulting mixture did not form a single phase, additional chloroform: methanol mixture (2:1 v/v) was added to form a single phase.

The single phase mixture was then filtered through Whatman No. 1 filter paper using a Buchner funnel and suction flask. The filter residue was washed twice with small volumes of the chloroform:methanol mixture. To this filtrate 0.2 volumes (2 ml for each 10 ml of filtrate) of aqueous sodium chloride solution (0.5 percent w/v) was added. After shaking this mixture was allowed to stand until two clear phases were visible. The lower chloroform phase (containing the total lipid extract) was carefully separated from the upper phase and concentrated *in vacuo* asing absolute ethanol to complete the evaporation of any residual water from the lipid. The extracted lipid was then diluted to a known volume with chloroform and stored in screw-cap culture tubes with teflon-lined caps under a nitrogen atmosphere.

Preparation of Fatty Acid Methyl Esters

Methyl esters were prepared by use of a boron trifluoride-methanol reagent (31). To an amount of lipid containing 300 ug of lipid phosphorous, 1 ml of boron trifluoride-methanol reagent was added. The mixture, sealed in a screw cap culture tube, was heated for twenty minutes in a boiling water bath. After cooling, 1 ml of water was added and the fatty acid methyl esters were extracted twice with 2 ml portions of n-hexane.

Fractionation of Lipids

Column chromatography. Silicic acid chromatography

(32) was used to separate neutral lipids from phospholipids. The silicic acid was activated by drying for 3 hours at 110 degrees centigrade prior to its use. This silicic acid was suspended in chloroform and slurried into a column of appropriate size for the amount of sample. Flow rates of 1-2 ml per minute were used. The loading factor was not over 25 mg of lipid per g of silicic acid. After the lipids were placed on the column in a small volume 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 methanol (at least 10 column volumes) until no lipid material could be detected by charring with concentrated sulfuric acid. Tests in this laboratory indicated that this procedure gives a quantitative separation of neutral lipids from phospholipids.

Rubber Membrane Dialysis. The lipid to be separated was dried and redissolved in n-hexane. This lipid solution was transferred to a latex rubber membrane (Perry Rubber Company, Massellon, Ohio) which was placed in 300 ml of the same solvent in a stoppered flask. The flask and rubber membrane were gassed with nitrogen. Dialysis was achieved after 24 hours at room temperature (33). During dialysis, the solvent in the flask was changed 3 times. The dialysate was analyzed for phosphorous to be certain no leakage occurred. The phospholipid fraction was removed from the rubber membrane by

pippette, dried in vacuo and stored in chloroform under nitrogen. The neutral lipids were recovered from the dialysate, evaporated to dryness and stored in chloroform under nitrogen.

Gas-Liquid Chromatography of Fatty Acid Methyl Esters

Separation. Fatty acid methyl esters derived from total lipid or fractionated lipid were separated and analyzed qualitatively and quantitatively by gas-liquid chromatography. Ester separations were effected on packed columns of ethylene glycol succinate polyester (EGS). Methyl ester standards of known composition were run periodically and under instrument conditions identical to those used for samples. Methyl esters in the samples were identified by comparison of their relative retention times (relative to palmitate) with those of the standard ester mixtures. Detector response was always linear with weight percent in the range of sample sizes used.

Standard instrument conditions were as follows:

Aerography Autoprep 700

Column	Aluminum length 4 meters inside diameter 7.5 millimeters
Packing	15 percent w/w EGS on Gas Chrom P 60/80 mesh
Temperatures	Column 185° C Injector 240° C Detector 240° C
Carrier Gas	Helium, Programmed flow rate from 100 ml/min to 300 ml/min

Sample size	2.0 ml of dry lipid
Perkin Elmer Model 881	
Column	Aluminum length 3 meters inside diameter 4.0 millimeters
Packing	15 percent w/w EGS on Gas Chrom P 60/80 mesh
Temperatures	Column 180° C Injector 230° C Detector 230° C
Carrier Gas	Nitrogen 60 ml/min
Sample size	Analytical 0.3 ul dry lipid Preparative 1.0 ul dry lipid

Fraction Collection. Fractions were collected from the chromatographs using a glass tube fitted with a Millipore filter (34) in a Swinney adaptor (Millipore Filter Corporation, Bedford, Massachusetts). Tests indicate this method has a collection efficiency better than 95 percent plus or minus 2 percent. Methyl esters were washed from the collectors with either chloroform or toluene.

For collecting samples from the Model 881, a stream splitter was inserted after the column so that 75 percent of the column eluent was diverted to the collector and only 25 percent entered the flame detector. A finely controlled vacuum was used to offset the resistence of the Millipore filter to gas flow.

Quantitation. Peak areas were determined by multiplying the peak height by the width of the peak at one-half its height. The absolute amount of methyl esters was assessed

either by determining the ester group equivalents (described below) or using a known amount of internal standard (behenic acid methyl ester) placed in the sample at the time of extraction of the lipid from the tissue.

Thin-layer Chromatography

Thin-layer chromatography was used to purify the fatty acid methyl esters collected from the gas-liquid chromatograph before an ester group analysis. Silica Gel G was slurried in water (20 g/50 ml), spread over clean glass plates in a uniform thickness (250 microns) and air dried. Methyl esters were spotted in a strip acrossone side of the plate. At one point a mixture of standards was applied. Development of the plate was carried out in a mixture of petroleum ether (B.P. 30-60° C): diethyl ether: acetic acid (70:30:1 v/v). After the solvent front had moved 15 cm the plate was removed from the solvent and dried in air. The side of the plate with the standard mixture was sprayed with Rhodamine-6-G (0.005 percent w/v in 95 percent ethanol) and observed under ultraviolet light. The fluorescent spots were marked and this was used as a guide for removal of the silicic acid containing the methyl esters from the plate. After scraping the methyl esters from the glass plate with a razor blade they were eluted from the silicic acid with small volumes of the chloroform-methanol mixture. For elution the silicic acid was retained by Whatman No. 3 filter paper in a small funnel.

The collected lipid was dried *in vacuo* and stored in a known volume of chloroform under nitrogen. Recoveries with selected test samples were better than 95 percent.

Radioactive Determinations

Liquid scintillation spectrometry was used to determine radioactivity in all lipid samples. Samples were counted in glass vials, dissolved in toluene containing 0.5 percent w/v PPO and 0.05 percent POPOP w/v. Counting efficiency was determined using an automatic external standard (AES). A standard curve for efficiency was made using labelled toluene of a known radioactivity and acetone as a quencher. All counting was done using balance-point operation. Dual labelling was counted by employing two instrument channels.

Preparation of Rat Liver Microsomes

Male rats were stunned by a blow on the head and exsaguinated by decapitation. The liver was removed, chilled with cold potassium phosphate buffer (0.15 molar, pH 7.5), blotted dry and weighed. Four ml of cold phosphate buffer (0° C) were added to each g of liver and the tissue homogenized using a teflon and glass tissue grinder. The homogenate was centrifuged in a refrigerated centrifuge at 8000 g for 15 minutes in a Spinco 30 rotor at 105,000 g 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 105,000 g. This washing process was re-

peated once. 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 (0.10 molar, 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.

Partial Hepatectomy

Partial hepatectomies were performed on male rats according to the method of Higgins and Anderson (35). The animals were anesthetized with ether and a mid-line abdominal incision was made. Through this incision the liver was exposed. A ligature was secured around the base of the medial and left lateral lobes (approximately 70 percent of the total liver wet weight) and these were removed by cutting. The incision was then closed with 4-0 silk sutures and the animal was allowed to revive. All surgical animals were maintained for 24 hours after the operation on 5 percent w/v sucrose in water after which they were returned to their regular diet. The animals tolerated this procedure very well. All ppera-tions were performed between 1 o'clock and 6 o'clock in the afternoon.

Assay for TBA Chromagen

Thiobarbituric acid (TBA) chromagen is formed in an *in vitro* system as a result of lipid peroxidation in microsomal

systems (11). The formation of the chromagen is used as a qualitative measure of enzymatic lipid peroxidation. A typical assay mixture contained per ml: 0.1 ml of microsomal suspension, 4.0 umoles of ADP-Fe^{+3} and 0.9 ml of Tris-HCl buffer (0.1 molar, pH 7.5). This was indubated with (Experimental) and without (Control) 0.3 umoles NADPH at 37° C in air. The enzyme reaction is terminated by the addition of 0.5 ml of 35 percent w/v trichloroacetic acid (TCA). One ml of 0.75 percent w/v TBA was added and the mixture was heated in a boiling water bath for 15 minutes according to the method of Ottolenghi (36). After cooling, 1 ml of 70 percent w/v TCA was added to each tube and swirled gently. The tubes were then centrifuged and the optical density of the clear pink supernatant was determined at 532 mu. In instances where a sample color was too dense to read directly it was 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.

Assay of L-Gulonolactone Oxidase Activity

Ascorbic acid is formed from L-gulonolactone by the action of L-gulonolactone oxidase found in rat liver microsomes. A typical assay mixture contained 0.1 ml of microsomal suspension, 0.9 ml phosphate buffer (0.10 molar, pH 7.5) and 46 umoles of L-gulonolactone. The mixture was incubated

in air for two hours at 37° C. The reaction was terminated by the addition of 2.5 ml of 6 percent w/v TCA. The mixture was centrifuged and the clear supernatant decanted into a large polyethylene tube and mixed with approximately 2 g of activated charcoal (Norite). This mixture is allowed to stand 15 minutes and then is filtered through Whatman No. 1 filter paper. To a 1.8 ml aliquot of the filtrate is added 0.05 ml of 10 percent w/v thiourea in 50 percent ethanol and 0.5 ml of 2 percent w/v 2,4-dinitrophenylhydrazine dissolved in 9 normal sulfuric acid. The mixture is heated at 57° C for 45 minutes. After cooling 2.5 ml of cold (0° C) 80 percent sulfuric acid is added slowly. After standing 30 minutes in the cold the solution is read at 520 mu. Standards of ascorbic acid are not incubated but are carried through the remainder of the procedure. The same incubation mixture may be used for assaying TBA chromagen formation from malondialdehyde as a result of the L-gulonolactone oxidase activity.

Chemical Analyses

General Considerations. All chemical analyses were done in duplicate. Appropriate standards of known concentrations were assayed with each sample.

Determination of Lipid Phosphorous. The chloroform was removed in vacuo from lipid samples containing 4 to 60 ug of lipid phosphorous. After drying the sample was mineralized

in sulfuric acid (1.2 ml of 5 Normal) and nitric acid (0.1 ml). The resulting inorganic phosphate was then determined by the method of Fiske and Subbarow (37).

Determination of Ester Equivalents. Lipid ester equivalents were determined by the method of Stern and Shapiro (38) as modified by Antonis (39). Chloroform was removed in vacuo from a sample containing the equivalent of 0.3 to 3.0 umoles of lipid ester and the sample was treated with alkaline hydroxylamine. The resulting hydroxamic acid was determined as a ferric complex which absorbed maximally at 520 mu. The optical density of the colored complex was compared with that developed by a standard of triolein.

Determination of Cholesterol. Cholesterol was determined by the Lieberman-Burchard reaction (40). Chloroform was removed in vacuo from a sample containing 50 to 200 ug of cholesterol and was redissolved in 5 ml of chloroform. Cold acetic anhydride-sulfuric acid reagent (4:1) was added. The mixture was allowed to stand at room temperature for 15 minutes and the optical density was immediately determined at 625 mu.

Determination of Protein. Protein was determined by the method of Lowry et al. (41). A sample containing 5 to 100 ug of protein was diluted to 1.0 ml with water. To the diluted sample an alkaline copper sulfate solution was added and the mixture allowed to stand at room temperature for 15 minutes. One-tenth ml of 1.0 Normal Phenol Reagent was added

and the resulting solution was mixed immediately on a vortex mixer. After standing at room temperature for 30 minutes the optical density of the colored solution was read at 520 mu. Bovine serum albumin was used as a standard.

For a comparison some protein samples were also determined by the biuret method (42). A sample containing 1 to 10 mg of protein was diluted to 1.0 ml and 4.0 ml of biuret reagent was added. This mixture was allowed to stand at room temperature for 30 minutes and then the optical density was determined at 550 mu. Bovine serum albumin was used as a standard. In all cases where both methods were used the results were in close agreement.

CHAPTER III

RESULTS

Fatty Acid Metabolism in Chickens Deprived of Tocopherol

Tocopherol Deprivation in Chicks

After twelve days on a tocopherol-deficient diet, one-half of a group of chickens exhibited signs of ataxia and head retraction characteristic of encephalomalacia. Onset of the symptoms was sudden and death of the affected chickens usually occurred within twelve hours if the animals were not used for an experiment. Examination of the brains from these chicks revealed a normal appearing cerebrum and swollen, hemorrhagic cerebellum. Livers from these animals appeared normal and no gross signs of necrosis were evident. There was no apparent evidence of muscle degeneration or exudative diathesis. Chicks grown on the same diet supplemented with 10 milligrams percent w/w of alpha-tocopheryl acetate had no symptoms after thirty days and were normal in growth and appearance.

Chicks maintained on diet for twelve days and which had not yet exhibited disease symptoms were selected for the

incorporation studies described below.

Acetate Incorporation into Chick Liver Fatty Acids

To determine the effect of tocopherol deprivation on acetate incorporation into fatty acids of the liver, two chicks deprived of tocopherol (Experimental) and two chicks with tocopherol supplement (Control) were injected intraperitoneally with 0.50 millicuries of acetate-1-^{14}C (29.0 millicuries/millimole) in 1.0 milliliter of normal saline.

Ninety minutes after the injection these animals were sacrificed by decapitation and their livers removed.

From these livers total lipids were extracted with chloroform-methanol. The lipid extracts from each animal were divided into three samples and these samples were analyzed separately as a check on the precision of the methods. Each lipid sample was methylated and extracted into hexane. A portion of each hexane extract was separated on thin-layer chromatography; the cholesterol and fatty acid spots were eluted from the silica gel and the radioactivity of each was determined. Table 3 shows the distribution of radioactivity between these two compounds. The precision of the multiple determinations on each lipid extract was very good. Variability between animals was large and different amounts of acetate incorporation into fatty acids are to be expected. No correlation could be made between tocopherol deprivation and acetate distribution between cholesterol and fatty acids from this data.

Fatty acid methyl esters were purified from the remainder of the hexane extract by thin-layer chromatography. These purified esters were then separated on a preparative gas-liquid chromatograph and the six major fatty acids collected from the gas effluent. The collected fatty acid esters were analyzed for radioactivity and micromoles (umoles) of ester. Specific activity was calculated from these measurements.

The specific activity of the total fatty acids are reported in Table 4.

The close agreement of the values for samples from the same animal indicates the predision of the procedure used. Each animal incorporated a different total amount of acetate into the fatty acids although the amounts are well within an order of magnitude of each other. This is probably due to individual animal variation in absorption of acetate or to an individual difference in distribution between classes of lipid.

Specific activities for the major individual fatty acids are reported in Table 5. The incorporation of ^{14}C into palmitic acid (16:0) appears greater in tocopherol-supplemented animals. Stearic acid (18:0) in the tocopherol-deprived animals has a higher amount of acetate incorporation than palmitic acid from the same animal, indicating the time of maximum incorporation for palmitic is shorter than ninety minutes. The same comparison of the specific activities of palmitic and stearic acids for the tocopherol-supplemented

TABLE 3

DISTRIBUTION OF ^{14}C FROM ACETATE-1-^{14}C BETWEEN CHOLESTEROL AND FATTY ACIDS FROM CHICK LIVER NINETY MINUTES AFTER INJECTION

Lipid Fraction	Percent Total DPM Incorporated[a]			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	% DPM		% DPM	
Animals	# 1	# 2	# 1	# 2
Cholesterol	27	16	46	16
	27	15	46	16
	27	16	46	15
Fatty Acids	73	84	54	84
	73	85	54	84
	73	84	54	84

[a] The total DPM are the sum of DPM in fatty acids and cholesterol of the sample eluted from TLC.

TABLE 4

INCORPORATION OF ^{14}C FROM ACETATE-1-^{14}C INTO TOTAL FATTY ACIDS OF CHICK LIVER NINETY MINUTES AFTER INJECTION

Determination	Specific Activity			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	DPM/umole		DPM/umole	
Animals	# 1	# 2	# 1	# 2
1	2,639	11,790	5,452	5,895
2	2,835	11,960	5,155	6,837
3	-----	12,720	-----	10,690

TABLE 5

SPECIFIC ACTIVITY OF MAJOR FATTY ACIDS FROM CHICK LIVER NINETY MINUTES AFTER INJECTION

Fatty Acid[a]	Specific Activity			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	DPM/umole		DPM/umole	
	#1	#2	#1	#2
16:0	4,879	13,920	4,556	4,934
	8,049	15,492	6,546	6,855
	-----	14,996	-----	2,830
16:1[b]		1,476		524
		166		123
		724		1,245
18:0	4,230	10,666	9,545	5,754
	4,531	12,300	8,539	7,029
	-----	9,457	-----	7,526
18:1	1,761	2,361	1,346	650
	1,019	2,333	1,806	305
	-----	2,263	-----	499
18:2	123	175	412	260
	118	213	704	258
	---	277	---	284
20:4	2,498	2,845	6,950	2,617
	2,639	3,055	6,251	2,425
	-----	2,974	-----	4,218

a Throughout this dissertation the following numbers will be used to represent the indicated fatty acids: 16:0, palmitic acid; 16:1, palmitoleic acids; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid.

b Animals #1 have the values for 16:1 included in the values given for 16:0

animals indicates that the time of maximum incorporation for palmitic is longer than ninety minutes. This suggests a more rapid turnover of liver fatty acids in tocopherol-deprived animals compared to the tocopherol-supplemented animals.

In order to show more definitely an increased turnover of liver fatty acids in tocopherol-deprived animals, the preceding experiment was repeated for incorporation times of 15, 45, and 90 minutes. Table 6 shows the distribution of ^{14}C between cholesterol and fatty acids from the livers of these animals. Once again the variation between individual animals is evident. In the earlier time periods most of the acetate incorporated into lipids appears in the fatty acids. By ninety minutes the cholesterol has received a large proportion of the acetate incorporated into the liver lipids. No correlation is evident between tocopherol deprivation and distribution of ^{14}C between these two lipids.

The specific activities of the total fatty acids are shown in Table 7. At 15 minutes there appears to be more acetate incorporation into liver fatty acids of tocopherol-deprived animals than in corresponding fatty acids from tocopherol-supplemented animals. The values for 45 minutes and 90 minutes indicate that fatty acids are metabolized more rapidly in the tocopherol-deprived animals than in the control animals. From this data it would appear that the turnover of fatty acids is more rapid in livers of animals deprived of tocopherol than in livers of animals with sufficient

TABLE 6

DISTRIBUTION OF ^{14}C FROM ACETATE-1-^{14}C BETWEEN CHOLESTEROL AND FATTY ACIDS FROM CHICK LIVER FOR THREE INCORPORATION TIMES

Lipid Fraction	Percent Total DPM Incorporated			
	15 Minute Incorporation Time			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	# 1	# 2	# 1	# 2
Cholesterol	16	15	9	21
Fatty Acids	84	85	91	79
	45 Minute Incorporation Time			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	# 3	# 4	# 3	# 4
Cholesterol	16	10	8	23
Fatty Acid	84	90	92	77
	90 Minute Incorporation Time			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	# 5	# 6	# 5	# 6
Cholesterol	38	50	44	24
Fatty Acids	62	50	56	76

TABLE 7

SPECIFIC ACTIVITY OF THE TOTAL FATTY ACIDS FROM CHICK LIVER FOR THREE INCORPORATION TIMES

Time After Injection	Specific Activity: Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	DPM/umole		DPM/umole	
	# 1	# 2	# 1	# 2
15 Minutes	7,237	13,582	65,397	19,995
45 Minutes	43,027	91,612	59,107	14,757
90 Minutes	122,575	67,641	8,948	18,970

tocopherol to prevent deficiency symptoms.

Table 8 shows the specific activities of the major individual fatty acids from these livers. At fifteen minutes after injection the tocopherol-deprived animals have a notably higher specific activity in the saturated and monounsaturated fatty acids than the same fatty acids in tocopherol-supplemented animals. By forty-five minutes the saturated fatty acids in the tocopherol-supplemented animals have approximately the same specific activity as the same fatty acids in the tocopherol-deprived animals, although the monounsaturated acids have a lower specific activity. Ninety minutes after the acetate-1-^{14}C injection, the saturated and monounsaturated fatty acids of the tocopherol-supplemented animals have a much higher specific activity than those in the tocopherol-deprived animals. This data supports the previously mentioned idea that the liver fatty acids are turning over more rapidly in tocopherol-deprived animals than in tocopherol-supplemented animals. Arachidonic acid, a polyunsaturated fatty acid, is approximately the same specific activity in tocopherol-supplemented and tocopherol-deprived animals for all the time periods. This indicates that arachidonic acid as a whole is not turned over more rapidly in tocopherol-deprived animals as are the saturated and monounsaturated fatty acids, but it does not eliminate the possibility that part of the arachidonic acid may turn over rapidly as a separate pool.

TABLE 8

SPECIFIC ACTIVITY OF MAJOR INDIVIDUAL FATTY ACIDS FROM CHICK LIVER FOR THREE INCORPORATION TIMES

Fatty Acid	Specific Activity: Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	15 Minutes After Injection			
	DPM/umole		DPM/umole	
	# 1	# 2	# 1	# 2
16:0	17,125	35,818	170,836	70,232
16:1	3,255	6,684	60,183	12,428
18:0	8,065	9,342	54,952	14,087
18:1	2,448	5,119	72,013	15,821
18:2	537	588	1,422	1,206
20:4	4,598	3,758	6,164	4,667
	45 minutes After Injection			
	DPM/umole		DPM/umole	
	# 3	# 4	# 3	# 4
16:0	121,857	127,222	191,452	28,837
16:1	15,160	1,730	74,467	5,328
18:0	79,134	511,054	103,993	18,644
18:1	36,395	16,789	115,665	12,268
18:2	903	177	1,505	------
20:4	17,523	2,493	9,200	4,148

TABLE 8 - Continued

	90 minutes After Injection			
	DPM/umole		DPM/umole	
	# 5	# 6	# 5	# 6
16:0	208,593	145,839	19,230	33,590
16:1	51,489	84,079	5,536	11,560
18:0	155,795	95,877	12,216	30,242
18:1	90,078	------	5,677	16,252
18:2	5,906	2,762	1,254	1,376
20:4	15,603	6,494	7,650	11,226

Acetate Incorporation into Chick Muscle Fatty Acids

The pectoralis muscle group of the chickens in the preceding experiment was removed at the same time as the liver. Total lipids were extracted, methylated and separated as in the liver.

The total muscle cholesterol was quantitatively determined and its specific activities measured for each animal. These values are shown in Table 9. During the first 15 minutes the tocopherol-deprived animals incorporated considerably more acetate into muscle cholesterol than did control animals. The data for the other time periods are inconclusive.

Total muscle fatty acid specific activities are shown in Table 10. The specific activities for the individual fatty acids from muscle are shown in Table 11. It is apparent from these figures that acetate is incorporated into muscle fatty acids much more slowly than into liver fatty acids. The data from the 15 and 45 minutes incorporation times shows no significant difference between tocopherol-supplemented and tocopherol-deprived animals. At 90 minutes it appears that more incorporation has occurred in the muscle fatty acids of animals supplemented with tocopherol. It would appear that the tocopherol-deprived animals have not reached the time of maximum specific activity since stearic acid is lower in specific activity than palmitic acid. It is apparent, however, that arachidonic acid has approximately the same specific activity in all three time periods in both tocopherol-supple-

TABLE 9

SPECIFIC ACTIVITY OF CHOLESTEROL FROM CHICK MUSCLE FOR THREE INCORPORATION TIMES

Time After Injection	Specific Activity: Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	DPM/umole		DPM/umole	
	# 1	# 2	# 1	# 2
15 Minutes	3,398	1,737	7,306	8,142
45 Minutes	3,092	10,360	10,698	4,592
90 Minutes	10,575	-----	5,707	8,521

TABLE 10

SPECIFIC ACTIVITY OF TOTAL FATTY ACIDS FROM CHICK MUSCLE FOR THREE INCORPORATION TIMES

Time After Injection	Specific Activity: Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	DPM/umole		DPM/umole	
	# 1	# 2	# 1	# 2
15 Minutes	1,040	754	3,365	894
45 Minutes	175	3,362	6,460	614
90 Minutes	6,660	3,998	1,121	1,095

TABLE 11

SPECIFIC ACTIVITY OF THE MAJOR INDIVIDUAL FATTY ACIDS IN CHICK MUSCLE FOR THREE INCORPORATION TIMES

Fatty Acid	Specific Activity: Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	15 Minutes Incorporation Time			
	DPM/umole		DPM/umole	
	# 1	# 2	# 1	# 2
16:0	-----	1,078	1,389	1,051
16:1	737	1,542	498	488
18:0	1,261	352	9,447	2,300
18:1	-----	229	368	1,343
18:2	892	128	95	106
20:4	1,449	660	-----	645
	45 Minutes Incorporation Time			
	DPM/umole		DPM/umole	
	# 3	# 4	# 3	# 4
16:0	3,754	6,525	9,668	1,334
16:1	302	2,468	1,491	286
18:0	3,753	6,653	-----	511
18:1	816	2,278	2,429	445
18:2	121	348	371	146
20:4	1,406	1,295	1,091	556

TABLE 11-CONTINUED

	90 Minutes Incorporation Time			
	DPM/umole		DPM/umole	
	# 5	# 6	# 5	# 6
16:0	18,444	9,365	1,646	2,200
16:1	5,443	2,823	1,009	688
18:0	10,664	5,552	1,353	1,558
18:1	349	3,672	1,389	894
18:2	642	268	151	167
20:4	1,646	1,132	1,381	1,810

mented and tocopherol-deprived animals. This is in agreement with the same observations in the liver fatty acids.

Regenerating Liver in Tocopherol-Deprived Rats

Liver Weight Gain in Tocopherol-Deprived Rats After Partial Hepatectomy

Two groups of rats were placed on the purified diet described in Table 1 as the Rat, Normal diet immediately after weaning. One group (Control) had 10 milligrams percent w/w dietary supplement of alpha-tocopherol acetate while the other group (Experimental) were unsupplemented. After one month on these diets, partial hepatectomies were performed on animals from both groups. Liver samples (Normal) removed at the time of surgery were homogenized and microsomes were prepared from the homogenates. At various times after surgery several animals from each group were sacrificed by decapitation and liver samples were removed, weighed and immediately homogenized for microsome preparation. From the weights of the animals, the original weight of each animal's liver was calculated according to the method of Higgins and Anderson (35). Using these calculated weights, the percentage of liver removed at surgery averaged 68 and 70 percent in control and experimental groups respectively. This agrees well with previously reported figures for removal of the specified lobes.

Table 12 gives the amount of liver weight increase at the times after partial hepatectomy. This figure was obtained

TABLE 12

INCREASE IN LIVER WEIGHT DURING REGENERATION;
EFFECT OF ALPHA-TOCOPHEROL

Hours of Regeneration	Liver Weight Increase*	
	Tocopherol-Supplemented Animals	Tocopherol-Deprived Animals
	grams	grams
24	1.47 (3)	2.19 (6)
30	2.04 (3)	1.76 (3)
45	2.46 (4)	2.79 (6)
54	3.65 (3)	2.08 (3)
96	3.44 (4)	3.99 (4)
144	4.15 (4)	5.13 (4)
192	4.58 (4)	4.36 (4)
240	4.08 (4)	6.11 (4)

* Number in parenthesis indicates number of animals used to obtain average number.

by subtracting the calculated weight of liver remaining after surgery from the weight of liver removed at sacrifice. The increase in liver weight of both groups proceeds at approximately the same rate. It would appear, therefore, that dietary tocopherol may not be required for the regeneration of liver tissue in the rat. The following evidence, however, suggests that processes which involve alpha-tocopherol may be diminished in regenerating liver. To determine the functioning of membrane-bound oxidative activities during regeneration, microsomes prepared from the above livers were assayed for L-gulonolactone oxidase activity and the NADPH-requiring phospholipid oxidation system. Table 13 gives the specific activities of gulonolactone oxidase in these livers at different times of incubation. Comparison of the values for non-regenerating liver from tocopherol-supplemented and tocopherol-deprived animals shows tocopherol deprivation decreases the enzyme activity only after 30 minutes of reaction time. Initial rates are the same, in agreement with previous work from this laboratory (43). Regeneration appears to have very little effect on gulonolactone oxidase activity, either in tocopherol-supplemented or tocopherol-deprived animals, at least during the liver growth occurring 4 to 10 days after surgery.

The NADPH-requiring phospholipid oxidation system can be qualitatively determined by measurement of one of the products of that reaction (malondialdehyde) with the thiobar-

TABLE 13

GULONOLACTONE OXIDASE ACTIVITY IN LIVER MICROSOMES FROM RATS FED A CORN OIL DIET[a]

Specific Activity[b]
Non-Regenerating Liver

	Tocopherol-Supplemented Animals			Tocopherol-Deprived Animals		
	mg a.a./gram protein			mg a.a./gram protein		
time	0′	30′	60′	0′	30′	60′
	3.19	35.5	49.4	3.16	24.9	30.0
	±1.1	±7.2	±11.7	±1.3	±9.1	±7.7

Specific Activity[b]
Regenerating Liver

Days	Tocopherol-Supplemented Animals			Tocopherol-Deprived Animals		
	mg a.a./gram protein			mg a.a./gram protein		
	0′	30′	60′	0′	30′	60′
4	4.4	35	49	3.8	23	21
6	3.4	23	40	3.6	20	25
8	1.7	39	57	2.3	34	44
10	5.5	25	40	5.5	18	23

[a] Rats fed the diet noted as Rats, Normal in Table 1

[b] Milligrams of ascorbic acid produced per gram of microsomal protein after incubations times indicated in minutes, days are the days of regeneration; non-regenerating values are means of 16 animals, other values are means of 4 animals

bituric acid reaction. Table 14 shows that liver microsomes from tocopherol-supplemented animals, whether from normal or regenerating liver, have low levels of activity. In tocopherol-deprived animals, however, microsomes from regenerating liver appear to have considerably lower activity, at least during the first six days when most of the liver growth after surgery is occurring.

Systems Promoting Lipid Oxidation in Microsomes from Normal and Regenerating Livers: Effect of Rat Restriction

Two groups of rats (tocopherol-supplemented and tocopherol-deprived) were placed on the fat-restricted diet described in Table 1. Partial hepatectomies were performed on these rats and the livers removed at surgery were used to determine activities in normal livers. Animals from each group were sacrificed at various times after hepatectomy. Microsomes were prepared from all individual liver samples. In Table 15 the gulonolactone oxidase activity of these microsomes is reported. The results for non-regenerating liver are the same as those for animals fed the corn oil diet, *iee.* gulonolactone oxidase activity becomes inhibited in microsomes from tocopherol-deficient animals. Regenerating liver microsomes from both tocopherol-supplemented and tocopherol-deprived groups have normal activity at 12 hours after partial hepatectomy but this is markedly reduced at 24 and 48 hours of regeneration. This reduction occurs to the same extent in both control and experimental animals.

TABLE 14

ACTIVITY OF THE NADPH-REQUIRING PHOSPHOLIPID OXIDATION SYSTEM IN LIVER MICROSOMES FROM RATS FED A CORN OIL DIET[a]

Specific Activity[b] Non-Regenerating Liver			
Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
O.D. units/mg protein		O.D. units/mg protein	
10'	30'	10'	30'
.111	.229	1.283	2.564
± .035	± .161	± .413	± .861

Days	Specific Activity[b] Regenerating Liver			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	O.D. units/mg protein		O.D. units/mg protein	
	10'	30'	10'	30'
4	.108	.129	1.085	1.270
6	.107	.177	.685	1.517
8	.106	.181	1.967	3.008
10	.222	.158	.994	1.300

[a] Rats fed the diet noted as Rats, Normal in Table 1

[b] Optical Density units per milligram microsomal protein after incubation times indicated in minutes, days are the days of regeneration; non-regenerating values are the means of 16 animals, other values are means of 4 animals

TABLE 15

GULONOLACTONE OXIDASE ACTIVITY IN LIVER MICROSOMES FROM RATS FED A FAT-RESTRICTED DIET[a]

Specific Activity[b]
Non-Regenerating Liver

Tocopherol-Supplemented Animals			Tocopherol-Deprived Animals		
mg a.a. / gm protein			mg a.a./gm protein		
0'	30'	60'	0'	30'	60'
3.36 ± .77	29.6 ± 5.3	58.1 ± 7.4	3.08 ±1.28	19.1 ± 3.8	31.1 ± 5.1

Specific Activity[b]
Regenerating Liver

Hours	Tocopherol-Supplemented Animals			Tocopherol-Deprived Animals		
	mg a.a./gm protein			mg a.a./gm protein		
	0'	30'	60'	0'	30'	60'
12	5.2	28	56	3.3	19	34
24	1.8	18	29	1.8	12	16
48	1.2	17	28	1.9	13	19

[a] Rats fed the diet noted as Fat-Restricted in Table 1

[b] Milligrams of ascorbic acid produced per gram of microsomal protein after incubations times indicated in minutes, hours are the hours of regeneration; non-regenerating values are means of 9 animals ± standard deviation, other values are means of 3 animals.

Lipid peroxidation (qualitatively measured by malondialdehyde formation)linked to gulonolactone oxidase activity is shown in Table 16 for non-regenerating liver. These values are in close agreement with results from other studies done in this laboratory for animalsoon a Corn Oil diet or on a commercial laboratory ration. It would appear that a Corn Oil diet does not lower *in vitro* peroxidation linked to gulonolactone oxidase in non-regenerating liver.

The activity of the NADPH-requiring phospholipid oxidation system in these microsomes is reported in Table 17. Assayed by measurement of malondialdehyde formation, microsomes from non-regenerating liver has only one-fourth the activity of microsomes from non-regenerating liver of animals fed the Corn Oil diet (Table 14). Regenerating liver has a reduced chromogen formation 24 and 48 hours after partial hepatectomy in both tocopherol-supplemented and tocopherol-deprived animals. The 48 hour microsomes have activity reduced so much that there is no difference in malondialdehyde formation between tocopherol-deprived animals and tocopherol-supplemented animals.

Fatty Acid Composition of Normal and Regenerating Rat Liver

Rat Fed a Fat-Restricted Diet

A group of experiments is reported here which were done, in part, to determine whether the marked changes in fatty acid composition of regenerating rat liver are affected

TABLE 16

GULONOLACTONE OXIDASE-CATALYZED MALONDIALDEHYDE FORMATION IN LIVER MICROSOMES FROM RATS FED A FAT-RESTRICTED DIET[a]

Specific Activity[b] Non-Regenerating Liver	
Tocopherol-Supplemented Animals	Tocopherol-Deprived Animals
.195 ± .162	.582 ± .040

[a] Rats fed the diet noted as Fat-Restricted in Table 1

[b] Optical density units per milligram microsomal protein, values are the means of 9 animals ± standard deviation

TABLE 17

ACTIVITY OF THE NADPH-REQUIRING PHOSPHOLIPID OXIDATION SYSTEM IN LIVER MICROSOMES FROM RATS FED A RAT-RESTRICTED DIET[a]

Specific Activity[b] Non-Regenerating Liver			
Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
O.D. units/mg protein		O.D. units/mg protein	
10'	30'	10'	30'
.155 ± .035	.229 ± .036	.337 ± .062	.521 ± .119

Hours	Specific Activity[b] Regenerating Liver			
	Tocopherol-Supplemented Animals		Tocopherol-Deprived Animals	
	O.D. units/mg protein		O.D. units/mg protein	
	10'	30'	10'	30'
12	.210	.440	.407	.368
24	.084	.100	.119	.178
48	.074	.098	.085	.057

[a] Rats fed the diet noted as Fat-Restricted in Table 1

[b] Optical Density units per milligram microsomal protein after incubation times indicated in minutes, hours are the hours of regeneration; non-regenerating values are the means of 9 animals, other values are means of 3 animals

by diet. Rats were maintained on a fat-restricted (described in Table 1) for 4 to 6 weeks. After this period of time, partial hepatectomies were performed on rats from the group. The portion of the liver removed at surgery was used as a normal liver sample. At various times after surgery, animals were sacrificed and the livers in various stages of regeneration removed immediately. A set of animals from the same group were subjected to the same operative procedure except that no liver was removed. These were sacrificed at the same time intervals as the hepatectomized rats and liver removed for analysis as a sham-operated control.

Total lipids were extracted from all liver samples and an internal fatty acid standard (behenic acid methyl ester) was added at the time of extraction to each liver sample. Methyl esters of fatty acids were prepared from the total lipid and analyzed by gas-liquid chromatography. Lipid phosphorous was determined for each lipid extract as well as the amount of protein in each liver.

The percent composition of the six major fatty acids are reported in Table 18. No difference in fatty acid composition was seen between normal livers and sham-operated controls at any time after surgery. These are reported as means of all the samples in each group. Fatty acid composition is the same as normal liver after 6 and 12 hours of regeneration. At 18 hours, however, palmitoleic (16:1) and oleic (18:1) acids are increased above normal while arachidonic

TABLE 18

FATTY ACID COMPOSITION OF WHOLE LIVERS FROM RATS FED A FAT-RESTRICTED DIET[a]

Regenerating Liver

Weight percent of total fatty acids

Hours after Partial Hepatectomy	16:0	16:1	18:0	18:1	18:2	20:4
6	26.5	6.44	16.2	24.7	6.83	19.3
	24.3	4.95	17.8	22.7	6.40	23.8
12	24.3	4.56	17.1	30.2	5.11	18.6
	25.4	5.50	18.3	26.8	6.92	17.1
18	25.9	10.3	11.5	32.5	9.24	10.5
	25.3	15.6	7.8	36.0	8.23	7.1
24	21.9	4.44	15.7	40.0	5.48	12.4
	24.3	8.14	10.9	43.3	5.69	7.7
36	24.1	11.7	10.6	37.9	6.61	8.96
	22.5	8.4	12.3	42.2	6.16	8.46
48	27.1	7.92	10.8	41.9	4.00	8.33
	25.3	7.23	12.6	36.2	6.20	12.4
60	31.8	3.76	18.9	32.6	6.45	7.12
	25.9	4.12	15.4	35.7	5.90	12.9
72	24.7	5.54	14.5	34.0	5.60	15.6
	25.8	6.02	12.5	39.1	6.56	10.0
96	25.8	5.28	17.6	28.0	4.04	19.2
	26.6	5.60	15.9	31.2	4.36	16.3

TABLE 18-Continued

Normal Liver						
Weight percent of total fatty acids						
n[b]	16:0	16:1	18:0	18:1	18:2	20:4
26	26.4	5.66	17.7	25.7	5.60	19.3
	± 2.58	± 1.20	± 1.87	± 2.89	± 1.38	± 3.17

Sham-Operated Liver[c]						
Weight percent of total fatty acids						
n[b]	16:0	16:1	18:0	18:1	18:2	20:4
	26.1	4.92	18.2	26.1	5.76	18.9
	± 2.54	± 1.24	± 1.78	± 4.15	± 1.14	± 3.76

[a] Rats fed diet noted as Fat-Restricted in Table 1

[b] Values for normal liver and sham-operated livers are means of n animals ± standard deviation.

[c] Sham-operated values are sum of sham-operated animals for all different times

(20:4) is lowered in terms of weight percent composition. Palmitic, stearic, and linoleic remain constant. By 24 hours after hepatectomy, palmitic acid returns to the control level but oleic and arachidonic acids remain elevated and lowered, respectively, until 96 hours after hepatectomy.

A quantitative analysis of fatty acids (in which absolute quantities of each fatty acid present is measured) from the same livers is reported in Table 19. Again no difference was seen between sham-operated and normal livers. In regenerating livers at 18 hours the palmitic, palmitoleic, oleic, and linoleic are all elevated above normal values. Stearic acid remains at control levels. By 24 hours all fatty acids return to normal levels except oleic acid which remains elevated until about 72 hours. It is also noted that arachidonic acid may be slightly lowered during the 96 hour period after regeneration begins.

Neutral lipids and phospholipids from some of the liver lipid samples were separated on silicic acid columns and the percent composition of these same fatty acids were analyzed by gas-liquid chromatography. These values are reported in Table 20. The means for thesellipid fractions from normal animals are shown in Table 21. These results show that the changes in fatty acid composition in regenerating liver occur only in the neutral lipid fraction and not in the phospholipid fraction.

The protein and lipid phosphorous content of these

TABLE 19

ABSOLUTE QUANTITIES OF FATTY ACIDS IN NORMAL AND REGENERATING LIVER OF RATS FED A FAT-RESTRICTED DIET

	Regenerating Liver					
	mg fatty acid/gram liver protein					
Hours after Partial Hepatectomy	16:0	16:1	18:0	18:1	18:2	20:4
6	25.8	6.24	15.8	24.1	6.68	18.7
	21.9	4.48	16.1	20.5	5.57	21.4
12	18.4	3.48	13.0	22.8	3.86	14.0
	22.3	4.83	16.0	23.4	6.10	15.0
18	40.5	16.1	18.0	50.9	14.4	16.4
	79.6	49.0	24.6	113.6	25.8	22.2
24	16.4	3.36	11.9	30.1	4.14	9.34
	43.8	14.8	19.7	78.4	10.3	13.9
36	15.9	7.96	7.21	25.7	4.49	6.09
	37.2	14.2	20.9	71.2	10.4	14.3
48	31.5	9.19	12.6	48.7	4.68	9.71
	20.5	5.87	10.3	29.4	5.03	10.1
60	23.1	2.71	12.6	29.2	4.81	10.6
	21.1	3.35	7.84	18.5	3.01	8.47
72	13.5	3.01	16.1	50.5	8.52	12.9
	33.5	7.81	11.7	18.7	2.71	12.8
96	17.1	3.51	11.7	18.7	2.71	12.8
	10.9	2.29	6.58	12.9	1.80	6.73

TABLE 19-Continued

Normal Liver

mg fatty acid/gram liver protein

n[b]	16:0	16:1	18:0	18:1	18:2	20:4
25	20.4	4.37	13.2	19.8	4.19	14.2
	± 7.0	± 1.38	± 5.3	± 6.6	± 2.16	± 6.4

Sham-Operated Liver[c]

mg fatty acid/gram liver protein

n[b]	16:0	16:1	18:0	18:1	18:2	20:4
23	20.3	3.70	14.0	20.0	4.61	14.9
	± 7.4	± 1.43	± 5.1	± 6.7	± 2.14	± 6.0

[a] Rats fed diet noted as Fat-Restricted in Table 1

[b] Values for normal liver and sham-operated livers are means of n animals ± standard deviation

[c] Sham-operated values are sum of sham-operated animals for all different times

TABLE 20

FATTY ACID COMPOSITION OF LIPID FRACTIONS FROM REGENERATING LIVER OF RATS FED A FAT-RESTRICTED DIET*

Neutral Lipid

weight percent of total fatty acids

Hours After Hepatectomy	16:0	16:1	18:0	18:1	18:2	20:4
18	17.6	10.8	6.5	42.8	8.4	7.5
	12.4	17.1	3.1	49.5	8.7	5.2
24	17.6	6.4	5.5	48.9	4.4	4.9
	13.0	11.1	6.1	53.4	6.3	6.3
36	19.9	6.2	11.2	25.1	6.4	18.9
	18.0	8.0	4.8	55.4	7.0	4.0
48	18.7	11.0	3.7	52.9	4.0	3.3
	26.1	13.5	2.3	27.3	6.6	7.2

Phospholipid

weight percent of total fatty acids

Hours After Hepatectomy	16:0	16:1	18:0	18:1	18:2	20:4
18	29.9	4.3	31.9	19.6	6.9	4.8
	20.4	4.4	24.6	17.0	10.2	18.2
24	20.4	2.8	19.8	24.6	6.6	16.8
	20.8	3.4	25.4	19.2	9.0	16.4
36	27.5	3.6	24.6	19.7	6.0	12.7
	18.5	2.3	24.6	20.7	8.2	17.5
48	25.0	4.2	20.4	24.5	6.6	8.5
	22.4	4.2	18.0	22.1	7.1	16.9

* Rats fed diet noted as Fat-Restricted in Table 1

livers is reported in Table 22. No difference was seen at any time after surgery between the three groups. This indicates that the amount of phospholipid and protein per gram of liver tissue do not change with injury or partial hepatectomy when the animals are fed a fat-restricted diet.

Rats Maintained on a Commercial Laboratory Ration

Another group of rats raised on a laboratory ration from weaning were treated in the same way as in the preceding experiment. The quantitative analyses of the six major fatty acids of livers from these rats is reported in Table 23. The livers from normal rats on ration contain much less fatty acid than normal livers from rats on a fat-restricted diet. Sham-operated livers appear to have slightly higher amounts of each fatty acid than normal livers but the difference is not of great significance. In the regenerating liver all the fatty acids except stearic and arachidonic are elevated above normal levels at 6 hours after partial hepatectomy. A peak in the amount of the same fatty acids as noted in the previous experiment are seen here at 18 hours. The amount of change in liver fatty acid content of regenerating liver is not nearly as great or as sharply defined in these animals as in the animals on a fat-restricted diet.

Table 24 gives the liver protein and lipid phosphorous values for the animals on a commercial laboratory ration. Once again no changes from the normal levels were seen in the sham-operated group or the partially hepatectomized related

TABLE 21

FATTY ACID COMPOSITION OF LIPID FRACTIONS OF NORMAL LIVER FROM RATS FED A FAT-RESTRICTED DIET[a]

	Fatty Acids[b]					
	weight percent of total fatty acids					
Lipid Fraction	16:0	16:1	18:0	18:1	18:2	20:4
Neutral	21.2 ± 1.8	6.5 ± 1.0	10.0 ± 2.4	28.5 ± 3.2	6.4 ± 0.8	17.0 ± 1.3
Phospholipid	24.1 ± 3.9	3.0 ± 0.7	24.8 ± 4.6	18.3 ± 3.6	5.1 ± 0.7	16.2 ± 4.2

[a] Rats fed diet noted as Fat-Restricted in Table 1

[b] Values are the means of 8 animals ± standard deviation

TABLE 22

PROTEIN AND LIPID PHOSPHOROUS IN NORMAL AND REGENERATING LIVER FROM RATS FED A FAT-RESTRICTED DIET[a]

	Protein		Lipid Phosphorous	
	mg protein/gram liver		umoles lipid P/gram liver	
State of Liver	n	mean[n]	n	mean[b]
Normal	25	136 ± 18.5	26	31.8 ± 6.0
Sham-Operated	23	143 ± 30.0	22	29.2 ± 8.6
Regenerating	23	144 ± 36.9	25	30.9 ± 5.3

[a] Rats fed diet noted as Fat-Restricted in Table 1

[b] Values are means of n animals ± standard deviation

TABLE 23

FATTY ACID CONTENT OF NORMAL AND REGENERATING LIVER FROM RATS FED A COMMERCIAL RATION[a]

Regenerating Liver

mg fatty acid/gram liver protein

Hours After Hepatectomy	16:0	16:1	18:0	18:1	18:2	20:4
6	18.4	2.45	13.9	14.0	25.6	13.8
	13.1	1.28	10.0	8.40	13.9	8.92
12	9.58	1.04	12.2	6.92	11.6	9.08
	18.9	2.05	14.8	14.7	22.9	17.1
18	23.6	----	14.5	20.0	25.9	14.0
	15.4	2.42	11.6	16.2	19.1	8.23
24	9.94	----	9.71	9.84	10.8	6.02
	17.2	1.21	11.1	18.2	25.4	9.57
36	19.7	0.82	14.7	16.5	24.7	14.0
	17.1	0.50	11.0	11.8	16.9	10.0

Normal Liver

mg fatty acid/gram liver protein

n[b]	16:0	16:1	18:0	18:1	18:2	20:4
10	10.8	0.73	10.3	6.35	10.2	13.6
	± 4.1	± .38	± 4.3	± 2.23	± 3.3	± 6.57

Sham-Operated Liver

mg fatty acid/gram liver protein

n[b]	16:0	16:1	18:0	18:1	18:2	20:4
10	12.3	2.0	12.3	8.13	12.5	13.2
	± 2.97	± .63	±2.6	± 2.95	± 2.8	± 4.4

[a] Rats fed a commercial laboratory ration described on page 10

[b] Values are means of n animals ± standard deviation

TABLE 24

PROTEIN AND LIPID PHOSPHORCUS IN NORMAL AND REGENERATING LIVER FROM RATS FED A COMMERCIAL RATION[a]

State of Liver	Protein		Lipid Phosphorous	
	mg protein/gram liver		umoles lipid P/gram liver	
	n	mean	n	mean
Normal	10	254 ± 25	10	22.5 ± 3.7
Sham-Operated	9	236 ± 28	9	28.6 ± 5.0
Regenerating	10	222 ± 22	10	31.4 ± 6.6

[a] Rats fed a commercial laboratory ration described on page 10

[b] Values are means of n animals ± standard deviation

to time after surgery. Liver protein is much higher in the normal animals maintained on a commercial ration than the same animals on the fat-restricted diet.

Lipid phosphorous values for normal livers are about one-third lower than the values for normal liver of animals maintained on a fat-restricted diet. Lipid phosphorous values of livers from animals on ration rise in regenerating and sham-operated liver to the values observed in animals fed the fat-restricted diet.

Comparison of the Two Diets

The most striking effect of feeding these two diets is that the livers of the animals on fat-restricted diet have much more total fatty acids than the livers of animals fed the commercial ration.

Regenerating liver of rats fed a commercial laboratory ration show an increase in four fatty acids (16:0, 16:1, 18:1, 18:2) at six hours after surgery. Livers from hepatectomized rats maintained on a fat-restricted diet do not show this increase until after twelve hours and when it occurs, it occurs in the same four fatty acids. The higher amounts of these four fatty acids in animals fed the laboratory ration remains at the elevated level for the first thirty-six hours while in the fat-restricted animals the amount shows a sharp maximum at eighteen hours with a return to normal levels for all fatty acids except oleic acid by twenty-four hours. This peak increase at eighteen hours may also be seen in the animals

fed the commercial ration but it is not as sharply defined as in the fat-restricted group.

Animals fed the commercial ration show an increase in the absolute amounts of fatty acids during regeneration which brings them temporarily to the level found in control animals on a fat-restricted diet. In normal liver the lipid phosphorous values in ration-fed animals is only about two-thirds the value in normal livers of animals fed the fat-restricted diet. Surgery and regeneration cause the lipid phosphorous of livers from animals fed the commercial ration to rise; this rise brings the lipid phosphorous values to the level of the normal liver of animals fed the fat-restricted diet. Surgery and regeneration do not cause a rise in liver lipid phosphorous in animals fed the fat-restricted diet.

Finally, it is observed that the protein per weight of liver is almost double in the animals fed laboratory ration than it is in the animals eating a fat-restricted diet. This difference cannot be explained by the increased amount of lipid in livers of animals fed the fat-restricted diet. It could be an increased amount of non-protein material in livers as a result of a low-fat, high carbohydrate diet.

CHAPTER IV

DISCUSSION

Tocopherol and Fatty Acid Metabolism

General Aspects of Fatty Acid Metabolism

Fatty acid synthesis in animals has been extensively studied using radioisotopes (44,45). The generalized pathways for the synthesis of the six major fatty acids considered in the present study are as follows: Palmitic acid is synthesized *de novo* from acetate, stearic acid is synthesized primarily through the elongation of fatty acid with acetate, palmitoleic and oleic acids are the result of a desaturation of palmitic and stearic acids respectively. Linoleic acid in animal tissues comes from the diet since it cannot be synthesized. Arachidonic acid is synthesized from linoleic acid through two pathways, both of which involve an elongation with acetate and two desaturation steps.

De novo synthesis occurs primarily in the soluble portion of fractionated tissues. The process of elongation occurs in the microsome and mitochondrial fractions. Desaturation occurs primarily in the microsomal fraction and involves part of the microsomal electron transport system and *in vitro*

requires O_2 and either NADH or NADPH (46). Desaturation of fatty acids is considered irreversible. Schlenk et al. have shown that possibly part of the polyunsaturated fatty acids may be degraded to shorter polyunsaturated fatty acids.

The general pathway for catabolism of all fatty acids is by successive removal of acetate units by the beta oxidation scheme. This acetate is then a part of the acetate pool for metabolism.

Effect of Tocopherol Deprivation on Fatty Acid Metabolism

The results of this study indicate that in tocopherol deprived chickens, an increased turnover of total fatty acids occurs as compared with control animals given tocopherol. When the specific activities of individual fatty acids are compared, there are differences between tocopherol-deprived and tocopherol-supplemented animals in the saturated and monounsaturated fatty acids. Since the specific activity of the stearic and palmitoleic acids is higher than that of palmitic in animals deprived of tocopherol 90 minutes after injection without a change in the absolute quantities of fatty acids, we conclude that fatty acids are turning over more rapidly in these animals. However, the specific activities of arachidonic acid was the same in the two groups of animals. It has been shown in this laboratory by Shires (48) and McCay (49) that no changes in fatty acid composition are observed after short term (several months) tocopherol depri-

vation in chickens and rats. This observation indicates both an increased catabolism and synthesis of fatty acids. Synthesis of arachidonic acid does not appear to be significantly different in these two groups of animals, although this is not conclusive.

The theory of tocopherol function which states that when tocopherol is limiting, polyunsaturated fatty acids are destroyed by peroxidation causing weakening or pathological changes in tissues, predicts that such destruction would result in a decrease of arachidonic acid or an increased synthesis of it, provided that no arachidonic acid was supplied in the diet. An increased synthesis of arachidonic is not observed in these experiments even when tocopherol deficiency is shown by disease symptoms. This disagreement with in vivo peroxidation theory is supported by the results of extensive studies of the subject by Green and coworkers (50-58). Their work fails to show an increased destruction of tocopherol in animals deprived of tocopherol or subjected to a dietary fat stress, even though such tocopherol destruction always accompanies in vitro lipid peroxidation. It is pointed out by these authors that there is no contradictory evidence to their observations reported in the literature.

Since the present study reports a change in saturated and monounsaturated fatty acid turnover but no change in the turnover of polyunsaturated fatty acid, it is possible that this change is occurring in the neutral lipid. It has been

reported in rats (1) and monkeys (59) that long term tocopherol deprivation results in a disappearance of adipose tissue and a decrease of neutral lipid in other tissues. This would support the possible increase in catabolism of neutral lipid of animals deprived of tocopherol.

If the proposal that lipid peroxidation does not occur in vivo as a result of tocopherol deprivation is accepted, the observations (60-62) that the feeding of large amounts of polyunsaturated fatty acids in the diet enhances the development of symptoms from tocopherol deprivation must be explained on the basis of something other than their "peroxidizability."

Brenner and Peluffo (63) report that the in vitro desaturation of stearic, oleic, and linoleic individually is inhibited by the presence of these same three fatty acids, the amount of inhibition being relative to the degree of unsaturation of the fatty acid involved. Their observations indicated that the three fatty acids were competing for the active site of dehydrogenation. Mohrhauer et al. (64) report that monounsaturated and polyunsaturated fatty acids can inhibit in vitro the elongation of linoleic acid, a step in the synthesis of arachidonic acid. It is further reported (45) that the feeding of oleate has a toxic effect on animals and can affect the metabolism of linoleic acid. The feeding of saturated fatty acids in large amounts increases the requirement for essential fatty acids.

Hill et al (65) and Bortz et al. (66) have shown that

hepatic lipogenesis is decreased in rats when fed a fat-supplemented diet. Pearce (67) has recently confirmed this for chickens. The inhibition is shown to occur at the level of acetyl CoA carboxylase, the enzyme which catalyzes the synthesis of malonyl CoA for the de novo synthesis of fatty acids. The inverse is also true, that is that the feeding of high carbohydrate diet increases hepatic lipogenesis. Desaturation of stearic and gamma-linolenic is increased by feeding a high carbohydrate diet (68).

From these observations it is reasonable to assume that if tocopherol deprivation increases fatty acid catabolism, and the feeding of fat decreases fatty acid synthesis, it would eventually result in a total decrease of tissue lipid. If such a situation were to persist it could result in an imbalance of necessary fatty acids. Such a change in composition of polyunsaturated fatty acids in rat liver has been reported by Witting and Horwitt (69) after a long-term tocopherol deprivation. The loss of adipose tissue might represent a transfer of lipid to essential organs such as liver.

What could be the possible site of tocopherol involvement in the above scheme? If lipid peroxidation does not occur in vivo then the observations of lipid peroxidation in vitro must result from changes occurring during the preparation of the tissue homogenate. May and McCay (20,21) have convincingly shown that the NADPH-requiring phospholipid oxidation in microsomes involves enzymatic activity of part of

the electron transport chain and only occurs when NADPH and O_2 are present; when the system has been treated inssuch a way as to inhibit electron transfer, denature protein, or remove the substrates or cofactors, lipid peroxidation does not occur.

At least part of this electron transport system may also be involved in the hydroxylation of drugs. This is suggested by the fact the activity of the lipid oxidizing system can be decreased if certain drugs are included in the incubation mixture (15,16). Carpenter (70) has reported that tocopherol deprivation results in a decrease of the *in vitro* drug hydroxylation activity.

Oshino *et al.* (46) have shown that a microsomal electron transport system is involved *in vitro* in the desaturation of stearic to oleic acid. This system will accept electrons from NADPH, NADH, and ascorbic acid. They also report that the addition of ferrous pyrophosphate, a substance which greatly facilitates NADPH-requiring phospholipid oxidation under certain circumstances, decreases the stearate desaturation by NADPH.

A possible explanation for this phenomena of NADPH-requiring phospholipid oxidation is that an electron transport component, involved in drug metabolism and fatty acid desaturation, becomes highly reduced when NADPH is present but its normal substrate is absent. If ferrous iron is added as a catalyst then polyunsaturated fatty acid radicals pres-

ent in the membrane can act as an electron acceptor, thereby oxidizing the component (after a free radical attack on the polyunsaturated fatty acids). Tocopherol *in vitro* then could exhibit its anti-oxidant characteristics by preventing the radical chain initiation and thereby destroying tocopherol.

Several other observations by other workers are in agreement with this explanation. Poyer (71) has shown that removal of iron from microsomal particles decreases lipid peroxidation relative to the amount of iron removed, even in the absence of tocopherol. Detwiler and Nason (72) have shown that tocopherol can react with cytochrome C in the presence of monounsaturated fatty acid to reduce cytochrome C, oxidize tocopherol and apparently have no effect on the fatty acid; this fatty acid is not polyunsaturated. This observation is an example of tocopherol interacting with a known electron transport component from mitochondria. *In vivo* tocopherol may be closely associated with an electron transport component as evidenced by its probable interaction *in vitro*. Its action *in vivo*, however, is not necessarily that observed *in vitro*.

Membrane-Bound Oxidative Activities in Regenerating Liver

Aspects of Regenerating Liver

The rapid liver growth after partial hepatic resection has been described quantitatively by Higgins and Anderson (35). During the first 24 hours after a partial hepatectomy,

no mitosis occurs in the liver. Approximately 24 hours after surgery the remaining liver has approximately doubled its weight. At this time a rapid mitosis occurs which continues for two to three days. After 4 days the liver has regained its original weight.

A large number of enzymatic activities are altered after partial hepatectomy. The liver may be described as a synchronous cell population during the first 24 hours until after the first wave of mitosis. Protein synthesis, RNA synthesis, and DNA synthesis occurs at approximately the same time in all the cells (reviews by Harkness (73) and Bucher (74,75)). Peters (76) has reported that the drug metabolism is lowered for the early period of liver regeneration. Since this is mediated in vitro by a microsomal electron transport system also associated with the NADPH-requiring phospholipid oxidation, this tissue was chosen to further study the membrane-bound oxidative systems in rat liver microsomes.

Effect of Tocopherol Deprivation

An empirical generalization from tocopherol function studies is that the symptoms of tocopherol deprivation are more pronounced in young, rapidly growing animals. If rapid growth itself is a factor determining the tocopherol requirement then regenerating liver should have a high requirement for tocopherol. The results reported in this study show that liver weight increase in tocopherol-deprived animals is not significantly different from tocopherol-supplemented animals.

It is then possible that the process which requires tocopherol is inhibited or absent in regenerating liver.

Activity of the NADPH-requiring phospholipid oxidation system in the microsomes is markedly reduced in regenerating liver 24 to 48 hours after surgery. This occurs in liver microsomes from both tocopherol-deprived or tocopherol-supplemented animals, fed either a fat-containing or a fat-restricted diet. The activity decrease appears to be a reduction in the extent of the reaction rather than the overall rate since the amount of malondialdehyde formed during the first 10 minutes of incubation is approximately the same as in non-regenerating liver.

Gulonolactone oxidase activity is also reduced in regenerating liver. This reduction is the same magnitude in both tocopherol-supplemented and tocopherol-deprived animals. The malondialdehyde formed in association with gulonolactone oxidase activity is not affected by liver regeneration. This, in contrast to the finding with NADPH-requiring phospholipid oxidation, indicated the oxidation of polyunsaturated fatty acids is by a different mechanism in these two activities. This has been confirmed by other work in this laboratory in which the gulonolactone oxidase system was separated from the NADPH-requiring phospholipid oxidation system.

These observations of the concomitant reduction of NADPH-requiring phospholipid oxidation and drug metabolism in regenerating liver is in agreement with the reports linking

NADPH-requiring phospholipid oxidation with the same microsomal electron transport system used for drug hydroxylation.

Effect of Dietary Fat Restriction

It is apparent from the study of NADPH-requiring phospholipid oxidation that this activity is reduced five-fold in non-regenerating liver microsomes from animals which have been fed a diet with no lipid except for the required linoleic acid. Since this activity of this system is so low, it may explain why alpha-tocopherol is not required by animals fed fat-restricted diets. Analysis of the fatty acids from these animals show that the concentration of arachidonic acid has not changed, as a result of the fat-restriction, so this low level of activity is not due to a lack of substrate for the reaction. (Compare normal liver in Table 19 with that in Table 23.) The lower activity may be a reflection of an alteration in the membrane structure resulting from the large increase in the amounts of less unsaturated fatty acids found in liver of animals fed a fat-restricted diet (compare Table 19 with Table 23). Note that the liver of fat-restricted animals have twice the amount of palmitic and three times the amount of oleic acid as does the commercial ration fed animals. In this same comparison, however, the liver of fat-restricted animals has only one-half the amount of linoleic acid as does the liver from animals fed the commercial ration.

Analysis of the protein and phospholipid content of these livers show that in all the microsomes where NADPH-

requiring phospholipid oxidation was reduced by regeneration following hepatectomy or by feeding a fat-restricted diet, the phospholipid content of the liver was approximately 30 micrograms of lipid phosphorous per milligram of liver weight. The normal animals on the laboratory ration (a diet which produces animals with a high activity of liver NADPH-requiring phospholipid oxidation) had a phospholipid content of approximately 23 micrograms per milligram liver weight. The present theory of in vivo lipid peroxidation does not account for the observed reduction in this activity when animals are red a fat-restricted diet. The lipid peroxidation as a result of gulonolactone oxidase activity is not reduced in the microsomes from non-regenerating livers of animals fed a fat-restricted diet. The data suggests that about four-fifths of the NADPH-requiring microsomal electron transport system which oxidizes phospholipids is lost in animals fed a fat-restricted diet and may alleviate the need for tocopherol. It is interesting to speculate that the activity of this system may be proportional to the amount of polyunsaturated fatty acids present in the diet. A possible explanation for this is discussed below.

Lipid Changes in Regenerating Liver

It has been reported that regenerating liver accumulates fat in the early stages of regeneration (68). This accumulation is also observed in the regenerating livers reported in this study. It seems evident from these results, how-

ever, that the diet markedly affects the extent and duration of this accumulation. That this change occurs only in the neutral lipid is consistent with results reported by other investigators (77,78). These observations show that the lipids are increased 6 hours after partial hepatectomy as observed both by chemical and microscopic techniques. This is in agreement with the observations in this report for animals fed a laboratory ration. However, when the animals are fed a purified diet with no fat except the required linoleic acid, no change is observed until after 12 hours. The change then is much larger and returns to normal more quickly than in animals on laboratory ration.

The studies by Camargo (79) and Fex and Olivecrona (80,81) show that the neutral lipid increase in regenerating liver is a result of transport of fatty acids from adipose tissue. Blood flow into the liver is greater per gram of liver in animals after a partial hepatectomy. Johnson and Albert (82,83) have indicated that liver fatty acid synthesis is decreased in regenerating liver. It is suggested then from the results reported in this study that some change occurs in liver from animals fed a fat-restricted diet which alters the transport of fatty acids into or out of the liver cell. This alteration probably occurs in cellular membrane.

The above results coupled with the changes in phospholipid content in regenerating livers fed a laboratory ration indicates that changes in dietary fat intake may cause altera-

tions in membrane structural components which may then affect the metabolic characteristics of any membrane-bound enzymatic system.

A Model for the Function of Tocopherol

The following hypothesis is proposed as a model for directing further study into the mechanism of *in vivo* tocopherol action:

"Tocopherol interacts with specific membrane enzymes to increase the proportion of each of those enzymes which exist in a particular conformation. Absence of tocopherol allows a different conformation of each enzyme to predominate which also has a different activity. The membrane enzyme-tocopherol interaction occurs because of tocopherol's lipid character and other similarly interacting lipids may substitute for tocopherol, including the proper ratio of naturally occurring lipids."

This hypothesis can account for many of the observations associated with both *in vitro* and *in vivo* activities of tissues from animals deprived of tocopherol.

First, in the case of an electron transport system, a free radical intermediate in the transport chain requiring NADPH for drug metabolism or phospholipid fatty acid oxidation could be impeded in its activity by a conformation change. Such a conformation change *in vivo* would alter the rate for these activities and the NADPH pool would change only slightly. *In vitro*, however, where the NADPH pool is

artificially large and the acceptor substrates are absent, a build-up of free radicals could occur where a normally unreactive substance may react, such as polyunsaturated fatty acid. In such a case the in vivo and in vitro activities would be markedly different.

The above effect does not have to occur with a free radical intermediate but could also occur with other enzymes. The synergism suggested here between in vivo and in vitro results with tocopherol deprivation suggest that tocopherol is associated with membrane components involved with oxidative reactions.

Second, this study suggests that the reason the requirement for tocopherol can be reduced or completely eliminated in animals fed a fat-restricted diet is due to the marked reduction in the activity of the enzymic phospholipid peroxidation system in microsomes. In vivo evidence for this same deletion of the tocopherol requirement has been presented by Bieri (84). He observed that chickens deprived of tocopherol from birth show no symptoms of encephalomalacia if they were fed a fat-restricted diet. The same diet with 15% lard caused chicks to develop encephalomalacia.

Third, the idea is presented here that changes in unsaturated fatty acid metabolism due to the feeding of high fat diet could cause the in vivo disease symptoms associated with tocopherol deprivation. Support for this comes from a description of the symptoms observed in essential fatty acid

deficiency (45):

1) In rats, sterility in male, resorption of the fetus in female
2) In chicks, pseudo-encephalomalacia
3) Skin problems, i.e. scaliness
4) Capillary fragility
5) Fatty liver (usually precedes liver necrosis)

These symptoms closely mimic those observed in animals deprived of tocopherol and given a high fat diet. A fat-restricted diet could relieve the tocopherol deficiency by removing the inhibition of the synthesis of polyunsaturated fatty acids.

Fourth, this hypothesis gives a reason for the substitution for tocopherol by some of the other antioxidants in vivo. If the antioxidant property is derived from the same structure that also gives tocopherol its enzyme interacting properties then other similarly acting antioxidants could partially substitute for tocopherol. This hypothesis does not explain the effect of structurally non-similar antioxidants on the in vivo symptoms observed in tocopherol deprivation.

The results reported in this study and by others mentioned in this discussion present doubts that the in vivo lipid peroxidation theory can account for the problems which arise in animals when they are deprived of tocopherol. The above hypothesis attempts to coalesce many of these observa-

tions into a meaningful relationship which can be tested by experiments.

Experimental Approaches

A test of the above hypothesis will involve showing:

1) That tocopherol interacts with specific membrane enzymes
2) That the above interaction results in a changed enzyme activity as a result of a conformation change
3) That other lipids or a change in ratio of lipids can cause the same effect as tocopherol deprivation

The test for interaction between enzyme and tocopherol could best be done with purified enzymes from membranes and would involve equilibrium binding studies using radioactively labelled tocopherol. A control could be run with several proteins to check for non-specific binding. At present there are no satisfactory methods for purifying membrane proteins although in this laboratory attempts have been made to purify gulonolactone oxidase (49) and the membrane-bound NADPH-requiring lipid oxidation enzyme (50) with partial success. These partially purified fractions would be sufficient to indicate whether ratioactive tocopherol would bind to them using a gel filtration technique.

Assuming that the interaction has been shown between a specific protein and tocopherol, the change in activity of an enzyme system could be measured *in vivo* in animals which had been rigorously deprived of tocopherol on a high fat diet.

This measurement could use a radioactive precursor of the substance to be synthesized. In the case of arachidonic acid, this could be a labelled linoleic acid. Using the deprived animals as controls, a change in activity should be noted a short time after injection of tocopherol into the animal. This type of experiment could be performed also on organs, tissue slices, and tissue fractions after homogenization.

The test for the substitution by other lipids for tocopherol could be performed as in the preceding paragraph except the lipid to be tested would be substituted for tocopherol.

To conclude, the study reported in this dissertation has indicated that a more unifying concept for the function of tocopherol is needed. The in vivo lipid peroxidation theory does not explain the observations in this report and the reports of other workers. A hypothesis is offered to present a different view of the problem and to suggest a different way for studying the reactions associated with tocopherol.

CHAPTER V

SUMMARY

The utilization *in vitro* of the polyunsaturated fatty acids in phospholipids of liver endoplasmic reticulum by an enzyme system requiring NADHH and O_2 has been previously demonstrated in this laboratory. The enzyme system is present in the endoplasmic reticulum. This activity is highest in microsomes from animals deprived of tocopherol. Feeding tocopherol to rats before microsome preparation produces a lag period in the utilization of the polyunsaturated fatty acids. It has been suggested that the function of tocopherol, a required nutrient for mammals, is involved in the control of the activity of this enzyme *in vivo* to prevent the excessive alteration of the phospholipids forming part of the membrane structure.

This hypothesis was tested in chicks by measuring the incorporation of acetate-1-^{14}C into the liver fatty acids in animals deprived of tocopherol. Compared to controls these animals had a faster turnover of total fatty acids in the liver. Analysis of individual fatty acids showed that this turnover occurred more in saturated and monosaturated fatty

acids than in polyunsaturated fatty acids. These results agree with observations that tocopherol affects total lipid metabolism. This effect cannot be explained simply by the increase of polyunsaturated fatty acid destruction.

Studies with rats were made on the effect of 1) dietary fat restriction and 2) the rapid growth occurring after partial hepatectomy, on the in vitro NADPH-requiring phospholipid oxidation. It was discovered that fat restriction (1.6% linoleic acid is the only lipid received in the diet) results in a marked reduction of lipid oxidation in microsomes from tocopherol-deprived animals. The microsomes from regenerating rat liver also showed a diminished activity of this enzyme. A study of the lipid changes in these livers show that coincident with the decrease in enzymatic activity there is a rise in phospholipid to protein ratio.

To determine if the decrease in the phospholipid oxidation system was the result of a decrease in the amount of arachidonic acid in the phospholipids of the liver of fat-restricted animals or in regenerating liver, the fatty acid composition of these tissues were determined. The content of arachidonate (the primary substrate of the NADPH-phospholipid oxidizing system) does not appear to be changed sufficiently to account for the large decrease in the activity of this enzyme system. Regenerating livers from rats on the fat-restricted diet show a four fold increase in specific fatty acids in the neutral lipids between 12 and 18 hours after

partial hepatectomy. By 24 hours this level has returned to normal.

The possibility that tocopherol functions to stabilize cellular membrane enzyme systems is discussed and an approach is suggested for a study of this hypothesis.

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