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THE METABOLISM OF 1,2-DIACYLGLYCERO-3-PHOSPHORYLETHANOLAMINE

by

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THE METABOLISM OF 1,2-DIACYLGLYCERO-3-PHOSPHORYLETHANOLAMINE

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Acyl-CoA:monoacylphosphoglyceride acyltransferases catalyze the transfer of fatty acids from their acyl-CoA esters to 1- or 2-acylglycero-3-phosphorylethanolamine (1- or 2-acyl-GPE) and 1- or 2-acylglycero-3-phosphorylcholine (1- or 2-acyl-GPC). The precise number of enzymes involved in the acyltransfer reactions is not known.

The predominant species of naturally occurring 1,2-diacyl-GPE have saturated acids esterified at the 1-position and unsaturated acids at the 2-position. The selectivities of the acyltransferases suggest that these enzymes may be involved in maintaining the fatty acid composition of naturally occurring phospholipids.

Acyltransferase rates can be followed by a continuous assay using an internal colorimetric reagent, 5,5'-dithiobis(2-nitrobenzoate), to measure CoA release. Isolation of the products from incubation mixtures containing radioactive acyl-CoA showed that the rate of CoA release corresponded to the amount of diacyl-GPE formed. Phospholipase A treatment of the ^{14}C-labeled diacyl-GPE showed that the 1- and 2-acyl-GPE preparations are relatively free of the other positional isomer.

Rat and pig liver microsomal acyltransferases preferentially transferred saturated and unsaturated acids having a <u>trans</u> double bond at carbon 9 to the 1-position of

monoacyl-GPE. Linoleic, arachidonic, and 8,11,14-eicosatrienoic acids were preferentially esterified to the 2-position of monoacyl-GPE.

Analysis of the acids esterified at the 2-position of diacyl-GPE and alkenylacyl-GPE from human erythrocyte stromata showed that both contained high proportions of polyunsaturated fatty acids. The composition of the fatty acids esterified to the 2-position of stromal diacyl-GPE could not be predicted from a consideration of only the abundance of the fatty acids in plasma and the acyltransferase rates.

No difference was observed in the rates of transfer of arachidonate to 1-acyl-GPE by acyltransferase preparations from male or female rat livers. Arachidonate was transferred faster than 8,11,14-eicosatrienoate to the 2-position of acyl-GPE whereas the trienoate was transferred faster to the 2-position of acyl-GPC.

To my Husband and Son

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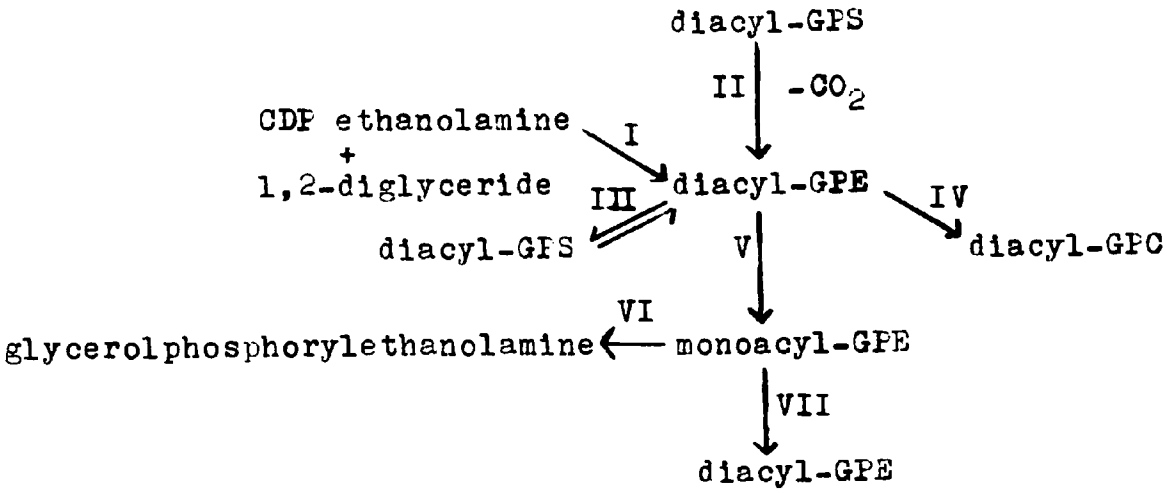
ABBREVIATIONS

ANSA	aminonaptholsulfonic acid
BHT	2,6-di-*t*-butyl-*p*-cresol
CDP	cytidine diphosphate
CoA	Coenzyme A
CPG	choline phosphoglyceride
DEAE-cellulose	diethylaminoethyl cellulose
DFP	diisopropylfluorophosphate
DOC	sodium deoxycholate
DTNB	5,5'-dithio-bis(2-nitrobenzoate)
EDTA	ethylenediaminetetraacetate
EPG	ethanolamine phosphoglyceride
GLC	gas-liquid chromatography
GPC	glycero-3-phosphorylcholine
GPE	glycero-3-phosphorylethanolamine
GPS	glycero-3-phosphorylserine
POPOP	*p*-bis[2-(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole
SPG	serine phosphoglyceride
TLC	thin layer chromatography
Tris-HCl	tris(hydroxymethyl)aminomethane chloride

INTRODUCTION

Ethanolamine phosphoglycerides represent 26 to 27% of the total phospholipids in rat liver microsomes (1,2), 30 to 40% in rat liver mitochondria (1,2), and 30% in erythrocytes (3). A large proportion of the phospholipids found in tissues and blood are present in membranes (4) and possibly function in maintaining the structural integrity of the membrane.

Reactions believed to be involved in the synthesis and metabolism of 1,2-diacylglycero-3- phosphorylethanolamine (diacyl-GPE) are shown below.



Enzymes:

I CDPethanolamine:1,2-diglyceride ethanolaminephosphotransferase (aminephosphotransferase)

II phosphatidylserine carboxy-lyase

III phosphatidylserine:ethanolamine phosphatidyltransferase

IV S-adenosylmethionine:phosphatidylethanolamine N-methyltransferase (N-methyltransferase)

V phosphatide acyl-hydrolase (phospholipase)

VI monoacylphosphoglyceride acyl-hydrolase (phospholipase B)

VII acyl-CoA:monoacylphosphoglyceride acyltransferase (acyltransferase)

Reaction I was first described by Kennedy and Weiss (5,6). Using whole liver homogenates they showed a conversion of phosphorylethanolamine, the precursor of CDP ethanolamine, to lipid. Bremer et al. (7) observed that when 3-^{14}C-serine was injected into rats (Reaction II), the specific activity of the ethanolamine phosphoglycerides was much greater than that of phosphorylethanolamine or CDIethanolamine. Further evidence for Reaction II was obtained by Wilson et al. (8) who showed that the incorporation of label from serine into phospholipid ethanolamine by liver microsomes was not reduced by adding unlabeled ethanolamine or phosphorylethanolamine. The relative importance of these two pathways in de novo synthesis of diacyl-GPE has not been determined. Furthermore the base exchange Reaction III has been observed by Dils and Hübscher (9). This reaction could influence the amount of de novo synthesis of diacyl-GPE from 1,2-diacylglycero-3-phosphorylserine (diacyl-GPS).

Diacyl-GPE can be converted to 1,2-diacylglycero-3-phosphorylcholine (diacyl-GPC) by Reaction IV (10). Although the direct methylation of diacyl-GPE by S-adenosylmethionine has not been demonstrated in mammalian tissues, the remaining steps from phosphatidylmonomethylethanolamine to phosphatidyldimethylethanolamine to phosphatidyl choline have been demonstrated in rat liver tissues (11).

Rat liver subcellular particles contain phospholipases (Reaction V) which catalyze the hydrolysis of exogenous phospholipids (12-15). Bjørnstad has studied the degradation

of endogenous phospholipids in rat liver microsomes (16) and mitochondria (17). Using subcellular fractions isolated from rats injected with 1,2-^{14}C-ethanolamine, methyl-^{14}C-L-methionine, or ^{32}P-orthophosphate he has shown that both preparations preferentially hydrolyzed endogenous diacyl-GPE. The main products in mitochondria were monoacyl-GPE and free fatty acids; while in microsomes the main products were glycerolphosphorylethanolamine and free fatty acids. Thus microsomes appeared to have more phospholipase B (Reaction VI) activity than mitochondria.

Monoacyl-GPE can either be further degraded to glycerolphosphorylethanolamine (Reaction VI) as described above (16-20) or re-esterified to form diacyl-GPE (Reaction VII) (21,22). The acyltransferase reaction (Reaction VII) has been observed <u>in vitro</u> (22) and <u>in vivo</u> (23).

Diacyl-GPE can have a different fatty acid composition depending upon the tissue from which the phospholipids were isolated. For instance, diacyl-GPE fractions isolated from rat liver microsomes and mitochondria (1,2) have very similar compositions while diacyl-GPE isolated from mouse liver (24), human plasma (25), or erythrocytes (3) have very different compositions.

Hydrolysis of phospholipids by phospholipase A from <u>Crotalus adamanteus</u> showed that saturated acids are located predominantly at the 1-position and unsaturated acids at the 2-position (15,26). Recently molecular species of diacyl-GPC (27-32) and diacyl-GPE (33) have been separated on the basis of the number of double bonds present in the molecule.

There seems to be a specific association of arachidonic acid with stearic acid and of oleic and linoleic acids with palmitic acid.

These differences in total fatty acid composition, in positional distribution of the fatty acids, and in molecular species could be maintained by a selectivity of the aminephosphotransferase system (Reaction I) for specific diglycerides. The specificity of the amine-phosphotransferase system has not been determined. These differences in fatty acid patterns could also be formed by hydrolysis of diacyl-GPE or diacyl-GPC (Reaction V) followed by re-esterification of the monoacyl-GPE or GPC (Reaction VII). Scherphof et al. (34) have reported that a mitochondrial phospholipase attacks the 2-position of diacyl-GPE and a microsomal enzyme attacks the 1-position. However, since they did not inhibit the microsomal lysophospholipase (16-20) their results do not exclude the possibility that phospholipases which attack both positions of diacyl-GPE are present in microsomes. Lands and Hart (22) have shown that the acyltransferase system preferentially esterifies unsaturated fatty acids at the 2-position and saturated acids at the 1-position. Thus the combined effect of a phospholipase and an acyltransferase could produce a selective turnover of fatty acids at either the 1- or 2-position.

The changes in fatty acid composition of erythrocyte and liver phospholipids under conditions of essential fatty acid deficiency suggest that arachidonic acid and the ethanolamine phosphoglycerides (EPG) containing this acid have a special metabolic role. In general, essential fatty acid deprivation leads to marked increases in oleic acid and to the appearance of 5,8,11-eicosatrienoic acid, while the levels of arachidonate and linoleate are decreased (4,35-37). No significant variation in the amount of individual phospholipids is observed (4,35-37).

In erythrocytes this change occurs in all fractions but is most evident in EPG and serine phosphoglycerides (SPG) which contain a high proportion of the polyunsaturated fatty acids (35). There appears to be an attempt to maintain a high degree of unsaturation in this EPG fraction either by retaining arachidonate (38) or replacing it with 5,8,11-eicosatrienoic acid (35).

Fischer and Glenn (37) have analyzed the fatty acid composition of individual liver phospholipids from rats fed a fat-free diet for 12 weeks. In the EPG arachidonic acid decreased gradually (19% decrease in 5 weeks), while in the choline phosphoglycerides (CPG) arachidonic acid decreased more rapidly (57%) in 5 weeks. The arachidonate was replaced by 5,8,11-eicosatrienoic acid in both phospholipid fractions. Yamamoto et al. (36) also observed a more rapid decrease of arachidonate in CPG than EPG using microsomes and mitochondria from livers of rats deficient

in essential fatty acids. This more gradual decrease in arachidonate in the EPG is paralleled by a slightly faster displacement of 5,8,11-eicosatrienoate in the EPG when either linoleate or arachidonate are fed to rats deficient in essential fatty acids (39). This retention of arachidonate in the EPG of both liver and erythrocytes suggests that the function of EPG in the cell membrane and in cell metabolism may differ from that of the CPG.

Sex differences have been observed in the fatty acid composition of phospholipids from rat liver (40-45), plasma and erythrocytes (38). The differences consisted of higher proportions of stearate and arachidonate in female rats and higher proportions of palmitate and linoleate in males. These sex differences in liver and plasma arachidonate levels were observed in the CPG, not the EPG fraction. Harris et al. (44), Collins (46) and Isozaki et al. (47) have shown that the stearyl arachidonyl species of diacyl-GPC had much lower specific activity than the palmityl linoleyl species when the incorporation of inorganic ^{32}P into diacyl-GPC was measured. Administration of methyl-labeled methionine resulted in a higher specific activity in the 1-stearyl-2-arachidonyl-GPC (Reaction IV) (45,47). Thus it appears that the species of diacyl-GPC originate by different pathways of synthesis. The role of estrogens in stimulating and androgens in inhibiting the transmethylation is indicated by studies with these hormones in normal

and gonadectomized animals (48-50). The differences in stearic and arachidonic acids could occur if female rats synthesized a higher proportion of their diacyl-GPC via methylation of diacyl-GPE than did males.

In this thesis the properties and specificities of acyltransferases from different sources are studied. The acyltransferases may be involved in determining the fatty acid composition observed in diacyl-GPE from erythrocytes. This enzyme appears to play a role in maintaining polyunsaturated fatty acids in diacyl-GPE.

METHODS

I. Preparation and Analysis of Substrates

A. Preparation of 1-Acylglycero-3-phosphorylethanolamine (1-Acyl-GPE)

Yolks from six eggs were mixed with 500 ml of ether and 5 ml of phospholipase A from Crotalus adamanteus venom (50 mg of phospholipase A, 20 µl of 1 M $CaCl_2$, 1 ml of 0.5 M Tris-HCl buffer in 4 ml of H_2O) for six hours. The reaction was stopped with 100 ml of methanol and the mixture taken to dryness on a rotary flash evaporator. Cold acetone was added to the dried lipoprotein and the suspension was homogenized in a Waring blendor and filtered. The precipitate was re-suspended in cold acetone, homogenized and filtered. The precipitate was suspended in chloroform:methanol (1:1) and stored for 12 hours at 0° C. The suspension was filtered and the precipitate washed several times with chloroform: methanol (1:1). The filtrate was dried and the residue suspended in chloroform. The phosphorus content of the acetone insoluble phospholipids was determined as described by Bartlett (51). The phospholipids isolated in this manner contained 290 mg of phosphorus.

The 1-acyl-GPE was separated from other phospholipids by silicic acid (Mallinckrodt, 100-200 mesh) chromatography (52). The column (4.5 x 32 cm) was prepared from 200 g of heat-activated silicic acid slurried in ether:benzene (1:1). The lipids (290 mg of phosphorus) were applied to the column in chloroform and eluted with 2000 ml of ethanol:ether (1:9) to remove neutral lipids and 3000 ml of ethanol:ether (1:1)

to remove any unreacted diacyl-GPE. Finally 150 ml fractions of ethanol were collected until spot tests for amino nitrogen were negative. The fractions were checked for purity by thin layer chromatography (TLC) on Silica Gel G (Brinkman) in chloroform:methanol:water (65:25:4) in which 1-acyl-GPE had an R_f of 0.3. The plates were allowed to dry and the lipids visualized as described on p. 14.

Ethanol fractions containing pure 1-acyl-GPE (usually fractions 8 to 20) were combined and the phosphorus content determined (51).

The methyl esters were prepared as described on p. 12. The 1-acyl-GPE had a molar ratio of ester to phosphate of 1.12 and contained 62.4 mole percent of stearic acid and 37.6 mole percent of palmitic acid.

The 1-acyl-GPE was stored in chloroform:methanol (1:1). Before use in the enzyme assays 10 μmoles of 1-acyl-GPE were dried under nitrogen and 2.5 ml of water or 0.05 M Tris-HCl (pH 7.4) were added. A suspension of 1-acyl-GPE in water or 0.05 M Tris-HCl (pH 7.4) was obtained by mixing and warming the solution in a 30-35° water bath.

B. Preparation of 2-Acylglycero-3-phosphorylethanolamine (2-Acyl-GPE)

1. Isolation of Ethanolamine Phosphoglycerides

Crude ethanolamine phosphoglycerides were isolated from pig heart phospholipids by silicic acid column chromatography (52). Fractions containing crude ethanolamine phosphoglycerides were combined, dried on a rotary evaporator

and dissolved in chloroform:methanol (9:1). Phosphorus content was determined (51) and about 20 mmoles were chromatographed on a column containing 25 g of DEAE-cellulose. The DEAE-cellulose column (2.5 x 15 cm) was prepared as described by Rouser (53). Crude ethanolamine phosphoglycerides were applied to the column and the column eluted with 2000 ml of chloroform:methanol (9:1) to remove nonacidic lipids except ethanolamine phosphoglycerides. Then 3000 ml of chloroform:methanol (7:3) were used to elute the ethanolamine phosphoglycerides (200 ml fractions were collected). These fractions were checked by TLC as described on p. 13 and 14. The plates were sprayed with 1% $HgCl_2$ in 95% acetic acid followed by fuchsin reagent (0.5 g fuchsin dissolved in one liter of water, filtered, and saturated with SO_2). A purple color was noted as a fuchsin-positive test for aldehydes.

Fractions containing ethanolamine phosphoglycerides were combined and the alkenyl ether content determined as described on p. 17. The combined ethanolamine phosphoglyceride fractions from DEAE-cellulose contained approximately 40% alkenylacyl-GPE and 60% 1,2-diacylglycero-3-phosphorylethanolamine (diacyl-GPE).

2. Preparation of 1-(1'-alk-1'-enyl)-2-acylglycero-3-phosphorylethanolamine

Alkenylacyl-GPE was separated from diacyl-GPE by phospholipase D treatment which removed the ethanolamine from diacyl-GPE at a faster rate than from alkenylacyl-GPE.

The procedure described by Lands and Hart (54) was used for phospholipase D treatment. DEAE-cellulose chromatography was used to separate the alkenylacyl-GPE from the 1,2-diacylglycero-3-phosphate formed. The yield of alkenylacyl-GPE was 82% based on the amount of alkenylacyl-GPE isolated on the first DEAE-cellulose column. The alkenylacyl-GPE preparation contained 85 μmoles of phosphorus (51), 77 μmoles of ester (55) and 80 μmoles of alkenyl ether* (p. 17).

3. Preparation of 2-Acylglycero-3-phosphorylethanolamine

The 2-acyl-GPE was prepared by treating 5-10 μmoles of alkenylacyl-GPE in 2 ml of chloroform:methanol (2:1) with 1 ml of 0.1 M Tris-HCl (pH 7.4) and 0.5 ml (8 μmoles) of aqueous I_2. After 20 min, 3 ml of ethanol were added, the excess I_2 was reacted with $Na_2S_2O_3$ and the chloroform solution was washed twice with H_2O to remove salts. The lipid extract was evaporated to dryness, treated with cold acetone to remove organic and inorganic iodides and then suspended in 5 ml of water.

The amount of 2-acyl-GPE** formed by treatment of alkenylacyl-GPE with aqueous iodine was determined by separation of the 2-acyl-GPE from any unreacted starting

---

* The alkenylacyl-GPE contains a small amount of diacyl-GPE which is not degraded by phospholipase D treatment. However, since alkenylacyl-GPE is the reactive component in the preparation of 2-acyl-GPE the fraction will be referred to as alkenylacyl-GPE.

** The 2-acyl-GPE preparation contains unreacted alkenylacyl-GPE and a small amount of diacyl-GPE. Since 2-acyl-GPE is shown to be the reactive component in the acyltransferase assays (p. 43) the fraction will be referred to as 2-acyl-GPE.

material. Thin layer chromatography (TLC) on Silica Gel G in chloroform:methanol:water (65:25:4) was used for the separation and the spots were visualized by spraying with molybdate spray (p. 14). The phosphorus content of the monoacyl-GPE and ethanolamine phosphoglyceride bands from TLC was determined as described on p. 14. From 280 mμmoles in the reaction mixture, 104 mμmoles were recovered in the monoacyl-GPE area and 161 mμmoles in the ethanolamine phosphoglyceride area. Thus only about 35% of the alkenyl-acyl-GPE was converted to 2-acyl-GPE.

C. Analysis of Ethanolamine Phosphoglycerides

1. Preparation of Methyl Esters

a. Methanolysis with Sodium Methoxide in Methanol

A known amount (180 mμmoles) of methyl pentadecanoate was added as an internal standard to each sample. The solvent was evaporated and 2 ml of 0.5 N sodium methoxide in methanol added. The reaction was stopped after 10 min with 0.2 ml of 6 N HCl and the resulting mixture extracted with 5 ml of petroleum ether and 5 ml of water. The petroleum ether layer was dried over Na_2SO_4:$NaHCO_3$, 2:1 (w/w) for at least 10 min, then decanted from the drying agent. Solvent was removed under a stream of nitrogen with gentle warming, and the methyl esters were taken up in 20-30 μl of CS_2. Portions of this solution were used for injection into the gas chromatograph.

b. Methanolysis with Sulfuric Acid in Methanol

A known amount (180 mμmoles) of methyl petadecanoate was added as an internal standard to each sample. The

solvent was evaporated and 5 ml of sulfuric acid:methanol (1:9) was added. The mixture was refluxed for 2 hours at 80°, allowed to cool to room temperature, and the methyl esters extracted as described above.

2. Gas-liquid Chromatography of the Methyl Esters

Gas chromatography of the methyl esters was done using a Barber-Colman instrument equipped with a hydrogen flame detector and a 1/4 in x 8 ft column packed with 10% ethyleneglycol succinate on Gas Chrom P. The temperature was maintained at 196°. Methyl esters were identified by comparing their retention times to those of a standard mixture of 16:0, 18:0, 20:0, and 22:0 methyl esters (56). The amount of each ester present was calculated from the product of the peak height and the retention time, measured in centimeters from the point of injection. This product, P, and the molecular weights, MW, of the methyl esters was used to calculate the fatty acid content as shown below.

$$\text{m}\mu\text{moles of acid} = \left(\frac{P_{\text{acid}}}{P_{15:0}}\right)\left(\frac{MW_{15:0}}{MW_{\text{acid}}}\right)(\text{m}\mu\text{moles 15:0})$$

3. Thin Layer Chromatography of Lipids

a. Solvent Systems for Separation of Lipids

(1) Silica Gel G plates were developed in chloroform: methanol:water (65:25:4) to separate mono- and diacyl-GPC, mono- and diacyl-GPE, and free fatty acid.

(2) Silicic acid (Mallinckrodt) plates were developed to approximately 6 cm from the origin in ether:petroleum ether (40-60°) (60:40), allowed to dry 15 min and developed to the top in ether:petroleum ether (12:90). This system

was used to separate monoglycerides, diglycerides, triglycerides, and free fatty acids.

(3) Plates spread with silicic acid slurried in 0.1 M Na_2CO_3 were developed in chloroform:methanol:1 N NH_4OH (80:36:2) to separate acyl-CoA, phosphatidic acid, and free fatty acid. Figure I shows the separations obtained in each of these solvent systems.

b. Detection of Lipids

(1) Ninhydrin [1% ninhydrin in acetone (w/v)] was used to detect amino nitrogen.

(2) Molybdate was used to detect phosphorus. The molybdate spray was prepared from two solutions. The first solution was prepared by adding 40.11 g of MoO_3 to one liter of concentrated sulfuric acid and heating gently until the MoO_3 dissolved. The second solution was made by dissolving 1.78 g of powdered molybdenum in 500 ml of the first solution and heating gently for 15 minutes. One ml of each solution was added to 6 ml of water to make the spray.

(3) Sulfuric acid [55% H_2SO_4 in saturated $K_2Cr_2O_7$ (v/v)] was used to detect compounds containing carbon.

(4) Iodine [1% iodine in methanol (w/v)] was used to detect lipids containing unsaturated acids.

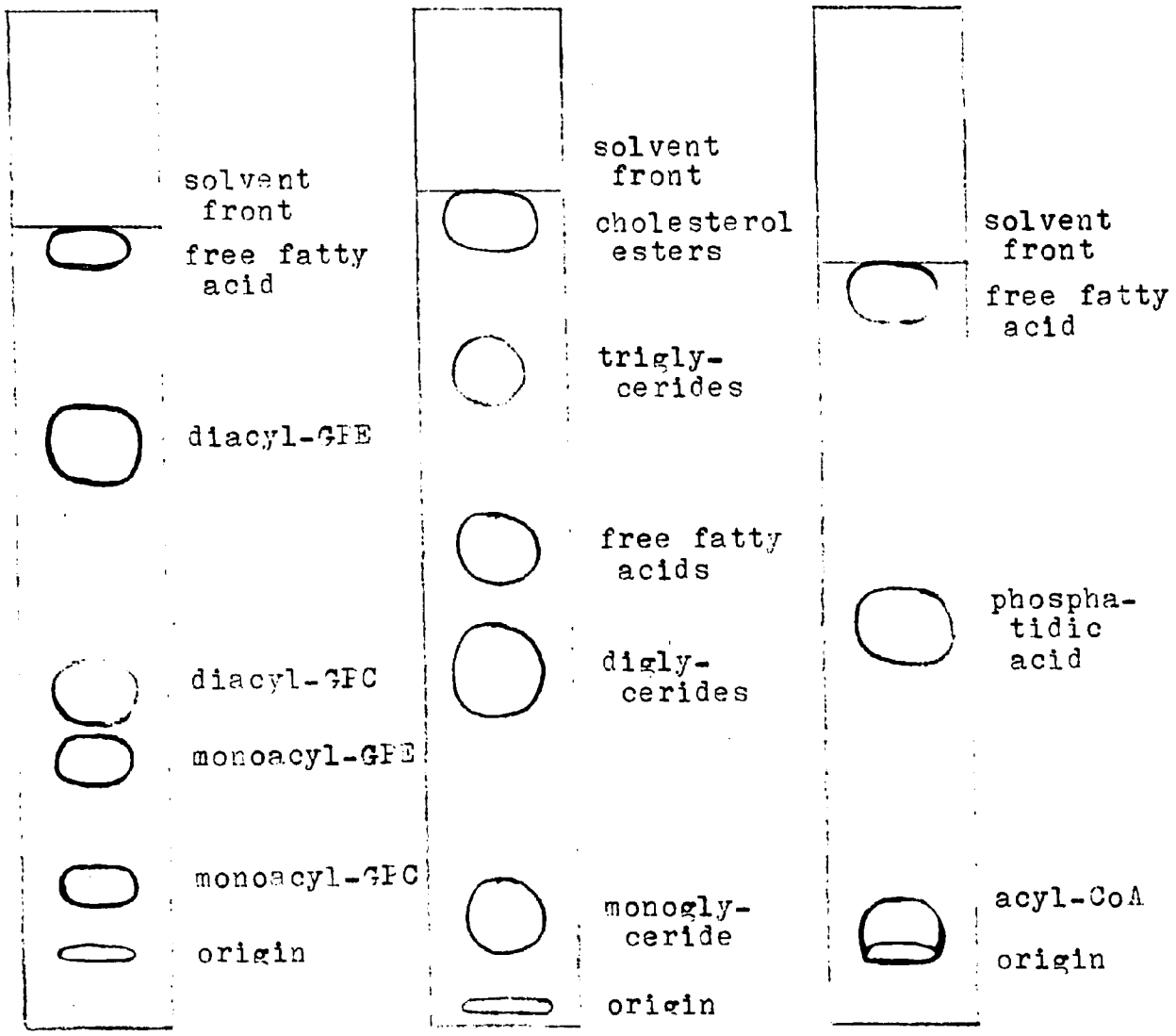
c. Quantitation of Phosphorus

The phospholipids are separated in solvent system 1 described above and the TLC plate sprayed with molybdate spray. Areas containing phospholipids were scraped into tubes, and 0.6 ml of 70% perchloric acid was added. This mixture was heated at 185° C for 2 hours and then cooled to

Figure I. The Separation of Lipids by Thin Layer Chromatography.

The separation of lipids was accomplished as described on p. 13. The lipid containing areas were identified as described on p. 14. The identification of the spots is shown to the right of each figure.

Figure I


solvent front
free fatty acid
diacyl-GPE
diacyl-GPC
monoacyl-GPE
monoacyl-GPC
origin
solvent front
cholesterol esters
triglycerides
free fatty acids
diglycerides
monoglyceride
origin
solvent front
free fatty acid
phosphatidic acid
acyl-CoA
origin

room temperature. Next, 5.0 ml of an ammonium molybdate solution (1.32 g ammonium molybdate per 500 ml water) and 0.2 ml of an aminonaphtholsulfonic acid solution (described below) were added. The samples were mixed and placed in a boiling water bath for 15 minutes. The samples were cooled, and the absorbance was read at 830 mμ. Tubes containing Silica Gel G and known amounts of phosphate were used as standards.

The aminonaphtholsulfonic acid solution was prepared from a dry mix, which was made by grinding 2.5 g of aminonaphtholsulfonic acid (ANSA), 142.5 g of $NaHSO_3$, and 5.0 g of Na_2SO_3 in a mortar. To 50 ml of water, 3.51 g of dry mix were added to make the ANSA solution used in phosphorus determinations.

4. Determination of Alkenyl Ethers

A modification of the procedure described by Gottfried and Rapport (57) was used to determine the alkenyl ether content of the phospholipids. A 2.5×10^{-4} N iodine solution in 3% potassium iodide (0.5 ml) was added to about 0.05 to 0.2 μmole of the sample and the tube mixed on a Vortex for about 10 seconds. The tubes were allowed to stand 15 min, and then 4 ml of 95% ethanol were added and the solution mixed. The absorbance was measured at 355 mμ. Standards containing known amounts of alkenylacyl-GPC were used.

II. Preparation of Acyl-CoA:Monoacylphosphoglyceride Acyltransferase

A. Preparation of Subcellular Fractions

Particles were isolated from rat liver homogenates in 0.25 M sucrose containing 10^{-3} M EDTA by collecting those that sedimented in 15 min between 5,000 and 15,000 x g (heavy particles) and those remaining in the supernatant that sedimented in 90 min at 78,000 x g (microsomes). The heavy particles were washed twice and finally suspended in 0.25 M sucrose with EDTA and stored at -15°. The microsomal particles were treated in the same manner, but washed only once. Protein was determined by the ratio of the absorbance measurements at 260 and 280 mμ (58).

B. Preparation of Erythrocyte Stromata

Hemolysis of red blood cells followed a slightly modified procedure of Dodge et al. (59). Erythrocytes were isolated from citrated whole blood by centrifugation at 1,000 x g for 20 min. The buffy layer of white blood cells and supernatant plasma were carefully removed. The erythrocytes were washed with a volume of isotonic phosphate (0.109 M sodium phosphate, pH 7.4) equal to the original volume of blood and centrifuged at 2,000 x g for 20 minutes. This washing was repeated twice. After the third centrifugation the cells were resuspended to the original volume of blood in isotonic phosphate. Hemolysis was performed by pipetting 2 ml of the washed cell suspension into 40 ml of hypotonic phosphate buffer (0.0072 M sodium phosphate), mixing gently and allowing the cells to sit at 0° for 15 min. The cells were then

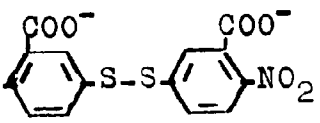
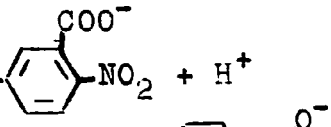
centrifuged for 40 min at 20,000 x g, the supernatant decanted carefully and the stromata resuspended in 40 ml hypotonic buffer and recentrifuged. This washing with hypotonic phosphate was repeated once. The stromata were resuspended in hypotonic buffer and spun at 30,000 x g for 40 min. The buffer was removed and the residue suspended in a volume of 0.1 M Tris-HCl equal to half the original volume of blood. The yield of stromata was 2.0 to 2.5 mg/ml whole blood.

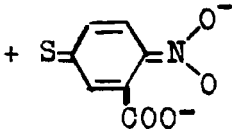
III. Acyltransferase Assays

A. Spectral Assay

The acyl-CoA:monoacylphosphoglyceride acyltransferase (acyltransferase) reaction was followed continuously in a spectrophotometer equipped with a Gilford multiple sample absorbance recorder using the internal colorimetric reagent, 5,5'-dithio-bis(2-nitrobenzoate) (DTNB) (60). A representative reaction mixture contained 0.2 mg of DFP-treated microsomal protein, 1.0 μmole DTNB, 400 mμmoles of 1-acyl-GPE, or 250 mμmoles of 2-acyl-GPE, 25-30 mμmoles of acyl-CoA, and 80 μmoles of Tris-HCl buffer (pH 7.4) in a total volume of 1.0 ml. The increase in 412 mμ absorbancy was followed as an indication of the release of CoA from acyl-CoA as shown below.

(I) $R-\overset{O}{\overset{\|}{C}}-SCoA + \text{1-acyl-GPE} \rightarrow \text{diacyl-GPE} + CoASH$

(II) $CoASH + NO_2$$S\text{-}S$$NO_2 \rightarrow CoAS\text{-}S$$NO_2 + H^+$



All rates were corrected for endogenous activity using a control containing no added monoacyl-GPE. This activity could be partially inhibited with diisopropyl-fluorophosphate (DFP). The microsomes are treated for 1 hour at room temperature with 10^{-3} M DFP. Before treatment the rate was 5 to 8 mμmoles CoA released/min/mg of protein and after treatment this rate was reduced to 1 to 2. DFP treatment lowered, but did not completely inhibit this endogenous activity and it did not affect the increase in rate observed when monoacyl-GPE was added. Addition of 1-acyl-GPE to incubation mixtures containing 20:4-CoA and microsomes gave an increase in rate of 12 mμmoles CoA released/min/mg of protein with or without DFP treatment of the microsomes.

B. Assay Using Radioactive Substrate

Assays using radioactive acyl-CoA were run as described on p. 19. The reaction was stopped at various time intervals by adding the reaction mixture to 10 ml of chloroform:methanol (2:1). This was washed with water two times and the chloroform layer dried under nitrogen. The lipids were redissolved in chloroform and separated by DEAE-cellulose column chromatography. Columns containing 1 g of DEAE-cellulose (1 x 5 cm) were prepared as described by Rouser (53). The samples were applied to the column in chloroform and eluted with the following solvents:

	Number of fractions collected	Volume of fractions	Lipids in fraction
		ml	
20 ml chloroform	1	20	cholesterol
60 ml chloroform:methanol (9:1)	2 1	25 10	lecithin, sphingomyelin
100 ml chloroform:methanol (7:3)	4 2	20 10	phosphatidyl ethanolamine
60 ml chloroform:acetic acid (3:1)	3	20	free fatty acid, altered diacyl-GPE, phosphatidyl serine
20 ml methanol	1	20	
120 ml chloroform:methanol (2:1) saturated with NH_4OH	6	20	phosphatidic acid, acyl-CoA

The fractions were dried and 3 ml of chloroform:methanol (1:1) added. A 1 ml aliquot was removed from each and added to scintillation vials, dried, and dissolved in 7 ml of scintillation fluid. The scintillation fluid contained 7 g of PPO (2,5-diphenyloxazole), 0.3 g of POPOP (p-bis[2-(5-phenyloxazolyl)] benzene), and 100 g of naphthalene made to 1 liter with dioxane and mixed 1 part scintillation fluid to 0.2 parts water just before use (61). The radioactivity of each vial was determined and another 1 ml aliquot was removed from those samples having significant radioactivity above background. These aliquots were dried and the components determined by TLC in one of the systems described on p. 13. The plates were dried, sprayed with 1% iodine in methanol to visualize the lipid-containing areas and the

silicic acid cut into 1 cm squares which were transferred to scintillation vials and the ^{14}C content determined in a liquid scintillation counter. The R_f values of the components were compared to those for known lipids to determine which components were labeled.

The position esterified by reaction with the labeled acyl-CoA was determined by phospholipase A treatment of the diacyl-GPE formed. Incubations and separation of components on DEAE-cellulose were performed as described above. Chloroform:methanol (7:3) fractions containing diacyl-GPE were combined, dried and redissolved in 5 ml chloroform: methanol (1:1). An aliquot was removed to determine the radioactivity, and the remainder dried after adding approximately 1 μmole diacyl-GPC. The lipids were dissolved in 10 ml of ether and treated with 0.1 ml of phospholipase A (25 mg of Crotalus adamanteus venom, 20 μl of 1 M $CaCl_2$, 1 ml of 0.5 M Tris-HCl in 4 ml of H_2O) and 2 μmoles of NH_4OH added. Control tubes were incubated without phospholipase A. The tubes were incubated 3 hours at room temperature and the reaction stopped with 5 ml methanol. The solvent was removed under a stream of nitrogen, chloroform:methanol (1:1) was added, and an aliquot removed to determine the radioactivity. The remainder was applied to a Silica Gel G plate and developed in solvent system 1 (p. 13). The spots were visualized with I_2 spray and 1 cm squares cut, scraped, and the radioactivity determined.

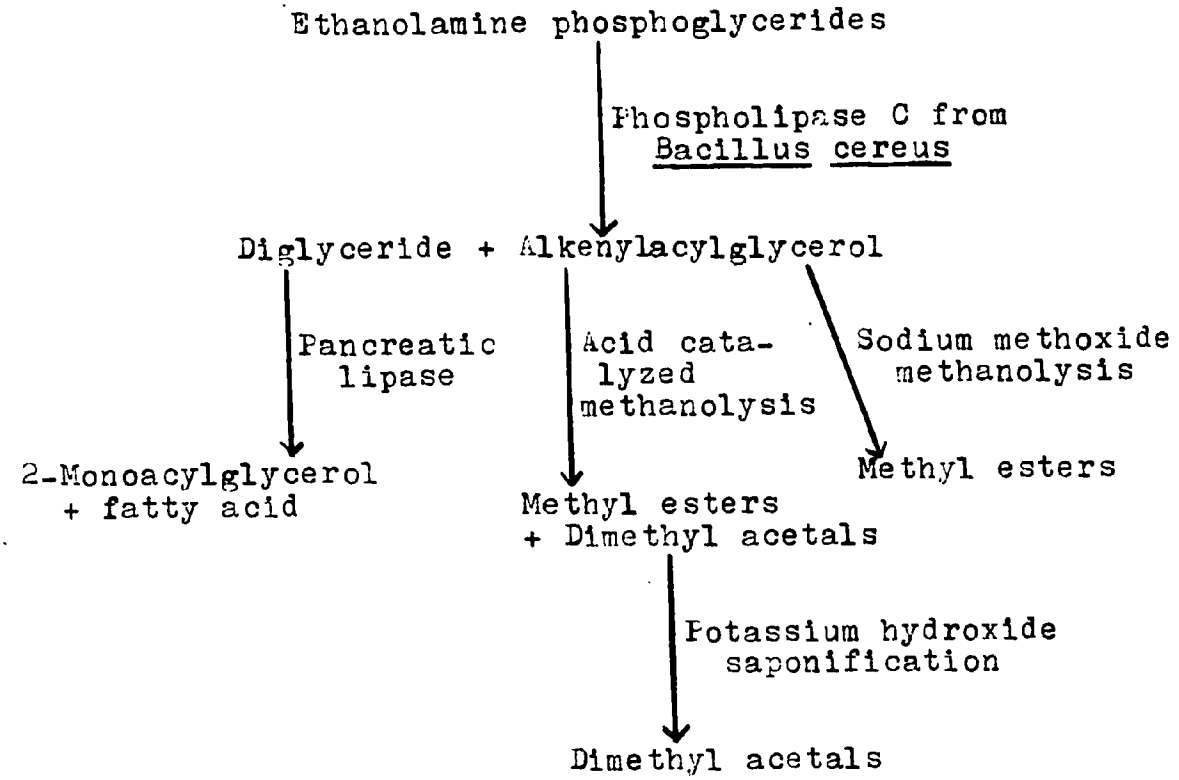
IV. Analysis of Lipids in Erythrocyte Stromata

A. Extraction and Purification of the Ethanolamine Phosphoglycerides

Lipids were extracted from stromata prepared as described on p. 18. The cells were added to absolute methanol (7 ml/ml cells). After 10 minutes, 14 ml of chloroform per ml of cells was added. All solvents used in the analysis of stromal lipids contained the antioxidant 2,6-di-t-butyl-p-cresol. The cells were allowed to sit about 8 hours in the chloroform:methanol (2:1) then filtered to remove protein. The cell extract was washed with water (3 ml per ml of cells used) and the chloroform layer dried on a rotary flash evaporator. The lipids were dissolved in chloroform:methanol (9:1) and the ethanolamine phosphoglycerides separated on a 5 g DEAE-cellulose column as described on p. 20 using 1/5 the amount of solvents given there. Purity of the ethanolamine phosphoglyceride fraction was determined by TLC on Silica Gel G in chloroform:methanol:water (65:25:4). The spots were visualized with ninhydrin, phosphate, and sulfuric acid spray as described on p. 14. The ethanolamine phosphoglycerides represented 25 to 35% of the total phospholipid and 56 to 65% of the ethanolamine phosphoglyceride fraction was 1-(1'-alk-1'-enyl)-2-acylglycero-3-phosphorylethanolamine.

B. Isolation of 1,2-Diacylglycerol and 1-(1'-alk-1'-enyl)-2-acylglycerol from Ethanolamine Phosphoglycerides

The scheme used to separate and analyze the diacyl-GPE and alkenylacyl-GPE is shown below.



The ethanolamine phosphoglycerides (1 to 3 μmoles phospholipid) were dried under nitrogen and 1 ml of ether added. *B. cereus* phospholipase C (2 mg) and 2 μl of 1.0 M $CaCl_2$ were added and the tubes incubated 5 min at room temperature with constant mixing. The lipids were extracted with ether, centrifuged, the ether layer removed and the water layer re-extracted 4 or 5 times. The ether layers were combined, dried under nitrogen and the products of the reaction (diacylglycerol and alkenylacylglycerol) separated by thin layer chromatography on Silica Gel HR in chloroform:acetone

(99:1). The bands containing diacylglycerol and alkenylacylglycerol were scraped into small columns and the lipids eluted with methanol:ether (0.05:9.5). The recovery from phospholipase C treatment was 95 to 98%.

C. Estimation of the Fatty Acids in 1,2-Diacylglycero-3-phosphorylethanolamine and 1-(1'-alk-1'-enyl)-2-acylglycero-3-phosphorylethanolamine.

The diacylglycerol was treated with pancreatic lipase (62) and the monoacylglycerol and free fatty acid separated in solvent system 3, p. 14. Methyl esters of the fatty acids in diacylglycerol and monoacylglycerol were prepared by methanolysis with sodium methoxide (p. 12). Methanolysis with sulfuric acid in methanol (p. 12) was used to prepare the methyl esters of the free fatty acids.

The aldehyde and fatty acid portions of the alkenylacylglycerol fraction were separated by acidic methanolysis followed by saponification. Acid catalyzed methanolysis will form both the dimethylacetal derivatives of the aldehydes and the methyl esters of the fatty acids. After extraction of the lipids from the reaction mixture (p. 12) the methyl esters were saponified by treatment with 0.5 N potassium hydroxide in methanol for 1 hour at 45°. The dimethylacetal derivatives were extracted with 5 ml petroleum ether and washed with 5 ml of water. Methyl palmitate (100 mμmoles) was added as an internal standard to each sample. The petroleum ether layer was evaporated and the dimethylacetals were taken up in 20-30 μl of CS_2. Portions of this solution were used for injection into the gas chromatograph.

Comparison of the retention times of the dimethylacetal derivatives to the retention times of known methyl esters was used to identify the aldehydes (63).

Methanolysis with sodium methoxide in methanol (p. 12) was used to prepare the methyl esters of the fatty acids in the alkenylacylglycerol.

RESULTS

I. Validity of the Spectral Assay and Products of the Acyl-CoA:Monoacylphosphoglyceride Acyltransferase Reaction

The acyl-CoA:monoacylphosphoglyceride acyltransferase (acyltransferase) is assayed using the colorimetric reagent DTNB. The validity of this assay for measuring incorporation of the acyl group into 1-acyl- or 2-acyl-GPE was determined by following the absorbance change and the incorporation of ^{14}C-labeled fatty acid into diacyl-GPE. The quantity of acyl-CoA incorporated into the product was calculated from the release of CoA followed spectrophotometrically and from the incorporation of ^{14}C into the diacyl-GPE isolated from the reaction mixture.

The quantity of CoA released in the complete reaction mixture and the control without added phospholipid agrees very well with the amounts of ^{14}C-labeled diacyl-GPE isolated from the reaction mixture (Figures II and III).

Figures IV and V show the amount of radioactivity in the lipid-containing fractions eluted from DEAE-cellulose. Total recovery of isotope was 85-95% of the radioactivity added as ^{14}C-labeled acyl-CoA.

Table 1 shows the results of the TLC analysis of the components in the chloroform:methanol (2:1) saturated NH_4OH fractions in solvent system 3 (p. 12). Radioactivity was localized in the area having the same R_f as acyl-CoA with only 5 to 10% (or 0.5 to 0.8 mμmole) in other areas. Thus

Figure II. Comparison of the Amount of ^{14}C-Labeled Diacyl-GPE Formed from 1-Acyl-GPE to the Amount of CoA Released from Acyl-CoA.

This figure compares the amount of ^{14}C-labeled diacyl-GPE recovered from the incubation mixture (chloroform: methanol 7:3 fraction from DEAE-cellulose) to the differences in absorbance between the complete reaction mixture (p. 19) and the controls without 1-acyl-GPE. The incubations and extraction of lipids were done as described on p. 19 using 400 mμmoles 1-acyl-GPE and 38 mμmoles ^{14}C-labeled 8,11,14-eicosatrienoic acid. (▨) Chloroform: methanol (7:3), (——) [spectral rate with 1-acyl-GPE]-[spectral rate without 1-acyl-GPE].

Figure III. Comparison of the Amount of ^{14}C-Labeled Diacyl-GPE Formed from 2-Acyl-GPE to the Amount of CoA Released from Acyl-CoA.

The same comparisons as shown in Figure II except 250 mμmoles 2-acyl-GPE and 36 mμmoles stearyl-CoA (2,200 cpm/mμmole) were used. (▨) Chloroform:methanol (7:3), (——) [spectral rate with 2-acyl-GPE]-[spectral rate without 2-acyl-GPE].

Figure II

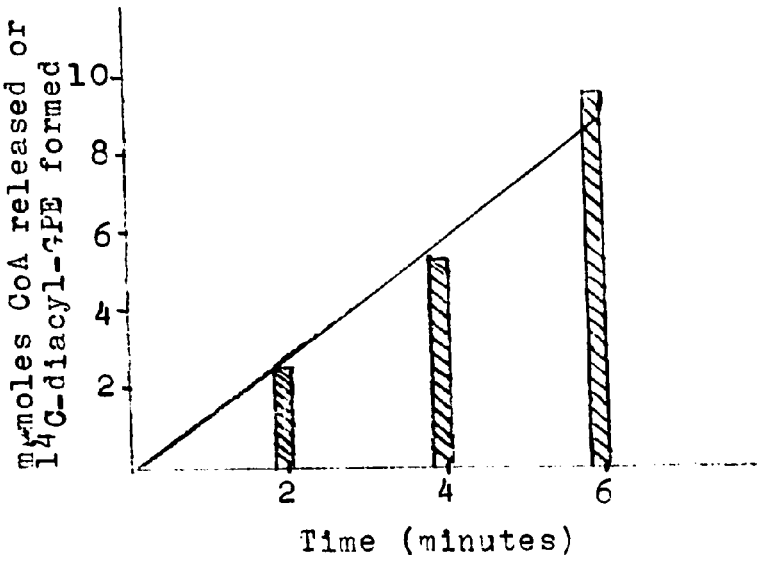


Figure III

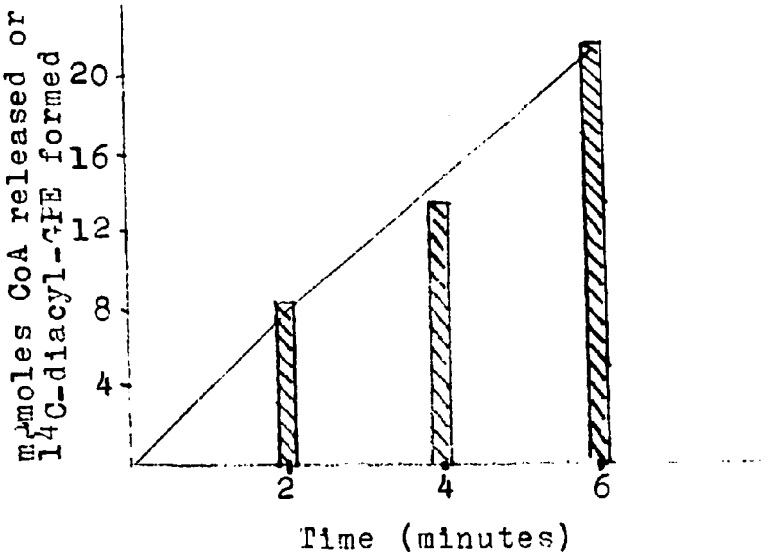


Figure IV. Incorporation of ^{14}C-Labeled 5,8,11-Eicosatrienoyl-CoA into Lipid Containing Fractions from DEAE-Cellulose.

The incubations and extraction of lipids were done as described on p. 20 using 400 mμmoles 1-acyl-GPE, 38 mμmoles of ^{14}C-labeled 8,11,14-eicosatrienoyl-CoA (160 cpm/mμmole) and 0.2 mg of microsomes for the times indicated. The specific activity of the acyl-CoA was used to convert the radioactivity incorporated into mμmoles for comparison to the spectral assay (see p. 21 for lipids in each fraction). (——) Spectral rate observed with 1-acyl-GPE, (---) spectral rate observed without 1-acyl-GPE, (▨) mμmoles in the chloroform:acetic acid (3:1) fraction, (□) mμmoles in the chloroform and chloroform:methanol (9:1) fraction, (▧) mμmoles in the chloroform:methanol (7:3) fraction, (■) mμmoles in the chloroform:methanol (2:1) saturated with NH_4OH fraction. NE signified the control where no microsomes were added to the assay tube.

Figure V. Incorporation of ^{14}C-Labeled Stearyl-CoA into Lipid Containing Fractions from DEAE-Cellulose.

The incubations and extraction of lipids were done as described on p. 20 using 250 mμmoles 2-acyl-GPE, 36 mμmoles stearyl-CoA (2,200 cpm/mμmole) and 0.2 mg microsomes for the times indicated. The specific activity of the stearyl-CoA was used to convert the radioactivity incorporated into mμmoles for comparison to the spectral assay. Symbols used are the same (see p. 21 for lipids in each fraction).

Figure IV

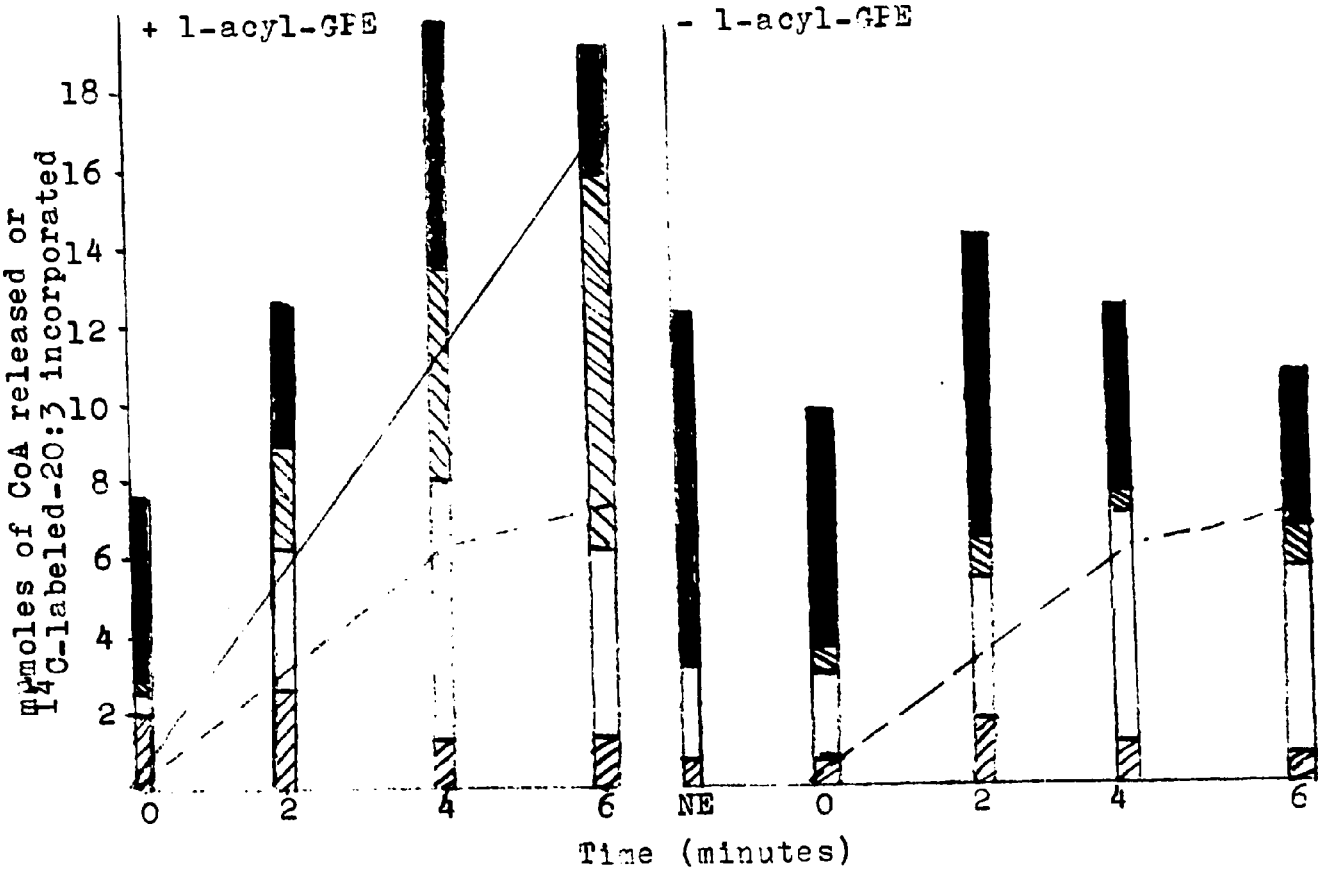


Figure V

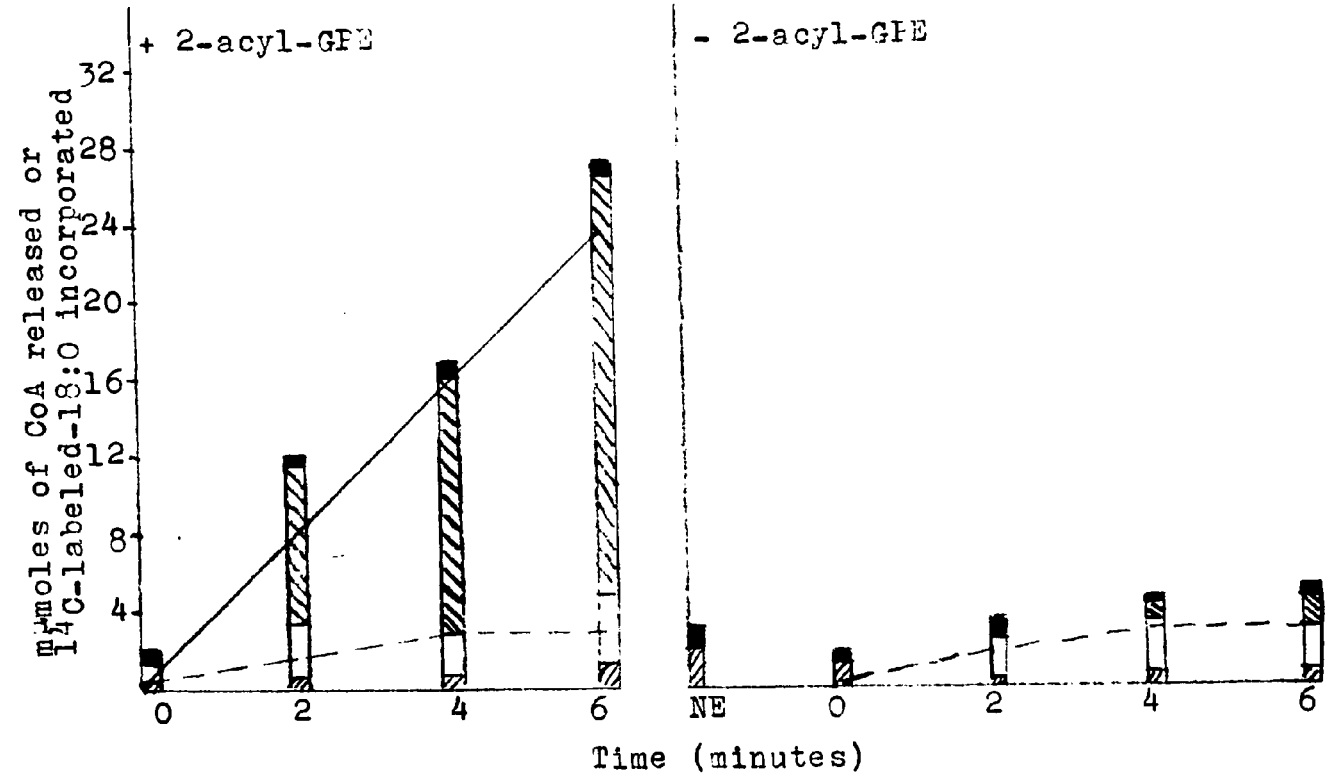


Table 1

TLC Analysis of the Acidic Lipid

Fraction from DEAE-Cellulose Chromatography

Band from TLC plate	Experiment 1		Experiment 2		Experiment 3		Known compounds with the same R_f
	cpm	mμm	cpm	mμm	cpm	mμm	
Solvent front	31	0.2	0	0	25	0.1	Free fatty acid
2	6	0	18	0.1	23	0.1	
3	6	0	2	0	20	0.1	
4	78	0.5	18	0.1	44	0.2	
5	14	0.1	41	0.3	14	0	Phosphatidic acid
6	78	0.5	48	0.3	138	0.6	
Origin 7	931	5.8	1135	7.1	367	1.6	Acyl-CoA
% Recovery	87	87	98	98	95	95	

In Experiment 1 and 2 the chloroform:methanol (2:1) saturated with NH_4OH fraction from incubation of 1-acyl-GPE with 1-^{14}C-(20:3)-CoA (160 cpm/mμmole) was used; in Experiment 3 the same fraction from incubation with 1-^{14}C-(18:2)-CoA (234 cpm/mμmole) was used. The standards which migrate to the same area as the bands are indicated on the right of the table. Solvent system 3 (p. 14) was used. The mμmoles were calculated from the specific activity of the 1-^{14}C-(20:3)-CoA (160 cpm/mμmole) and the 1-^{14}C-(18:2)-CoA (234 cpm/mμmole).

this fraction appears to contain unreacted acyl-CoA which was not removed by water washing. Attempts to completely remove the acyl-CoA by further washings were not successful.

The fractions eluted with chloroform:methanol (9:1) and chloroform:acetic acid (3:1) contained more radioactive material than could be accounted for by the rate observed in the spectral assay when no 1-acyl-GPE was added. In solvent system 1 (p. 13) the radioactivity from both fractions was located at the solvent front in the area to which free fatty acids or neutral lipids migrated. As seen in Table 2 the radioactivity in the chloroform:methanol (9:1) fractions was not localized in any area having an R_f corresponding to the non-polar lipid standards. The chloroform:acetic acid (3:1) fraction had radioactivity at the origin and in the area corresponding to free fatty acids. Controls without added 1-acyl-GPE (Figure IV) showed this lack of correlation between the rate observed in the spectral assay and radioactivity incorporated into the chloroform:methanol (9:1) fraction. Therefore, the lack of correlation was not due to a contaminant in the 1-acyl-GPE. The control without microsomes (NE) and the zero time control had radioactivity in both of these fractions from DEAE-cellulose columns. As seen in Table 2, acyl-CoA alone had radioactivity in the same areas of the TLC plate as the fractions from DEAE-cellulose. The effect of varying levels of acyl-CoA on the incorporation of radioactivity into these fractions was determined (Figure VI). Increasing levels of acyl-CoA gave greater incorporation of radioactivity into both fractions. These results indicate

Table 2

TLC Analysis of Fractions from DEAE-Cellulose Chromatography

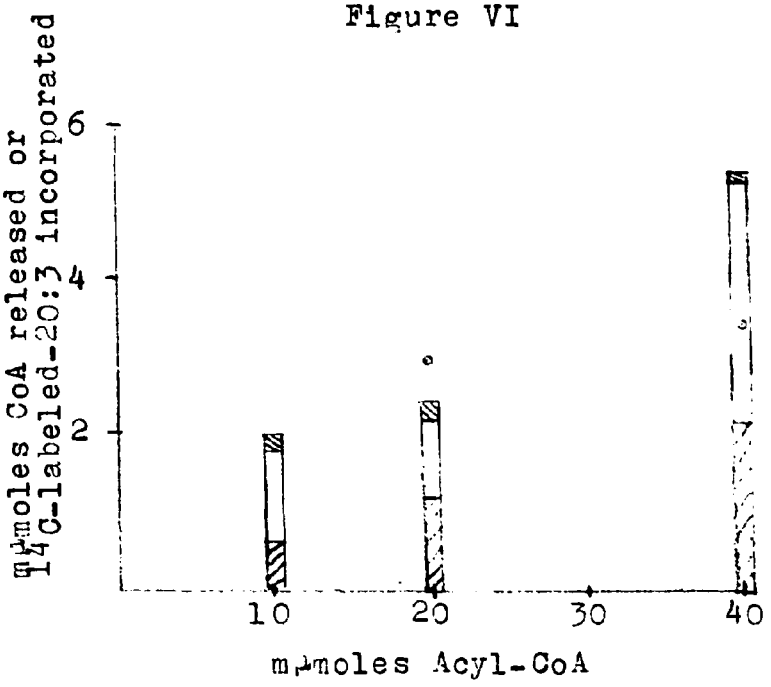
Band from TLC plate	Experiment 1	Experiment 2	Experiment 3	20:3 CoA	Known compounds with the same R_f
	cpm	cpm	cpm		
1 origin	1372	1841	150	6261	origin
2	280	229	38	2229	
3	100	94	18	720	monoglyceride
4	42	36	18	247	
5	13	31	23	42	
6	16	0	23	26	diglyceride
7	14	11	20	51	diglyceride
8	28	28	40	67	
9	150	169	288	273	fatty acid
10	53	55	90	129	
11	38	48	20	41	triglyceride
12	45	7	0	78	triglyceride
13	46	0	0	4	
14 solvent front	0	16	0	0	cholesterol ester

In Experiments 1 and 2 the chloroform and chloroform: methanol (9:1) fractions and in Experiment 3 the chloroform: acetic acid (3:1) fraction from DEAE-cellulose from incubation of 1-acyl-GPE with 1-^{14}C-(20:3)-CoA were used. Also the 1-^{14}C-(20:3)-CoA was used without any incubation in the assay system. The column on the right gives the standards which migrate to the same area as the bands in solvent system 2 (p. 13).

Figure VI. Effect of Acyl-CoA Concentration on the Incorporation of Radioactivity into Lipid Containing Fractions from DEAE-Cellulose

The incubations were carried out as described in the Methods section for 2 min. No 1-acyl-GPE was added to these incubations and the amount of ^{14}C-labeled-(20:3)-CoA was varied. Symbols are the same as in Figure IV (p. 31). (•) represents the amount of CoA released as observed in the spectral assay.

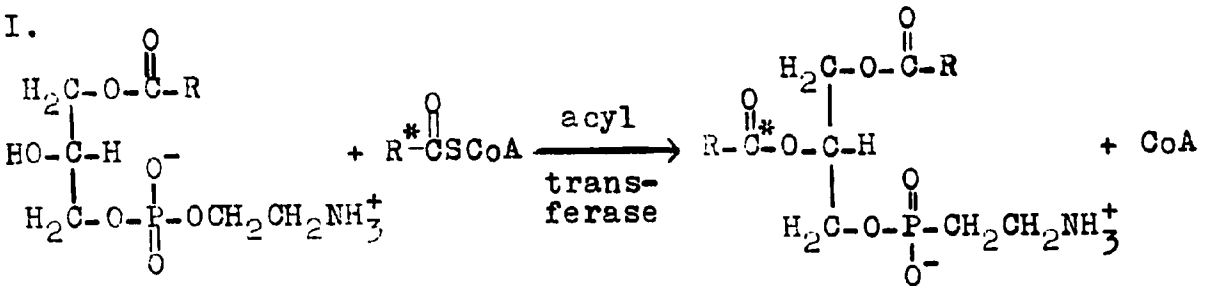
Figure VI


mμmoles CoA released or
14C-labeled-20:3 incorporated
2
4
6
10
20
30
40
mμmoles Acyl-CoA

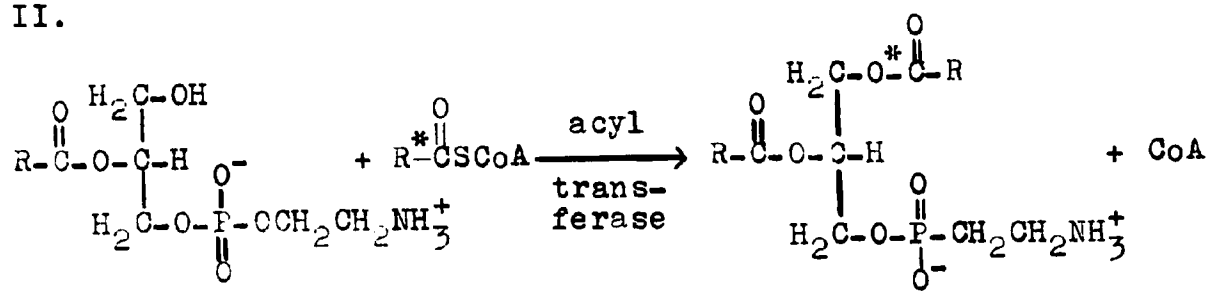
that the acyl-CoA may contain a contaminant which is eluted in either or both of these fractions.

The diacyl-GPE formed when 1-acyl-GPE or 2-acyl-GPE was added to the incubation mixture was further analyzed to determine the position acylated. The products formed with each phospholipid are shown in equations I and II.

I.



II.



$R^{*}CSCoA$ = ^{14}C-labeled acyl-CoA

Phospholipase A has been shown to remove the fatty acid from the 2- (or β-) position of diacyl-GPE. Therefore phospholipase A treatment will remove the ^{14}C-labeled fatty acid from the product of reaction I; while in reaction II the 1-acyl-GPE will retain the label after phospholipase A treatment. Products of phospholipase A hydrolysis of the ^{14}C-labeled-diacyl-GPE formed in the acyltransferase reaction were separated by TLC as described on p. 22 and the radioactivity determined. The results are presented in Tables 3 and 4.

Table 3

Phospholipase A Treatment of the Diacyl-GPE Formed in the Acyltransferase Reaction with 1-Acyl-GPE

TLC Fraction	Amount of Labeled Fatty Acid in Fraction					
	Expt. 1		Expt. 2		Expt. 3	
	Phospholipase A		Phospholipase A		Phospholipase A	
	-	+	-	+	-	+
	mμmoles†					
Origin	0.4	0.5	0.7	0	0	0
	0	0	0	0	0	0
Monoacyl-GPE	0	0.4	0	0.4	0	0.54
Diacyl-GPC	0.2	0	0.2	0	0.02	0.05
	0.2	0	0	0	0	0
Diacyl-GPE	6.9	0.1	7.0	0	0.76	0.04
	0.7	0.5	0.7	0.5	0	0
Fatty acid	1.2	8.6	0.9	8.2	0.06	0.16

† Results are expressed as mμmoles which were calculated from the specific activity of the acyl-CoA used.

Incubations were carried out as described in the Methods section using 460 mμmoles 1-acyl-GPE and 38 mμmoles 1-^{14}C-(20:3)-CoA (160 cpm/mμmole) in Expt. 1 and 2 and 36 mμmoles 1-^{14}C-(18:0)-CoA (2,200 cpm/mμmole) in Expt. 3. The diacyl-GPE was isolated, divided in 2 parts, and half treated with phospholipase A and the products separated on TLC, while the other half was not treated with phospholipase A but run on TLC. The % recovery calculated from the mμmoles in the diacyl-GPE fraction isolated from the DEAE-cellulose column (Expt. 1, 11.5 mμmoles; Expt. 2, 11.1 mμmoles; Expt. 3, 0.97 mμmole) was 80-90%.

Table 4

Phospholipase A Treatment of the Diacyl-GPE Formed in the Acyltransferase Reaction with 2-Acyl-GPE

TLC Fraction	Amount of Labeled Fatty Acid in Fraction			
	Expt. 1		Expt. 2	
	Phospholipase A		Phospholipase A	
	-	+	-	+
	mμmoles			
Origin	0.1	0.5	0	0
	0.1	0.1	0	0.1
Monoacyl-GPE	0.6	5.8	1.3	21.4
Diacyl-GPC	0	0	0.2	0.2
	0	0	0	0
Diacyl-GPE	5.2	0.6	20.3	0.9
	0.1	0.2	0.2	0.1
Fatty Acid	0.5	1.0	1.7	1.9

Experimental conditions and symbols are as seen in Table 3 except 250 mμmoles 2-acyl-GPE and 38 mμmoles 1-^{14}C-(18:0)-CoA (2,200 cpm/mμmole) were used in both Experiments 1 and 2.

As seen in Table 3, incubation of microsomes with 1-acyl-GPE gave diacyl-GPE having the label in the 2-position, since 90% or more of the radioactivity was recovered in the fatty acid fraction after phospholipase A treatment. Incubation of microsomes with 2-acyl-GPE gave diacyl-GPE with the label in the monoacyl-GPE as shown in Table 4.

Since 18:0-CoA is preferentially incorporated into the 1-position of diacyl-GPE by the acyltransferase (Table 17), use of this acyl-CoA with 1-acyl-GPE as the acyl group acceptor would result in the maximum incorporation into any 2-acyl-GPE present in the 1-acyl-GPE preparation. As seen in Table 3, 0.5 mμmole of diacyl-GPE was formed from a total of 460 mμmoles of 1-acyl-GPE added to the incubation mixture. Thus less than 0.1% 2-acyl-GPE is present in the 1-acyl-GPE.

The monoacyl-GPE or fatty acid found in the fractions not treated with phospholipase A was probably due to some decomposition of the diacyl-GPE on the Silica Gel G plate. This decomposition is observed when the diacyl-GPE band is scraped, eluted, and applied again to a TLC plate. In all cases some radioactivity was observed in the fatty acid or monoacyl-GPE or both.

II. Properties of the Acyl-CoA:Monoacylphosphoglyceride Acyltransferase (Acyltransferase)

A. Requirements of the Enzyme for Maximal Activity

Tables 5 and 6 show the dependence of the acyltransferase reaction on added phospholipid. These rates were all

Table 5

Effect of 1-Acyl-GPE Concentration on the Acyltransferase Reaction

1-Acyl-GPE Added	Rate of Reaction
mμmoles	mμmoles/ml/min
29.3	1.5
58.5	2.2
117.0	3.3
234.0	3.7
351.0	4.0
468.0	4.6
702.0	4.4
936.0	4.6

The 1 ml reaction mixture contained 1 μmole of DTNB, 80 μmoles of 0.1 M Tris-HCl (pH 7.4), 30 mμmoles of 18:2-CoA, 0.2 mg of DFP-treated rat liver microsomal protein, and different amounts of 1-acyl-GPE as indicated. The apparent K_m for 1-acyl-GPE calculated from the above values is 75 μM.

Table 6

Effect of 2-Acyl-GPE Concentration on the Acyltransferase Reaction

2-Acyl-GPE Added	Rate of Reaction
mμmoles	mμmoles/ml/min
52 (112)	2.0
104 (225)	3.3
156 (300)	4.0
208 (450)	4.0
416 (900)	4.0

The 1 ml reaction mixture contained 1 μmole of DTNB, 80 μmoles of 0.1 M Tris-HCl (pH 7.4), 25 mμmoles of 18:0-CoA, 0.2 mg of DFP-treated rat liver microsomal protein, and different amounts of 2-acyl-GPE as indicated. The numbers in parentheses represent the total amount of lipid-phosphorous added to the incubation mixture. The numbers on the left indicate the amount of 2-acyl-GPE present (see p. 43). The apparent K_m for 2-acyl-GPE calculated from the above values is 50 mM.

corrected for any endogenous activity by subtracting values for controls without added phospholipid.

In the case of 2-acyl-GPE the phospholipid added contains unreacted alkenylacyl-GPE, diacyl-GPE, and 2-acyl-GPE. These cannot be separated because of migration of the acyl group from the 2-position to the 1-position of 2-acyl-GPE in the presence of acid. This excludes the usual techniques of purification such as TLC, or column chromatography on silicic acid or DEAE. An approximate value for the amount of 2-acyl-GPE present in a preparation can be determined by separation on TLC and quantitation of the phosphate present in the monoacyl-GPE area (p. 11 and 14). These values are given in Table 6. The numbers in parentheses represent the total amount of lipid-phosphorus added to the incubation mixture. The 2-acyl-GPE was shown to be the active component by assaying the alkenylacyl-GPE preparation before treatment with aqueous iodine. The alkenylacyl-GPE (300 mμmoles) gave a rate of 0 in the assay system before 2-acyl-GPE was added; addition of 250 mμmoles of the 2-acyl-GPE preparation gave a rate of 3.8 mμmoles/ml/min.

The concentration of phospholipid required to saturate the acyltransferase varies with the substrate used. About 400 μM 1-acyl-GPE and about 150 μM (total phospholipid concentration 300 μM) 2-acyl-GPE were required to give a maximal velocity. The apparent K_m for 2-acyl-GPE (50 μM)

was slightly lower than that for 1-acyl-GPE (75 μM). Lands and Hart (16) have shown that 100 μM 1-acyl-GPC was sufficient to attain maximal velocity and that the K_m was 20 μM.

Table 7 shows the effect of acyl-CoA concentration on the acyltransferase reaction. In the case where 1-acyl-GPE is the acyl group acceptor the K_m value for acyl-CoA ($K_m < 5 \times 10^{-6}$ M) was so low that the rates of reaction were essentially independent of the thiol ester concentrations used in these experiments. Zahler et al. (64) have determined the critical micellar concentration (CMC) of acyl-CoA esters (16:0-CoA = 2 to 4 μM). The concentrations of acyl-CoA used (5 mμmoles per ml was the lowest concentration that could be used in the spectral assay) were above this CMC and since the rate appeared to be independent of the acyl-CoA concentration, the enzyme may require free acyl-CoA as substrate. Once the CMC has been reached the concentration of free substrate should be constant (64) and thus no dependence on acyl-CoA concentration would be observed.

When 2-acyl-GPE is used as the acyl group acceptor an inhibition of the rate is observed as the acyl-CoA concentration is increased above 24 mμmoles per ml. This inhibition was also observed by Lands and Hart (22) with 2-acyl-GPC as the acyl group acceptor. The inhibition could be relieved by boiled microsomes or albumin.

Table 7

Effect of Acyl-CoA Concentration on the Acyltransferase Reaction

	Acyl-CoA Added	Rate of Reaction
	mμmoles	mμmoles/ml/min
A.	5.5	3.7
	11.0	4.0
	16.5	4.0
	22.0	4.2
	27.5	4.2
	33.0	4.2
	44.0	4.6
B.	9.6	6.5
	19	12.5
	24	13.0
	36	11.5
	48	10.5
	120	6.0

A. The assay was run as in Table 5 with 400 mμmoles of 1-acyl-GPE and varying amounts of 18:2-CoA as indicated.

B. The assay was run as in Table 6 with 300 mμmoles of 2-acyl-GPE and varying amounts of 18:0-CoA as indicated.

As shown in Figure VII, the rate of reaction with 2-acyl-GPE as the acyl group acceptor did not show the same dependence on protein concentration at low levels of protein as it did at higher levels. At low protein concentrations acyl-CoA may be inhibiting the reaction. Because of this inhibition by acyl-CoA the assays with 2-acyl-GPE must be run in the range of protein concentration where the specific activity shows a linear increase with increasing protein concentration. With 1-acyl-GPE as the acyl group acceptor, a linear dependence on protein concentration was seen over the range from 0.03 to 0.4 mg (Figure VII).

The acyltransferase had a broad pH optimum over the range from pH 6.0 to 7.5. Changes in the ionic strength (0.05 to 0.5 M KCl in 0.1 M Tris, pH 7.4) did not affect the rate of reaction.

B. Effect of Various Chemical and Physical Treatments

Microsomes were prepared with and without EDTA (10^{-3} M) to determine the effect of metals on the acyltransferase reaction. No significant difference in activity was observed when EDTA was omitted from the sucrose. Attempts to add various metal salts such as $CaCl_2$ or $MgCl_2$ to the assay system (10^{-3} M final concentration) using microsomes prepared without EDTA resulted in the formation of a precipitate which could not be resuspended.

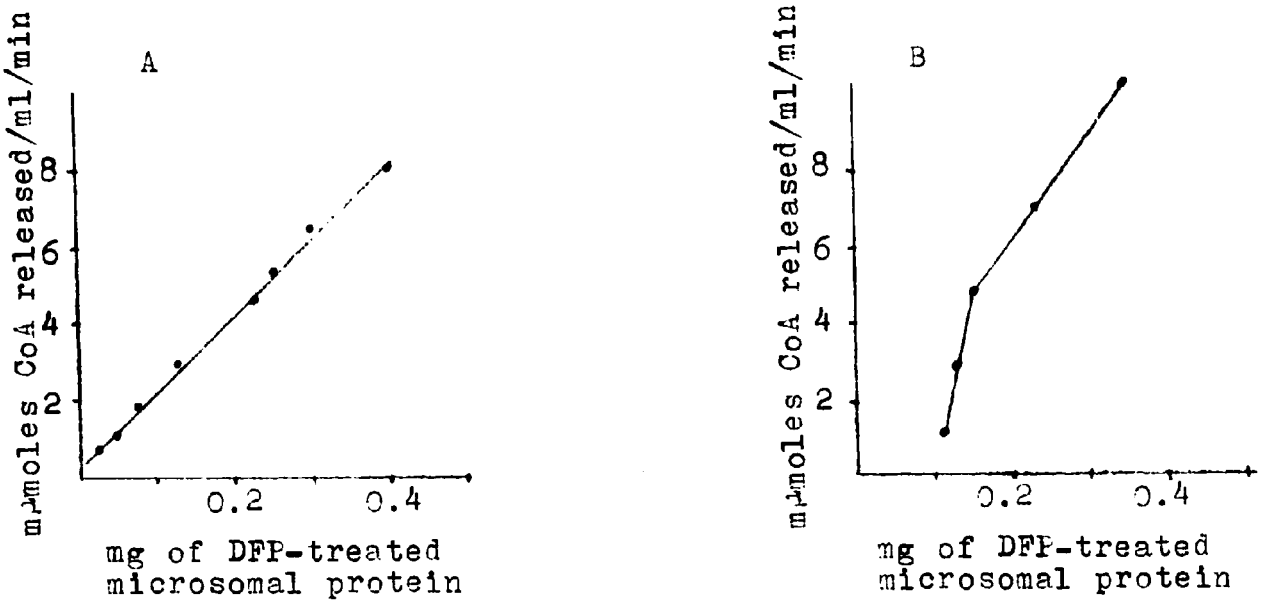
The effect of treatment of microsomes at high and low pH, heating and freezing was determined as shown in Table 8. Low pH and heating lowered the activity toward both

Figure VII. Dependence of the Acyltransferase Reaction on Protein Concentration.

A. The 1 ml reaction mixture contained 1 μmole of DTNB, 80 μmoles of 0.1 M Tris-HCl (pH 7.4), 30 mμmoles of 20:4-CoA, 400 mμmoles of 1-acyl-GPE, and different amounts of DFP-treated rat liver microsomal protein as indicated.

B. The same as A. except 250 mμmoles of 2-acyl-GPE and 25 mμmoles of 18:0-CoA were used.

Figure VII


A
mμmoles CoA released/ml/min
2
4
6
8
0.2
0.4
mg of DFP-treated
microsomal protein
B
mμmoles CoA released/ml/min
2
4
6
8
0.2
0.4
mg of DFP-treated
microsomal protein

Legend for Table 8

The assays were run as described on p. 19. The 1-acyl-GPE was assayed with 25 mμmoles of 20:4-CoA and the 2-acyl-GPE was assayed with 25 mμmoles of 16:0-CoA.

Experiment I: The microsomes were prepared as described on p. 18 except part of the liver was homogenized in 0.25 M sucrose without EDTA.

Experiment II: The microsomes were treated at pH 5.6 using 0.1 M sodium acetate or pH 10.0 using 0.1 M Tris for 10 min then titrated back to pH 7.0 for assay.

Experiment III: The microsomes were heated at approximately 55° for 10 min in a water bath before assaying.

Experiments IV and V: The microsomes were frozen for 24 hours at concentrations of 15 and 32 mg/ml, thawed for assay and refrozen for 48 hours, thawed and assayed.

Table 8

The Effect of Various Treatments on the Acyltransferase from Rat Liver Microsomes

Treatment of Microsomes	Rate of Reaction	
	1-Acyl-GPE	2-Acyl-GPE
	mμmoles/min/mg	
I. Microsomes with EDTA	14.6	29.0
Microsomes without EDTA	12.7	28.0
II. Microsomes untreated	15.1	30.4
Microsomes pH 5.6	4.7	5.5
Microsomes pH 10.0	16.3	30.4
III. Microsomes untreated	22.1	31.2
Microsomes 10 min at 55°	3.0	0
IV. Microsomes unfrozen 15 mg/ml	22.1	31.2
Microsomes frozen 24 hours	23.0	32.0
Microsomes frozen 72 hours	12.0	34.0
V. Microsomes unfrozen 32 mg/ml	22.1	31.2
Microsomes frozen 72 hours	21.0	33.0

1-acyl-GPE and 2-acyl-GPE as the acyl group acceptor. Some precipitation of protein was observed with both of these treatments. This was removed by centrifugation at 20,000 x g before assaying the supernatant. The pellets were resuspended in 0.25 M sucrose containing 0.001 M EDTA and assayed. Neither pellet had any activity for either acyl group acceptor.

Freezing of microsomes for 72 hours at concentrations of 32 mg/ml or higher did not result in a loss of activity with either 1-acyl-GPE or 2-acyl-GPE as the acyl group acceptor. However, freezing for 72 hours at 15 mg/ml resulted in the loss of about 50% of the activity with 1-acyl-GPE. The microsomes frozen at 32 mg/ml did not lose activity as quickly as the more dilute solution, but after 2 to 3 weeks they also showed a decrease of 50 to 75% of the activity for 1-acyl-GPE without a significant loss of activity for 2-acyl-GPE. Freezing and thawing of microsomes five times over a period of two hours resulted in a loss of only 10 to 15% of the activity. Thus it appears that the microsomes needed to be in the frozen state for longer than 24 hours before a lowering of activity was observed. This loss of activity is probably associated with structural changes in the microsomes similar to changes observed in frozen mitochondria (65,66). The frozen microsomes could not be reactivated by addition of 0.5 to 2.0 μmoles of micellar lecithin.

Table 9 shows the effect of mono-, di-, and trihydroxy alcohols on the acyltransferase activity. As the length of

Table 9

Effect of Alcohols on the

Acyltransferase Assay System

Alcohol Added	Rate of Reaction	
	1-Acyl-GPE	2-Acyl-GPE
	mμmoles/min/mg	
None	16.0	20.0
10% Methanol	12.0	17.0
10% Ethanol	3.0	--
5% n-Propanol	0	--
5% n-Butanol	0	0
None	17.0	15.0
10% Ethylene glycol	15.0	15.0
None	9.5	15.0
10% Propylene glycol	10	15.0
10% Glycerol	10.5	15.0

The 1 ml reaction mixtures contained 1 μmole of DTNB, 0.2 mg of pig liver microsomal protein, 400 mμmoles of 1-acyl-GPE or 250 mμmoles of 2-acyl-GPE, 70 μmoles of Tris-HCl (pH 7.4), and the alcohols as indicated above. The reaction was started by adding 25 mμmoles of linoleyl-CoA (18:2-c9,c12-CoA) to the cuvettes containing 1-acyl-GPE or 25 mμmoles of linoelaidyl-CoA (18:2-t9,t12-CoA) to the cuvettes containing 2-acyl-GPE.

the carbon chain increased in proportion to the number of hydroxyl groups the activity decreased.

C. Sonication of Microsomes

Microsomes were sonicated with and without sodium deoxycholate in an attempt to solubilize the acyltransferase (Table 10). The recovery of activity was 85 to 100%. Addition of 0.1% DOC and centrifugation without sonication solubilized only 10% of the activity; whereas, sonication of the microsomes treated with 0.1% DOC gave solubilization of 50 to 60% of the activity. When 1% DOC was used 90% of the microsomal protein was in the 100,000 x g supernatant without sonication, but the acyltransferase was inhibited 80% and could not be reactivated by dialysis against 0.25 M sucrose, 0.1 M Tris-HCl (pH 7.4) or 5% ammonium sulfate in 0.1 M Tris-HCl (pH 7.4). Attempts to use other detergents such as Triton X-100 or Cutscum did not solubilize the enzyme any better than 0.1% DOC.

D. Further Attempts to Purify the Acyl-CoA:Monoacylphosphoglyceride Acyltransferase

Ammonium sulfate (25 to 45%) precipitated acyltransferase activity from the 100,000 x g supernatant obtained by sonication of microsomes containing 0.1% DOC (Table 11). About 35% of the activity was lost and essentially no purification was obtained.

Column chromatography was used in an attempt to purify the acyltransferase remaining in the 100,000 x g supernatant after centrifugation of sonicated microsomes which contained 0.01% DOC (0.01% DOC supernatant). No

Legend for Table 10

The assays were run as described on p. 19 using 18:3-CoA with 1-acyl-GPE and 18:0-CoA with 2-acyl-GPE.

Experiment I: The microsomes (9.6 mg/ml) were sonicated for three 20-second intervals and centrifuged 1 hour at 100,000 x g.

Experiment II: The microsomes (28 mg/ml) were diluted 1 to 2 (14 mg/ml) and 1% sodium deoxycholate (DOC) added to a final concentration of 0.01%. The microsomes were then sonicated for three 20-second intervals and centrifuged 1 hour at 100,000 x g.

Experiment III: Like Experiment II except the DOC was added to a final concentration of 0.1%.

Table 10

Sonication of Microsomes

Fraction	Total Volume	Protein	Specific Activity		Total Activity		% Solubilization	
			1-acyl-GPE	2-acyl-GPE	1-acyl-GPE	2-acyl-GPE	1-acyl-GPE	2-acyl-GPE
	ml	mg/ml	mμmoles/min/mg					
I.								
Microsomes	1.5	9.6	22.9		330.0			
100,000 x g supernatant from sonicated microsomes	3.4	0.1	0		0		0	0
II.								
Microsomes	2.5	28	6.5	11.7	455	820		
100,000 x g supernatant from sonication of microsomes in 0.01% DOC	6.0	2.0	12	27.6	144	332	31	40.5
III.								
Microsomes	2.5	28	6.5	11.7	455	820		
100,000 x g supernatant from sonication of microsomes in 0.1% DOC	6.0	4.0	9.9	23.1	238	555	52	67.6

Table 11

Precipitation of Acyltransferase Activity by Ammonium Sulfate

Ammonium sulfate fraction	Total volume	Total protein	Specific activity 1-acyl-GPE	Total activity	Yield
	ml	mg/ml	mμmoles/min/mg		
0.1% DOC supernatant	20	2.1	13.1	550	100
0.25%	1.0	0.25	0	0	0
25-35%	4.8	3.2	15.7	241.8	44
35-45%	2.0	2.8	19.8	111.0	22
45% supernatant	28.5	0.9	0	0	0

The 100,000 x g supernatant from sonication of rat liver microsomes containing 0.1% DOC was treated with solid ammonium sulfate to the percent saturation indicated and mixed for 40 min. After centrifugation at 15,000 x g for 20 min the pellet was suspended in 0.05 M sodium phosphate (pH 7.4) and dialyzed overnight before assaying. The assays were run as described on p. 19 using 1-acyl-GPE and 20:4-CoA.

acyltransferase activity was eluted from DEAE-cellulose by 1.0 M KCl:0.1 M Tris-HCl, pH 8.0. Addition of 0.01% DOC or 5% methanol to the buffer, or raising the pH of the buffer did not elute the acyltransferase activity. The recovery of protein was 70%. The enzyme had either been inactivated or remained tightly bound to the DEAE-cellulose. The acyltransferase activity was eluted in the void volume from Sephadex G-200 and Bio-Gel A-50M (molecular weight exclusion limit 50 x 10^6). Use of eluting buffers (0.2 M NaCl:0.1 M Tris-HCl, pH 8.0) with or without 0.01% DOC did not change the elution pattern. Results of recentrifugation of the 0.01% DOC supernatant at 100,000 x g for another hour are shown in Table 12.

Reaggregation occurred in the presence and absence of potassium chloride, at low ionic strength and in the presence of sucrose. This suggests that sonication with DOC did not solubilize the acyltransferase but rather the microsomes were broken up into smaller fragments which reaggregate.

Inactivation of the acyltransferase by treatment of the microsomes with acetone suggested a lipid requirement. Treatment of microsomes with phospholipase C will degrade microsomal phospholipids (67,68). Therefore inactivation by phospholipase C treatment could indicate a requirement for phospholipids. Table 13 shows the disappearance of activity with phospholipase C treatment. The disappearance

Table 12

Recentrifugation of the 100,000 x g Supernatant from Sonication of Microsomes Containing 0.01% DOC

Fraction	Total Volume	Protein	Specific Activity	Total Activity
	ml	mg/ml	mμmoles/min/mg	
I.				
Supernatant	9.7	0.35	1.3	4.4
Precipitate	1.6	6.2	7.1	70.3
II.				
Supernatant	9.5	0.65	0.7	4.3
Precipitate	1.2	3.2	36	136.8
III.				
Supernatant	9.7	0.75	1.2	8.8
Precipitate	0.7	6.2	9.9	42.6
0.01% DOC Supernatant*	1.0	13.6	7.9	106.4

* This is the supernatant from sonication of microsomes containing 0.01% DOC and centrifuged at 100,000 x g for 1 hour. It was used for the centrifugations described by experiments I to III.

Experiment I: The 0.01% DOC supernatant* (1.0 ml) was diluted to 10 ml in 0.1 M Tris-HCl (pH 7.9) and 0.2 M NaCl and centrifuged 1 hour at 100,000 x g.

Experiment II: The 0.01% DOC supernatant* (1.0 ml) was diluted to 10 ml in 0.003 M Tris-HCl (pH 7.9) and centrifuged 1 hour at 100,000 x g.

Experiment III: The 0.01% DOC supernatant* (1.0 ml) was diluted to 10 ml in 0.25 M sucrose containing $1C^{-3}$ M EDTA and centrifuged 1 hour at 100,000 x g.

The fractions were assayed as described on p. 19 using 1-acyl-GPE and 20:4-CoA.

Table 13

Phospholipase C Treatment of Microsomes

Time	Phosphorus Disappearing	Diglyceride Formed	Acyltransferase Rate	% Inhibition
	μmoles/mg protein	μmoles/mg protein	mμmoles/min/mg	
0	0	0	11.0	0
5	0.4	0.36	5.9	46
15	0.63	0.68	2.5	77
30	1.1	0.83	2.5	77

Microsomes (15 mg/ml) in 0.25 M sucrose containing 10^{-3} M EDTA were diluted with an equal volume of 0.05 M Tris-HCl (pH 7.4). Clostridium welchii phospholipase C (6 mg) and 60 μl of 0.1 M $CaCl_2$ were added to the diluted microsomes. Aliquots were removed at 0, 5, 15 and 30 minutes and added to 0.5 M EDTA (pH 7.4) to stop the reaction. The microsomes were assayed for acyltransferase activity as described on p. 19 using 1-acyl-GPE and 18:2-CoA. The amount of diglyceride formed and phosphate disappearing was determined by extracting the lipids from aliquots of the reaction mixture with chloroform:methanol (2:1). The extracted lipids were separated by TLC on Silica Gel G in solvent system 1, p. 13. The diglyceride was visualized with dichlorofluorscein, the band was put into a small column and the lipid eluted with 10 ml of 5% methanol in ether. The diglyceride formed was measured by analysis for esters (55). The TLC plate was then sprayed with molybdate spray (p. 14) to detect phosphorus. The amount of phosphorus disappearing was determined as described on p. 14.

of lipid-soluble phosphorus and appearance of diglyceride indicate that microsomal phospholipid was being degraded. In Table 14 the effect of exogenous diglyceride and diacyl-GPC on the assay is shown. The decrease in activity observed with increasing levels of exogenous lipid could be due to changes in the solubility or micellar form of the substrate, non-specific binding of lipid to the enzyme or turbidity effects on the spectral assay. A comparison of the percentage inhibition by exogenous diglyceride and by phospholipase C showed approximately 1.5 times higher inhibition by phospholipase C treatment than by a comparable amount of exogenous diglyceride per mg protein. This difference could be due to the endogenously formed diglyceride being more readily available to combine non-specifically with the protein or it could be that the enzyme is actually being inactivated as lipid is being removed from the lipoprotein.

The microsomes were treated with trypsin and urea (Table 15) in an attempt to break up the microsomal membrane and possibly solubilize the enzyme. Treatment with 3 M urea destroyed about 20% of the activity. The remaining activity was found in the pellet after centrifugation at 100,000 x g. Trypsin treatment inactivated the enzyme almost completely and on centrifugation at 100,000 x g the remaining activity was found in the pellet.

Table 14

Effect of Diglyceride and Diacyl-GPC on the Acyltransferase Reaction

Lipid Added	Rate	% Inhibition
μmoles/mg protein	mμmoles/min/mg	
I. Diglyceride		
None	14.5	0
0.39	9.3	35
0.78	6.5	55
1.56	6.0	59
2.35	4.5	69
II. 1,2-Diacyl-GPC		
None	9.0	0
0.22	8.5	5
0.44	7.5	17
1.31	5.5	39

The 1 ml reaction mixture contained 1 μmole of DTNB, 0.23 mg of rat liver microsomal protein, 25 mμmoles of 18:2-CoA, 70 μmoles of Tris-HCl (pH 7.4), 400 mμmoles of 1-acyl-GPE and diglyceride or 1,2-diacyl-GPC as indicated.

Table 15

Urea and Trypsin Treatment of Microsomes

Treatment of Microsomes	Total Volume	Protein	Specific Activity		Total Activity	
			1-acyl-GPE	2-acyl-GPE	1-acyl-GPE	2-acyl-GPE
	ml	mg/ml	mμmoles/min/mg			
I.						
Microsomes	1	14.0	15.1	15.2	211.4	212.8
1 M Urea	1.0	14.0	15.3	17.6	214.2	246.4
3 M Urea	1.0	14.0	11.5	12.9	161.0	180.6
100,000 x g supernatant	9.3	0.3	0	0	0	0
100,000 x g pellet	2	4.4	20	22.2	176.0	195.4
II.						
Microsomes	1	14.0	13.4	11.0	187.6	154.0
Trypsin	1	14.0	3.1	0	43.4	0
100,000 x g supernatant	9.6	0.4	0	0	0	0
100,000 x g pellet	2	3.8	5.5	0	41.8	0

The microsomes were assayed as described on p. 19 using 20:4-CoA with 1-acyl-GPE and 18:0-CoA with 2-acyl-GPE.

Experiment I: The microsomes were made 1 M or 3 M in urea and after 1 hour at 0° were centrifuged 1 hour at 100,000 x g.

Experiment II: The rat liver microsomes (14 mg) were treated with 3 mg of trypsin for 1 hour and then centrifuged 1 hour at 100,000 x g.

III. Specificity of the Acyl-CoA:Monoacylphosphoglyceride Acyltransferase

A. Comparison of Acyl-CoA:Monoacylphosphoglyceride Acyltransferase Specificity from Rat Liver 5,000 to 15,000 x g and 15,000 to 78,000 x g Particles

The 5-15,000 x g (heavy particles) and the 15,000 to 78,000 x g (microsomes) particles were isolated from rat liver as described on p. 18. The distribution of activity between the cell fractions is shown in Table 16.

The specificity of the subcellular fractions from rat liver for the transfer of different fatty acids to 1-acyl-GPE and 2-acyl-GPE is shown in Table 17. With 2-acyl-GPE as the acyl group acceptor microsomes had 1-1/2 to 2-1/2 times higher specific activity than heavy particles. Both subcellular fractions preferentially esterified saturated acids and unsaturated acids having a trans configuration to the 1-position (i.e. 2-acyl-GPE was the preferred acyl group acceptor). With 1-acyl-GPE as the acyl group acceptor microsomes had up to 4 times higher specific activity than heavy particles. Both subcellular fractions preferentially esterified to the 2-position those acids which had two or more cis double bonds (i.e. 1-acyl-GPE was the preferred acyl group acceptor).

B. Pig Liver Microsomal Acyl-CoA:Monoacylphosphoglyceride Acyltransferase Specificity

Pig liver microsomal acyltransferase preferentially esterified saturated acids and unsaturated acids with a trans configuration at carbon 9 to the 1-position (i.e.

Table 16

Acyltransferase Activity in Subcellular Fractions of Rat Liver

Fraction	Total Volume	Protein	Specific Activity		Total Activity	
			1-Acyl-GPE	2-Acyl-GPE	1-Acyl-GPE	2-Acyl-GPE
	ml	mg/ml	mμmoles/min/mg			
heavy particles	41.8	22.0	5.0	8.7	4600	8004
microsomal particles	9.9	45.0	14.0	18.4	6244	8206

The 1 ml reaction mixture contained 1 μmole of DTNB, 80 μmoles of Tris-HCl (pH 7.4), 250 mμmoles of 2-acyl-GPE and 25 mμmoles of 18:0-CoA, or 400 mμmoles of 1-acyl-GPE and 25 mμmoles of 18:2-CoA, and 0.2 mg of DFP-treated protein as indicated.

Table 17

Acyltransferase Activity of Rat Liver Subcellular Particles

Acyl-CoA Added	Rate of Reaction			
	heavy particles		microsomal particles	
	1-Acyl-GPE	2-Acyl-GPE	1-Acyl-GPE	2-Acyl-GPE
	mμmoles/min/mg			
16:0	2.8	10.5	2.5	22.0
18:0	2.5	9.1	2.1	20.3
18:1c	2.8	7.1	4.0	9.6
18:1t	1.4	12.7	4.1	17.9
18:2cc	5.1	4.4	12.9	6.7
18:2tt	0.8	14.1	3.4	35.3
18:3ccc	4.9	--	5.9	--
20:3ccc	3.2	--	6.9	--
20:4cccc	4.3	2.0	10.2	3.7

The 1-ml reaction mixture contained 1 μmole of DTNB, 80 μmoles of Tris-HCl (pH 7.4), 250 mμmoles of 2-acyl-GPE, or 400 mμmoles of 1-acyl-GPE, 0.2 mg of DFP-treated 5,000-15,000 x g or 15,000-78,000 x g particles, and 25 to 30 mμmoles of acyl-CoA as indicated. These reaction rates are the average obtain with three different preparations of subcellular particles.

2-acyl-GPE was the preferred acyl group acceptor) (Table 18). However, when the double bond is at carbon 11 rather than carbon 9 the enzyme did not distinguish between cis and trans configuration.

The pig liver microsomal acyltransferase preferentially transferred linoleate and 8,11,14-eicosatrienoate to the 2-position. However, the pig liver enzyme did not distinguish between 18:2cc and 18:2tt when esterifying 1-acyl-GPE.

IV. Acyl-CoA:Monoacylphosphoglyceride Acyltransferase from Human Erythrocyte Stromata

A. Attempt to Correlate Fatty Acid Composition of the 2-Position of 1,2-Diacylglycero-3-phosphorylethanolamine from Human Erythrocyte Stromata with the Acyl-CoA: Monoacylphosphoglyceride Acyltransferase Rate

Waku and Lands (69) have shown that the acyltransferase from human erythrocyte stromata could control the fatty acid composition at the 2-position of diacylphosphatidylcholine (diacyl-GPC) in those cells. By measuring the unesterified fatty acid pool available to the enzyme and the rate of the acyltransferase they were able to predict the fatty acid composition of the 2-position of diacyl-GPC. Since Robertson and Lands (70) had shown that human erythrocyte stromata could transfer fatty acids to 1-acyl-GPE as well as 1-acyl-GPC, an attempt was made to predict the composition of the 2-position of diacyl-GPE from human erythrocyte stromata (Table 19). The acyltransferase rates were measured using 1-acyl-GPE as the acyl group acceptor and the

Table 18

Specificity of Pig Liver Microsomal Acyltransferase

Acyl-CoA Added*	Rate of Reaction	
	1-Acyl-GPE	2-Acyl-GPE
	mμmoles/min/mg	
16:0	---	14.0
18:0	1.3	6.5
18:1-c9	2.2	4.5
18:1-t9	3.8	14.5
18:1-c11	2.0	15.5
18:1-t11	2.5	19.0
18:2-cc	7.5	2.0
18:2-tt	7.4	20.0
20:3-ccc	6.0	2.0

* The numbers after the hyphen represent the position of the double bond and the letters represent the configuration.

The 1 ml reaction mixture contained 1 μmole of DTNB, 80 μmoles of Tris-HCl (pH 7.4), 250 mμmoles of 2-acyl-GPE or 400 mμmoles of 1-acyl-GPE, 0.2 mg of pig liver microsomal protein, and 25 to 30 mμmoles of acyl-CoA as indicated. The position of the double bond in the monoenes is indicated by the number after the hyphen.

Table 19

Comparison of the Composition of the 2-Acyl Group in Stromal Diacyl-GPE to the Composition of the Plasma Unesterified Fatty Acids and Acyltransferase Rate

Fatty Acid	Acyltransferase Rate			Plasma Acids	(1) x (2)			Composition of the 2-Acyl Group					
								Predicted			Observed		
	1	2	3		1	2	3	1	2	3	1	2	3
	mμmoles/min/mg			mole %				mole %			mole %		
16:0	1.8	1.3	2.5	24.8	44.6	32.2	62.0	19.5	13.3	18.4	10.5	6.6	14.7
18:0	1.8	1.3	2.5	17.0	30.6	22.1	42.5	13.4	9.1	12.6	7.4	8.0	6.6
18:1	1.8	2.6	4.0	34.1	61.4	88.7	136.4	26.8	36.5	40.5	37.6	25.2	24.8
18:2	4.4	5.0	4.5	14.4	63.4	72.0	64.8	27.7	29.6	19.2	11.3	7.2	9.1
20:4	3.1	3.2	4.0	5.5	17.1	17.6	22.0	7.5	7.2	6.5	22.4	21.8	18.4
16:1	4.4	3.8	3.5	2.7	11.9	9.5	10.3	5.2	2.8	4.2	3.9	---	---

The acyltransferase rates were determined as described on p. 19 using 0.1 mg of stromal protein, 400 mμmoles of 1-acyl-GPE and 25 mμmoles of the acyl-CoA esters as indicated. The composition of the 2-acyl esters was determined as described on p. 25. Experiment 1 represents the acyltransferase rates and composition from a pooled blood sample; experiment 2 represents the rates and composition from an individual male and experiment 3 from an individual female.

composition of the 2-position of diacyl-GPE was determined as described on p. 23. The concentration of free fatty acids in plasma reported by Waku and Lands (69) are in agreement with the values obtained by Dole (71).

Assuming that the composition of the acyl-CoA pool in stromata is related to the pool of precursor acids the predicted mole % of a particular fatty acid present in the 2-position of diacyl-GPE was calculated by multiplying the relative abundance of the fatty acid (mole %) by its rate of incorporation into diacyl-GPE (mμmoles/min/mg). As can be seen from Table 19 the predicted mole % and observed mole % for the 2-position of diacyl-GPE did not correlate. Therefore other factors besides the relative abundance of a particular fatty acid and its maximal rate of esterification to 1-acyl-GPE must be involved in determining the composition of the 2-position of diacyl-GPE.

B. Fatty Acid Composition of the Total Ethanolamine Phosphoglyceride Fraction from Human Erythrocyte Stromata

Since the ethanolamine phosphoglyceride fraction from stromata contains around 65% alkenylacyl-GPE, total fatty acid composition would represent fatty acids from the 1- and 2-position of diacyl-GPE and fatty acids from alkenylacyl-GPE. Total fatty acid composition and the composition of diacyl-GPE and alkenylacyl-GPE fractions are given in Table 20. The components of the ethanolamine phosphoglyceride fraction were separated as described on p. 24.

Table 20

Fatty Acid Composition of Ethanolamine Phosphoglycerides from Human Erythrocyte Stromata

Fatty Acid	Ethanolamine Phosphoglycerides	1,2-Diacyl-GPE		Alkenylacyl Glycerol 2-Acyl Group
		1-Acyl Group	2-Acyl Group	
	mole %	mole %	mole %	mole %
16:0	16.6 (17.5)	41.3 (41.0)	6.6 (14.7)	6.1 (6.8)
18:0	6.9 (14.4)	23.6 (26.5)	8.0 (6.6)	5.4 (2.3)
18:1 (n-9)	25.1 (20.6)	35.1 (25.8)	25.2 (24.8)	14.3 (9.9)
18:2 (n-6)	10.6 (8.3)	-- (6.7)	7.2 (9.1)	4.0 (2.9)
20:3 (n-6)	0.9 (3.1)	--	2.3 (3.7)	0.9 (9.5)
20:4 (n-6)	34.7 (23.8)	--	21.8 (18.4)	65.3 (42.3)
22:5 (n-6)	-- (0.7)	--	--	--
22:5 (n-3)	2.0 (8.1)	--	13.3 (14.9)	1.7 (14.9)
22:6 (n-3)	3.3 (2.7)	--	15.7 (7.7)	2.3 (5.2)
24:4 (n-6)	-- (1.7)	--	--	-- (5.4)

Each set of values represents duplicate analysis. The values on the left were from a male and the values in parentheses were from a female.

C. Comparison of Acyl-CoA:Monoacylphosphoglyceride Acyltransferase Rates of Human Erythrocyte Stromata from Individual Donors

Acyltransferase rates with stromata from individual blood donors were compared using 1-acyl-GPE and 1-acyl-GPC as the acyl group acceptors (Table 21). No transfer of fatty acids to 2-acyl-GPE was observed with human stromata. Also no endogenous activity was observed in the controls without added 1-acyl-GPE or 1-acyl-GPC. The K_m for 18:2-CoA was less than 5×10^{-6} M. Table 22 presents a comparison of the rates of transfer of arachidonyl-CoA (20:4-CoA) and linoleyl-CoA (18:2-CoA) to 1-acyl-GPE and 1-acyl-GPC. This ratio shows a higher value ($P < 0.0001$) in females than males with 1-acyl-GPE as the acyl group acceptor. However, the absolute values for the rates (Table 21) do not show a statistically significant difference between males and females (18:2-CoA, $P = 0.25$ and 20:4-CoA, $P = 0.35$).

V. Comparison of the Rate of Transfer of Fatty Acids to 1-Acylglycero-3-phosphorylcholine and 1-Acylglycero-3-phosphorylethanolamine by Male and Female Rat Liver Microsomes

The rate of transfer of fatty acids to 1-acyl-GPE and 1-acyl-GPC was determined using livers from male and female rats. No significant difference was noted between male and female rats with 1-acyl-GPC or 1-acyl-GPE as the acyl group acceptor. However, the specificity for the fatty acid esterified to 1-acyl-GPE is different than that observed

Table 21

Rates of Transfer of Fatty Acids to 1-Acyl-GPE and 1-Acyl-GPC by Erythrocyte Stromata

Acyl-CoA Added	Phospholipid Added	Rate of Reaction*								
		F_1	F_2	F_3	F_4	F_5	M_1	M_2	M_3	M_4
		mμmoles/min/mg								
18:1	1-acyl-GPE	4.0	0.3	2.3	2.5	4.4	0.9	2.6	2.6	2.8
18:2	1-acyl-GPE	4.5	1.9	2.4	2.7	4.9	2.4	5.6	5.0	3.4
20:4	1-acyl-GPE	4.0	1.9	2.8	2.7	4.8	1.7	3.5	3.2	2.4
18:1	1-acyl-GPC	--	2.6	4.3	5.2	8.0	2.6	--	--	5.3
18:2	1-acyl-GPC	10.0	5.0	5.5	5.7	8.9	4.9	11.3	10.1	7.0
20:4	1-acyl-GPC	--	2.7	3.5	3.3	6.2	3.0	--	--	4.6

*F = Female; M = Male

The 1 ml reaction mixture contained 1 μmole DTNB, 0.1 mg stromal protein, 100 mμmoles 1-acyl-GPC or 350 mμmoles 1-acyl-GPE, 25-30 mμmoles acyl-CoA and 70 μmoles Tris-HCl (pH 7.4).

Table 22

Comparison of the Rate of Transfer of 20:4 and 18:2 by Erythrocyte Stromata

Stromata*	Rate of reaction with 20:4-CoA / Rate of reaction with 18:2-CoA	
	1-Acyl-GPE	1-Acyl-GPC
F_1	0.89	--
F_2	1.0	0.54
F_3	1.2	0.64
F_4	1.0	0.58
F_5	0.98	0.70
M_1	0.71	0.61
M_2	0.63	--
M_3	0.64	--
M_4	0.70	0.66

* F = Female, M = Male

The rates of reaction from Table 21 are compared by calculating the ratio of the rate of reaction using 18:2-CoA to that using 20:4-COA with 1-acyl-GPE or 1-acyl-GPC as the acyl group acceptor.

for esterification to 1-acyl-GPC. With 1-acyl-GPE arachidonate (20:4) was esterified faster than 8,11,14-eicosatrienoate (20:3), while with 1-acyl-GPC 20:3 was esterified faster than 20:4. Also the difference between the rates of esterification of linoleyl-CoA (18:2-CoA) and oleyl-CoA (18:1-CoA) was somewhat larger when 1-acyl-GPE was the acyl group acceptor than when 1-acyl-GPC was the acyl group acceptor.

Table 23

Rates of Fatty Acid Transfer to 1-Acyl-GPE and 1-Acyl-GPC by Male and Female Rat Liver Microsomes

Acyl-CoA Added	Phospholipid Added	Rate of Reaction*			
		Experiment 1		Experiment 2	
		F	M	F	M
		mμmoles/min/mg			
18:1	1-acyl-GPC	31.8 (30.5-34.7)	42.0 (36.7-48.7)	18.6	21.3
18:2	"	36.2 (34.0-38.2)	49.7 (41.9-60.2)	26.2	30.8
20:3	"	51.1 (45.8-58.5)	60.8 (49.2-75.0)	32.8	28.0
20:4	"	38.6 (32.0-44.1)	50.8 (44.5-59.9)	22.3	23.6
18:1	1-acyl-GPE	6.9 (4.6-9.0)	11.1 (10.5-11.8)	7.0	8.3
18:2	"	15.8 (13.5-17.5)	16.2 (13.1-18.1)	11.6	13.3
20:3	"	14.6 (11.1-17.7)	18.9 (14.8-21.1)	7.3	8.4
20:4	"	22.1 (20.8-24.5)	24.3 (20-27.3)	12.2	10.3

* F = Female, M = Male

The assays were run as described under Table 17, p. 65.

Experiment 1: 100 to 125 g rats were used and the values are the average of 4 females and 3 males.

Experiment 2: 180 to 250 g rats were used and the values are the average of 2 females and 2 males.

The rates in parentheses indicate the range of values obtained for individual rats.

DISCUSSION

I. Properties of the Acyl-CoA:Monoacylphosphoglyceride Acyltransferase

Neither sonication nor treatment with 0.1% sodium deoxycholate (DOC) solubilized the acyltransferase. By combining these treatments of the microsomes, about 50% of the acyltransferase activity remained in the 100,000 x g supernatant after centrifugation for 1 hr. Further attempts to purify the enzyme which remained in the supernatant resulted in reaggregation. Thus it appears that sonication disrupted the microsomes making the lipoproteins available to combine with deoxycholate. This caused the microsomes to break into smaller fragments which appeared to be solubilized enzyme. Similar results have been obtained with the cytochrome oxidase system (72,73) where sodium cholate or deoxycholate was used to solubilize the enzymes from mitochondria. Any treatment such as ion exchange chromatography which removed the DOC, led to precipitation of the enzyme.

The loss of about 20% of the activity in 3 M urea suggests hydrophobic bonds are important in maintaining the proper conformation of the enzyme. This requirement for the proper microenvironment was also demonstrated by the treatment of microsomes with alcohols. An increased ratio of carbon chain length to number of hydroxyls decreased the activity, suggesting that hydrophobic and hydrophilic interactions of the enzyme and substrate (or both) are important in retaining enzyme activity.

The complete loss of activity for 2-acyl-GPE on trypsin treatment; the loss of activity for 1-acyl-GPE but not 2-acyl-GPE on freezing; and the difference in the effect of acyl-CoA concentration all suggest that different enzymes are involved in the acylation of 1- and 2-acyl-GPE. Further evidence supporting the idea that different enzymes are involved was obtained from studies on the specificity for the fatty acid esterified.

II. Comparison of Acyl-CoA:Monoacylphosphoglyceride Acyltransferase Specificity of Rat Liver Subcellular Particles and Pig Liver Microsomes

Both the microsomal and heavy particles preferentially esterified saturated fatty acids and unsaturated acids having *trans* double bonds to the 1-position. Linoleate and arachidonate were preferentially esterified to the 2-position (Table 17, p.65). Analysis of rat liver and egg phospholipids (15,26) has shown that saturated acids are located predominantly at the 1-position and unsaturated acids at the 2-position. Analysis of lecithins from livers of rats fed *cis* and *trans* octadecadienoates has shown that the *trans* acid is preferentially incorporated into the 1-position (74). The correlation between the specificities of the acyltransferase and the distribution of fatty acids in phospholipids suggests that this enzyme may be involved in the formation of phospholipids having specific fatty acid patterns (1,2).

The existence of different acyltransferases in microsomes and heavy particles is suggested by a comparison of the specific activities with different acyl-CoA esters. For example with 1-acyl-GPE as the acyl group acceptor and oleyl-CoA (18:1c) as the acyl group donor the microsomes have 1.4 times higher specific activity than the heavy particles; whereas, with linoleyl-CoA (18:2cc) the microsomes have 2.5 times higher specific activity. These differences in the rate of reaction with different acyl-CoA esters suggest that the enzymes from the subcellular fractions are not the same. Either the acyltransferases in microsomes and heavy particles may have different relative specificities for the acyl-CoA esters or separate enzymes for each acyl-CoA are present in varying amounts.

Pig liver microsomes showed selectivity similar to that found in rat liver microsomes for acylation of the 1-position with saturated acids or unsaturated acids having a trans double bond at carbon 9, and acylation of the 2-position with linoleate or 8,11,14-eicosatrienoate (Table 18).

The pig liver microsomal enzyme which catalyzes esterification at the 1-position discriminated between geometric isomers at carbon 9 but not at carbon 11 of octadecenoates. Both cis- and trans-11-octadecenoates were transferred to the 1-position at about the same rate as trans-9-octadecenoate suggesting that the position of the double bond rather than the configuration

influenced the rate with acids having a double bond at carbon 11. The effect of position of the double bond on the rate of transfer of fatty acids to monoacyl-GPC and GPE has been shown by Reitz et al. (75). They found that cis octadecadienoates with the first double bond at carbons 8, 10, 12 or 14 were transferred to 2-acyl-GPC and 2-acyl-GPE at a faster rate than the acids having the first double bond at carbon 9.

III. Human Red Blood Cell Stromal Acyl-CoA:Monoacylphosphoglyceride Acyltransferase

A. Attempt to Correlate Fatty Acid Composition of the 2-Position of 1,2-Diacylglycero-3- phosphoryl-ethanolamine from Human Erythrocyte Stromata with the Acyl-CoA:Monoacylphosphoglyceride Acyltransferase Rate

Dietary studies have indicated that the fatty acid patterns of phospholipids in circulating erythrocytes are subject to change (35,76-79). This change in composition could occur by de novo synthesis of phospholipids (6). The uptake of ^{32}P was found to be of a much lower order than the incorporation of ^{14}C-fatty acids (80-83) and was restricted mainly to diacylglycerophosphate (80). Also L-3-glycerophosphate did not enhance the incorporation of fatty acids into lipids (84-86). Therefore the de novo synthesis of phospholipids is probably of minor importance in determining the fatty acid composition observed in erythrocytes.

Robertson and Lands (70) have shown that human erythrocyte stromata can transfer ^{14}C-labeled fatty acids to 1-acyl-GPE and 1-acyl-GPC. Treatment with phospholipase A from snake venom showed that those ^{14}C-labeled acids were incorporated into the 2-position. Although erythrocytes do not appear to have any appreciable phospholipase activity (70,86) they could acquire monoacyl derivatives from the plasma (86,87). Thus the turnover of phospholipids in erythrocytes may involve the acyltransferase reaction using monoacyl derivatives and unesterified fatty acids acquired from the plasma.

Waku and Lands (69) have found that neither the acyltransferase rate alone nor the concentration of unesterified fatty acids available in plasma was a reliable indicator of the fatty acid composition at the 2-position of stromal diacyl-GPC. However, consideration of the two together gave predicted compositions which were very close to the observed composition of the 2-position of stromal diacyl-GPC. Attempts to show a similar type of predictability for the 2-position of stromal diacyl-GPE did not show the same dependence on acyltransferase rate and relative abundance of unesterified fatty acids (Table 19, p. 68).

Consideration of the Michaelis constant may be necessary to predict the composition of the 2-position of diacyl-GPE. The K_m for 18:2-CoA was less than 5 μM. Exact K_m values for the different acyl-CoA esters could not be determined as rates with acyl-CoA concentrations below 5 μM could not be measured in the assay system. However, if the

K_m values for the different acyl-CoA esters influenced the observed composition we should be able to calculate relative K_m values. The composition predicted from consideration of the rate of reaction and the abundance of the fatty acids in plasma differed from the observed composition by a factor (F). If we assume a factor of 1 for 18:1-CoA, relative factors for different acyl-CoA esters can be calculated from the following equation.

$$\frac{F_{18:2}}{F_{18:1}} = \frac{\left(\frac{\text{predicted composition 18:2}}{\text{observed composition 18:2}}\right)}{\left(\frac{\text{predicted composition 18:1}}{\text{observed composition 18:1}}\right)}$$

Values for 18:2-CoA and 20:4-CoA are shown below.

Acyl-CoA	Relative F values		
	Experiment		
	1	2	3
18:1	1.0	1.0	1.0
18:2	3.5	2.8	1.3
20:4	0.5	0.2	0.2

If we assume that the concentration of the acyl-CoA esters are much less than their Michaelis constants these factors would represent relative K_m values for the different acyl-CoA esters. The mole percent of 20:4 in the 2-position of diacyl-GPE was about 3 times higher than predicted and the relative K_m for 20:4 was about 1/5 the value for 18:1-CoA. These values indicate that consideration of K_m values may be necessary to predict the composition of the 2-position of diacyl-GPE.

The composition of stromal diacyl-GPE could also be influenced by acquisition of phospholipids from plasma lipoproteins. Some exchange of plasma phospholipids with erythrocyte phospholipids has been reported (88-92). Serum ethanolamine phosphoglycerides (EPG) contain about the same mole % of 20:4 as erythrocyte EPG but a lower mole % of 18:1 (93). However, EPG represents only about 2.5% of the total serum phospholipids (93) so the contribution to the total composition of stromal EPG may be small.

B. Comparison of Acyl-CoA:Monoacylphosphoglyceride Acyltransferase Rate of Erythrocyte Stromata from Males and Females

Monson et al. (38) have reported that females have a higher proportion of 20:4 in their erythrocyte phospholipids than males and they tend to maintain this higher proportion under conditions of essential fatty acid deficiency. Since the EPG fraction contains a high percentage of the total 20:4 (3) it could be the phospholipid which retains this fatty acid. The higher relative rate of esterification of 20:4 to 1-acyl-GPE by erythrocyte stromata from females (Table 22) suggests that the acyltransferase reaction may be involved in the retention of 20:4 by females.

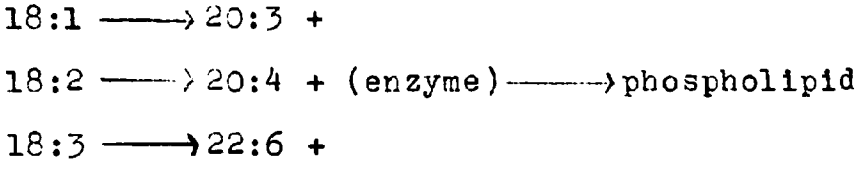
IV. Comparison of the Rate of Transfer of Fatty Acids to 1-Acylglycero-3-phosphorylcholine and 1-Acylglycero-3-phosphorylethanolamine by Male and Female Rat Liver Microsomes

Sex differences have been observed in the fatty acid composition of rat liver phospholipids (43-45). These

differences consist of a higher proportion of 1-stearyl-2-arachidonyl-GPC in females. This particular species of diacyl-GPC is believed to be synthesized by the transfer of methyl groups from S-adenosyl methionine to diacyl-GPE (46-49). Recent studies on phospholipid biosynthetic pathways have indicated that female rats can incorporate more ethanolamine into diacyl-GPE as well as more methionine methyl into diacyl-GPC (94). Thus it appears that estrogens somehow enhance both diacyl-GPE synthesis and its methylation to diacyl-GPC. Comparison of the rate of esterification of arachidonate to 1-acyl-GPE by rat liver microsomes from male and female rats did not show any significant difference in the rates (Table 23). Thus estrogens probably do not affect the acyltransferase system directly.

Although no sex difference in the acyltransferase(s) rate was found, a difference in specificity for the esterification of 8,11,14-eicosatrienoate (20:3) and arachidonate (20:4) to 1-acyl-GPC and 1-acyl-GPE was observed (Table 23). This difference in specificity could be due to selectivity of the acyltransferase(s) catalyzing the esterification of 1-acyl-GPE for the double bond at carbon 5 or to selectivity by the number of double bonds in the fatty acid. Since Reitz and Lands (75) did not show preferential esterification of 5,8-octadecadienoyl-CoA to 1-acyl-GPE the acyltransferase(s) probably selects by the degree of unsaturation of the fatty acid.

Livers from rats deficient in essential fatty acids retained 20:4 longer in the diacyl-GPE fraction than in the diacyl-GPC (37,39) and on refeeding essential fatty acids the 20:4 is incorporated into this fraction faster (39). Mohrhauer and Holman (95) showed that arachidonate was three times as effective as linoleate in relieving the symptoms of essential fatty acid deficiency. According to Dhopeshwarkar and Mead (96) and Holman and Mohrhauer (97) these essential fatty acids compete for the enzymes involved in their transformation into polyunsaturated fatty acids. It has been proposed that the order of affinity of the enzyme sites depends on the degree of unsaturation of the fatty acid [i.e. linolenate (18:3) > linoleate (18:2) > oleate (18:1)] (98,99). If this were the case, arachidonate (20:4) would probably inhibit its own synthesis. Brenner and José (100) have proposed that competition between 5,8,11-eicosatrienoate (20:3) and 20:4 may take place in their incorporation into the 2-position of phospholipids and that this competition may be interrelated with polyunsaturated fatty acid synthesis. They suggested that control is exerted at both steps 1 and 2 in the scheme below.



Evidence for the involvement of both enzyme systems in replacement of 20:3 was obtained by studies of the kinetics of this replacement (39). Incorporation of both 18:2 and

20:4 into total phospholipids of rat liver followed an exponential curve and evoked an exponential decrease in 20:3. In the case of 20:4 the increasing curve corresponded to the decrease in 20:3. This suggested that 20:4 replaced 20:3 directly as well as inhibited the synthesis of 20:3 from 18:1 (95). Brenner proposed that this replacement of 20:3 occurs by specific incorporation of 20:4 into the 2-position of phospholipids (39).

The effect of dietary linoleate was somewhat different. Although it prevented desaturation and elongation of 18:1 into 20:3 and was incorporated into the 2-position of phospholipids, it did not appear to replace 20:3 directly. Also 18:2 is converted to 20:4 in rat liver and this 20:4 was found in the 2-position of the phospholipids. However, 20:4 did not seem to replace 20:3 directly in this case. Johnson et al. (101) have studied the changes in liver and plasma phospholipid fractions at earlier time periods (up to 24 hr) after feeding linoleate. At these shorter time periods they showed that the increase in 18:2 in phospholipids paralleled the decrease in 20:3. No significant increase in 20:4 occurred until 14 to 19 hours after feeding. Johnson et al. (101) concluded that 18:2 did not inhibit conversion of 18:1 to 20:3 at early time periods because no 20:4 was incorporated into phospholipids at this time. Thus the effect of 18:2 appears to be complex and may involve both an early phase of direct replacement of 20:3 and a later phase where the combined effects of 20:4 incorporation

into phospholipids and inhibition of the conversion of 18:1 to 20:3 occurs.

The specificity of the acyltransferase(s) for acylation of 1-acyl-GPE with 18:2 and 20:4 rather than 20:3 (Table 23) suggests that the acyltransferase(s) is important in the production of phospholipids containing the essential fatty acids. The enzyme(s) may play a principle role in the direct replacement of 20:3 by 18:2 and 20:4 (100).

SUMMARY

Acyl-CoA:monoacylphosphoglyceride acyltransferase catalyzes the transfer of fatty acids from their acyl-CoA esters to the free position of 1- or 2-acyl-GPE. Whether one or more enzymes possessing this activity are present in microsomes is not known. The rates of acyltransfer are determined by a continuous assay using an internal colorimetric reagent, DTNB, to measure CoA release. Isolation of the products from incubation mixtures containing a ^{14}C-labeled acyl-CoA showed that the rate observed spectrally corresponds to the amount of diacyl-GPE formed. Phospholipase A treatment of the radioactive diacyl-GPE formed showed that the 1- and 2-acyl-GPE preparations are relatively free of the other positional isomer.

The apparent K_m of 1-acyl-GPE was 75 μM and for 2-acyl-GPE, 50 μM. The activity of the transferase with 2-acyl-GPE is inhibited by higher concentrations of acyl-CoA esters. Freezing for 72 hours at a protein concentration of 15 mg/ml destroys most of the activity for 1-acyl-GPE. Sonication of DOC-treated microsomes did not solubilize the transferase activity with either 1- or 2-acyl-GPE.

Particles from rat liver which sediment between 5,000 and 15,000 x g (heavy particles) and between 15,000 and 78,000 x g (microsomes) both had transferase activity but the latter particles had a higher specific activity with either 1- or 2-acyl-GPE. Both the subcellular fractions preferentially transferred saturated and unsaturated fatty

acids containing trans double bonds to the 1-position of the acceptor. Linoleic, 8,11,13-eicosatrienoic and arachidonic acids were preferentially transferred to the 2-position.

The pig liver microsomal acyltransferase which catalyzes esterification at the 2-position did not discriminate between cis and trans isomers of octadecadienoate, but transferred them both more rapidly than the saturated acids. The enzyme system which acts at the 1-position discriminated between geometric isomers at carbon 9 and preferentially transferred trans octadecenoate and octadecadienoate as well as saturated acids to the 1-position. However, the enzyme did not discriminate as markedly between geometric isomers at carbon 11 of octadecenoate and transferred both isomers to the 1-position at about the same rate.

Analysis of the fatty acids esterified to the 2-position of diacyl-GPE and alkenylacyl-GPE from human erythrocyte stromata showed that both contained high proportions of polyunsaturated fatty acids. The composition of the fatty acids esterified to the 2-position of stromal diacyl-GPE could not be predicted from consideration of the abundance of the fatty acids in plasma and the acyltransferase rates.

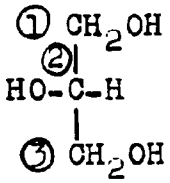
No difference was observed in the rates of transfer of arachidonate to 1-acyl-GPE by acyltransferase preparations from male or female rat livers. However, a difference in the specificity of the enzyme catalyzing the esterification

of 1-acyl-GPE and 1-acyl-GPC was observed. The enzyme catalyzing esterification of 1-acyl-GPC transferred 8,11,14-eicosatrienoate faster than linoleate or arachidonate; while the enzyme catalyzing esterification of 1-acyl-GPE transferred arachidonate faster.

APPENDIX I. NOMENCLATURE

A. Phospholipid Nomenclature

The nomenclature used in this thesis is based on the stereospecific numbering system suggested by Hirschmann (102). The carbons are numbered as shown in the structural formula which has been drawn according to the Fischer convention with the central hydroxyl on the left.



The compound 1,2-diacylglycerol-3-phosphorylethanolamine is referred to as diacyl-GPE; 1,2-diacylglycerol-3-phosphorylcholine is referred to as diacyl-GPC. The plasmalogen, 1-(1'-alk-1'-enyl)-2-acylglycerol-3-phosphorylethanolamine is referred to as alkenylacyl-GPE. The mixture of diacyl-GPE and alkenylacyl-GPE isolated from natural sources is referred to as ethanolamine phosphoglyceride (EPG). Serine phosphoglycerides (SPG) contain diacyl-GPS and alkenylacyl-GPS and choline phosphoglycerides (CPG) contain diacyl-GPC and alkenylacyl-GPC.

B. Fatty Acid Nomenclature

A fatty acid represented as 18:2 (n-6) contains 18 carbon atoms and 2 double bonds; (n-6) indicates the first double bond occurs at the sixth carbon atom from the terminal methyl carbon. The fatty acids have double bonds which are separated by a single methylene group.

Fatty acids can also be represented as 18:2-cc. The number notations indicate the chain length (18) and

the number of double bonds (2). Configuration of the double bond is indicated after the hyphen. For example 18:2-c9,c12 has the first double bond at the ninth carbon (measured from the carboxyl end) in the cis configuration. The acyl-CoA esters used in the acyltransferase assay system are listed below with their common and systematic names.

Acyl-CoA	Common Name	Systematic Name
16:0	palmitic	n-hexadecanoic
18:0	stearic	n-octadecanoic
18:1-c	oleic	cis-9-octadecenoic
18:1-t	elaidic	trans-9-octadecenoic
18:1-c	vaccenic	cis-11-octadecenoic
18:1-t	---	trans-11-octadecenoic
18:2-cc	linoleic	cis,cis-9,12-octadecadienoic
18:2-tt	linoelaidic	trans,trans-9,12-octadecadienoic
18:3-ccc	linolenic	cis-9,12,15-octadecatrienoic
20:3-ccc	---	cis-8,11,14-eicosatrienoic
20:4-cccc	arachidonic	cis-5,8,11,14-eicosatetraenoic

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