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THE CONTROL OF FATTY ACID COMPOSITION IN GLYCEROLIPIDS OF THE ENDOPLASMIC RETICULUM*

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SUMMARY

A functional relationship between constitutive enzymes of the endoplasmic reticulum that are involved in lipid metabolism and the proteins and phosphoglycerides constituting those membranes was examined by altering the protein and fatty acid composition. The specific activity of alkenyl glycerylphosphoryl choline hydrolyase did not change upon fasting, fasting and refeeding or administration of phenobarbital, whereas the specific activities of both acyl-CoA hydrolyase and acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase(s) increased upon alteration of the nutritional state. Other enzymic activities also showed changes following the treatments. The constant specific activity of 1-alkenyl glycerylphosphoryl choline hydrolyase suggests that it is a true constitutive enzyme of the endoplasmic reticulum and a useful indicator of membrane biogenesis.

Alteration in the fatty acid composition of microsomal phosphoglycerides by maintenance of animals under different dietary conditions, by *in situ* incorporation of fatty acids into microsomal phosphoglycerides, or by the addition of exogenous, micellar lecithins did not produce adaptive changes in the specificity for esterification of fatty acids to Position 2 of lecithins. Partial removal of microsomal phosphoglycerides by treatment with phospholipases failed to demonstrate a functional requirement for diacyl phosphoglycerides in acyltransferase activity. These considerations indicate that a functional role of phosphoglycerides in acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase activity is limited to the requirement of phosphoglycerides for the integrity of the membrane.

INTRODUCTION

Biomembranes contain large amounts of lipid, especially those surface-active lipids which possess both hydrophobic and hydrophilic moieties. Phosphoglycerides are the major surface-active lipids in membranes from mammalian cells. These phosphoglycerides exhibit an asymmetric distribution of fatty acids in which saturated fatty acids are esterified mainly to Position 1 of the glycerol moiety and unsaturated

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fatty acids are esterified predominantly at Position 2 (refs. 1 and 2). Various enzymes, which catalyze the incorporation of fatty acids into phosphoglycerides, are present in the particulate fractions of cells. For example, acyl-CoA:phosphoglyceride acyltransferases, which preferentially transacylate 1-acyl glycerylphosphoryl choline and 2-acyl glycerylphosphoryl choline with saturated and unsaturated fatty acids respectively[3–5], are found only in the microsomal fraction of rat liver[6]. These acyltransferases may be important in achieving and maintaining the asymmetric distribution of fatty acids in membranes and thereby be significant in the function and maintenance of the membranes. The question of whether certain combinations of fatty acids are capable of endowing phosphoglycerides with certain physical characteristics which are in turn required for the functional integrity of the membrane remains unanswered.

Fasting rats for 2 days followed by refeeding of a fat-deficient diet for 2 days leads to marked changes in the fatty acids of liver lipids[7]. These changes in fatty acid composition are reflected by the altered species of liver lecithins[8,9]. Other studies indicate that such dietary regimes not only change the fatty acid composition of membrane lipids but also lead to alterations in activity of various membranous enzymes. The specific activity of glucose-6-phosphatase increased upon fasting for 48 h (refs. 10 and 11), whereas stearoyl-CoA desaturase activity was negligible in fasted rats, but increased to levels above normal in rats refed a high-carbohydrate diet[12]. Phenobarbital also produces alterations of enzymic activities of microsomal enzymes[13] and in the turnover of microsomal phosphoglycerides[13,14].

In the present study, we have examined the interrelationship between acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase activity and the phosphoglycerides of rat-liver endoplasmic reticulum while considering the following questions: (1) Does the activity or specificity of acyl-CoA:acyltransferase change with different nutritional states which are known to markedly alter the fatty acid composition of liver lipids? (2) Does the activity of acyl-CoA:acyltransferase increase under conditions known to increase the synthesis of the endoplasmic reticulum? (3) Does a structural interrelationship exist between the acyltransferase activity of the endoplasmic reticulum and its component phosphoglycerides? (4) Does the specificity or activity of acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase change when the lipid composition of the membrane is altered?

METHODS AND MATERIALS

Maintenance of animals

Male rats (150–170 or 230–240 g) were maintained for at least 3 days on a Rockland complete mouse/rat diet. Animals in different nutritional states were obtained by dividing nine or twelve rats of the same weight range into three groups of three or four. Fat deficient, refed rats were fasted for 48 h and then refed a fat-deficient test diet (General Biochemicals, Chagrin Falls, Ohio). Fasted rats were fasted for 48 h, and control rats were maintained on the stock diet. The animals from the different nutritional states were sacrificed at the same time, and groups of livers were pooled.

Preparation of microsomes from animals of different nutritional states

Method A. Rat livers were minced and homogenized in 2 vol. of 0.25 M sucrose in 1 mM EDTA using a Dounce ball-type homogenizer. The homogenate was centri-

fuged at 20000 × *g* for 20 min, and the pellet was homogenized in 2 vol. of the sucrose solution and centrifuged again at 20000 × *g* for 20 min. The combined supernatants were centrifuged at 85000 × *g* for 60 min, and the pellet was resuspended in 2 vol. of 0.25 M sucrose in 1 mM EDTA and centrifuged again at 85000 × *g* for 60 min. The resultant pellet was designated as the microsomal fraction, and it was resuspended in 2 vol. of 0.25 M sucrose in 1 mM EDTA. These microsomes were treated with diisopropylfluorophosphate prior to assaying for acyltransferases[5].

Method B. Rat-liver microsomes were prepared as described by EIBL *et al.*[6]. In this method, animals were fasted overnight, and the livers were perfused *in situ* with ice-cold 0.154 M NaCl prior to preparing microsomes.

Phospholipase treatment of microsomes

Hydrolysis of microsomal phosphoglycerides was performed in an incubation mixture containing 0.2 mg of phospholipase A (crude venom from *Crotalus adamanteus*, Ross Allen Reptile Farms, Silver Springs, Fla.), 0.5 µmole $CaCl_2$ and 5 µmoles Tris–HCl buffer (pH 7.4) per mg of microsomal protein. For hydrolysis of microsomal phosphoglycerides with phospholipase C (ref. 15), the incubation mixture contained 0.16 mg of phospholipase C, 0.3 µmole $CaCl_2$ and 3 mmoles of Tris–HCl buffer (pH 7.4) per mg of microsomal protein. Both hydrolyses were conducted at room temperature, and the final protein concentration of microsomes was 10 mg/ml. Aliquots of the incubation mixture were removed at indicated times for assay of lipid phosphorus and acyltransferase activity. Aliquots for enzymic assay were treated with EDTA (5 moles per mole of calium) and aliquots for lipid analysis were added to 20 vol. of chloroform–methanol (2:1, by vol.).

Enzyme assays

The activity of acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase (acyltransferase) was assayed in a reaction mixture containing 25 nmoles of acyl-CoA, 175 nmoles 1-acyl glycerylphosphoryl choline, 90 µmoles Tris–HCl buffer (pH 7.4), 1 µmole 5,5′-dithio-bis-(2-nitrobenzoic acid) and 0.20 mg of protein in a final volume of 1.0 ml (ref. 5). For assaying acyl-CoA hydrolyase activity, the same system was used with 40–60 nmoles of acyl-CoA in the absence of 1-acyl glycerylphosphoryl choline. The activity of the 1-(1′-alk-1′-enyl) glycero-3-phosphoryl choline alkenyl ether hydrolase (alkenyl glycerylphosphoryl choline hydrolase) was measured as described by ELLINGSON AND LANDS[16]. D-Glucose-6-phosphate phosphohydrolase (EC 3.1.3.10) (glucose-6-phosphatase) rates were determined by a modified procedure of HARPER[17]. The enzymic reaction was performed in an incubation mixture containing 16.7 mM sodium citrate buffer (pH 6.5); 32 mM glucose 6-phosphate (pH 6.5); and about 1 mg microsomal protein in 1.5 ml total volume. At timed intervals, 0.3-ml aliquots were removed and analyzed for inorganic phosphate.

The activity of stearoyl-CoA desaturase was assayed in an incubation mixture containing 400 µmoles of NADPH, 40 µmoles of sodium thioglycollate, 80 nmoles of [^{14}C]stearoyl-CoA (2200 counts/min per nmole) and about 4 mg of microsomal protein in a final volume of 4.0 ml. At timed intervals, 1.0-ml aliquots of the reaction mixture were added to 2.5 ml of 5% $HClO_4$. After centrifugation, the pellet was dispersed in 0.4 ml of 0.5 M KOH and heated for a few minutes in a sand bath maintained at 50°. The fatty acids were converted to methyl esters by adding 0.5 ml

of BF_3 in methanol (14 %, w/v). The methyl esters were extracted twice with 2 ml of *n*-hexane, and aliquots of the methyl esters were resolved into saturated, monounsaturated and polyunsaturated by chromatography on silicic acid-impregnated paper with a solvent system of *n*-hexane–benzene (100:6.5, by vol.). The methyl esters were detected by spraying with 1 % I_2 in methanol. After I_2 was removed by gently heating, the chromatogram was divided into zones which were cut into small pieces and placed in scintillation vials. Individual zones were assayed for radioactivity in 10 ml of scintillation fluid (4 g 2,5-diphenyl oxazole and 100 mg *p*-bis-[5-phenyl oxazolyl]-benzene per l of toluene) using a liquid scintillation counter.

The N-demethylation of ethylmorphine (Merck, Sharp and Dohme) was assayed in a 5-ml incubation mixture containing 160 μmoles NADP, 18 μmoles of glucose 6-phosphate, 10 μmoles $MgCl_2$, 38.5 μmoles semicarbizide, 0.2 mmole of potassium phosphate buffer (pH 7.4) and 4.0 μmoles ethyl morphine. The mixture was incubated for 12 min at 37°, and the reaction mixture was stopped by the addition of 0.4 ml of 70 % $HClO_4$. Formaldehyde content was assayed by the method of NASH[18].

Analysis of fatty acids in microsomal lipids

Microsomes (100–150 mg of protein) were added to 19 vol. of chloroform–methanol (2:1, by vol.) containing 20 μl of a 1 % solution of 2,6-di-(*tert.*-butyl)-*p*-cresol. The mixture was subjected to three 30-sec homogenizations in a Waring Blender at 1-min intervals. The mixture was allowed to stand for 30 min at room temperature with intermittent swirling. The mixture was then washed with 0.2 vol. of water, and the chloroform layer was evaporated under vacuum. Conversion of glyceryl esters to methyl esters by transesterification with sodium methoxide and quantitative gas–liquid chromatographic analyses have been described in a previous communication[15].

Estimation of protein and RNA

Protein and RNA were estimated by the procedure of WARBURG AND CHRISTIAN[19]. Spectrophotometric measurements were made in the presence of 1 % sodium deoxycholate to solubilize the particulate proteins.

Estimation of phosphoglycerides in phospholipase-treated microsomes

The total lipid extract, which was prepared according to FOLCH *et al.*[20], was applied to a thin-layer plate coated with 0.37 mm of silica gel HR. The plate was developed in chloroform–methanol–acetic acid–water (50:30:8:4, by vol.), and the phosphoglycerides were visualized by spraying with molybdate reagent[21]. The plate was divided into bands which were scraped into test tubes, and 0.6 ml of 70 % $HClO_4$ was added. Digestion of organic matter was completed at 180°, and phosphorus was estimated by the method of BARTLETT[22]. Standards and blanks were prepared in the presence of silica gel HR.

Preparation of sonicated lipids

Synthetic phosphatidyl cholines[23] were dispersed in 0.1 M Tris–HCl buffer (pH 7.4, 2–3 μmoles/ml) with a ball-type homogenizer. The suspension was sonicated for 5–15 min with a Branson 75 SL sonifier at full power. An ice bath and intermittent sonication were used to maintain the temperature at less than 20°. The final concen-

tration was determined by phosphorus analysis, and the samples were freshly prepared for each experiment.

RESULTS

Fasted and refed animals

Table I presents the specific activities (nmoles/min per mg) of various microsomal enzymes from rats maintained on different diets. The specific activity of glucose-6-phosphatase increased 2–3-fold upon fasting for 48 h and the specific activity of stearoyl-CoA desaturase underwent a dramatic increase upon refeeding the fat-deficient diet. The specific activities of alkenyl glycerylphosphoryl choline hydrolyase and acyl-CoA hydrolyases did not change significantly with different nutritional conditions. The total acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase activity increased upon fasting for 48 h and remained elevated in the fasted and refed animals. An increase in specific activity (35–40 %) was observed with all four acyl-CoA's tested, and the relative rates of esterification for these thiol esters were unchanged.

TABLE I

THE SPECIFIC ACTIVITIES OF RAT-LIVER MICROSOMAL ENZYMES IN RATS UNDER DIFFERENT NUTRITIONAL STATES

Specific activities are the average value for three separate experiments using rat-liver microsomes prepared from three pooled livers. The values in parenthesis represent a separate group of pooled livers. Preparation of microsomes (Method A), enzyme assays and conditions for different nutritional states are described under METHODS AND MATERIALS.

Enzyme	*Specific activity (nmoles/min per mg)*		
	Control	*Fasted*	*Refed*
Glucose-6-phosphatase	57 (61)	122 (152)	152 (120)
Stearoyl-CoA desaturase*	19 (14)	5 (13)	1540 (1640)
Alkenyl glycerylphosphoryl choline hydrolase	32 (35)	34 (32)	32 (33)
Acyl CoA:1-acyl glycerylphosphoryl choline transferase			
with 20:4 acyl-CoA	37 (31)	41 (44)	47 (45)
with 18:2 acyl-CoA	28 (26)	31 (35)	36 (40)
with 18:1 acyl-CoA	25 (21)	39 (31)	29 (32)
with 16:1 acyl-CoA	10 (9)	13 (13)	11 (17)
Acyl-CoA hydrolase			
with 20:4 acyl-CoA	21 (17)	26 (26)	24 (24)
with 18:2 acyl-CoA	24 (20)	25 (23)	24 (20)
with 18:1 acyl-CoA	12 (8)	10 (8)	12 (9)
with 18:0 acyl-CoA	21 (14)	16 (15)	17 (27)

* The specific activity of this enzyme is expressed in pmoles/min per mg.

Table II shows the changes in fatty acid composition of microsomal lipids which are evoked by fasting and refeeding. In agreement with earlier studies[7], linoleic, arachidonic and stearic acids decreased while oleic and eicosatrienoic acids increased upon starving and refeeding. Treatment of the animals with phenobarbital, however, produced no significant change in fatty acid composition.

TABLE II

FATTY ACID COMPOSITION OF MICROSOMAL LIPIDS

Preparation of microsomes (Method A), extraction of lipids from microsomes, and preparation of methyl esters are described under METHODS AND MATERIALS. The values (in mole %) are the averages from three separate experiments, each using microsomes prepared from three pooled rat livers. The values in parentheses represent results from the control microsomes for a separate group of rats injected with phenobarbital.

Acid	*Nutritional state*			
	Control	*Fasted*	*Refed*	*Phenobarbital*
16:0	18.7 (20.7)	22.5	27.1	20.8
16:1 (n-7)	1.9 —	0.8	9.3	—
18:0	18.8 (21.3)	24.4	11.6	23.3
18:1 (n-9)	10.0 (12.4)	7.1	23.4	10.0
18:2 (n-6)	18.9 (17.3)	5.2	4.5	18.3
20:3	0.9 (1.3)	0.5	3.0	1.0
20:4 (n-6)	21.3 (21.3)	28.9	14.4	21.7
22:6 (n-3)	7.3 (5.6)	8.9	5.1	5.3
Minor polyunsaturates	2.2 —	1.7	1.3	—

TABLE III

ENZYMATIC ACTIVITIES OF TOTAL CELL HOMOGENATE AND MICROSOMAL FRACTION

Each total homogenate and microsomal fraction was prepared from three livers combined. The total recovery in subcellular fractions of activity for all enzymes was 85% or greater. Assay conditions are described in the text.

Enzyme	*Nutritional state*					
	Control		*Fasted*		*Refed*	
	*Specific activity**	*Total activity***	*Specific activity**	*Total activity***	*Specific activity**	*Total activity***
Glucose-6-phosphatase						
Homogenate	18.0	72 000	51.0	82 000	17.0	102 000
Microsomes	58.0	24 000	104.0	22 000	101.0	35 000
Alkenyl glyceryl-phosphoryl choline hydrolase						
Homogenate	5.4	30 000	5.5	18 000	4.2	34 000
Microsomes	35.0	14 000	32.0	7 000	33.0	12 000
Acid phosphatase						
Homogenate	5.1	28 000	5.5	18 000	4.2	34 000
Microsomes	5.0	2 000	5.2	1000	5.2	2 000
Succinate–cytochrome *c* reductase						
Homogenate	35.0	108 000	37.0	88 000	30.0	107 000
Microsomes	1.2	600	2.8	450	2.0	600

* Measured in nmoles/min per mg.
** Measured in nmoles/min.

Table III shows the specific and total activities of several enzymes in the homogenate and the microsomal fraction. The total activity of the glucose-6-phosphatase in the total homogenates was increased in fasted and refed animals. On the other hand, the total activity of the alkenyl glycerylphosphoryl choline hydrolase was lower in the homogenate and in the microsomes obtained from the starved rats. The total recovery of each enzymatic activity studied was in the range of 85–95 %.

Contamination of the microsomes by other cell fractions was determined by assaying for enzymes known to be associated with other cellular components. The microsomal fraction was enriched in glucose-6-phosphatase and alkenyl glycerylphosphoryl choline hydrolase, two enzymes which have been previously shown to be associated with the microsomal fraction[24,25]. The isolated microsomes contained less than 0.5 % of the succinate–cytochrome *c* reductase, an activity known to be associated exclusively with the mitochondria. Using β-glycerophosphate as a substrate, the microsomes contained about 5–10 % of the total measured acid phosphatase activity. This enzymatic activity is associated with the lysosomes, and the percent of this activity found in the microsomes was about the same in all three nutritional conditions. Contamination of the microsomes by soluble enzymes was probably negligible because the microsomes were centrifuged for 1 h at 85000 $\times$ *g* and resuspended twice in 2 vol. of 0.25 M sucrose, 0.001 M EDTA.

Phenobarbital-treated rats

Table IV shows the specific activities of microsomal enzymes from phenobarbital-treated and control rats. In agreement with a previous report[26], the specific

TABLE IV

SPECIFIC ACTIVITIES OF RAT-LIVER MICROSOMAL ENZYMES FROM PHENOBARBITAL-TREATED RATS

Rats were injected intraperitoneally with phenobarbital (4.0 mg/kg of body wt.) every 24 h for 4 days. Control rats were injected with physiological saline. Animals were sacrificed 24 h after the final injection, and microsomes were prepared by Method A. Values for control animals represent two separate experiments, and the values for phenobarbital-treated rats represent the average of three separate experiments, each with three pooled livers. Experimental details are described under METHODS AND MATERIALS.

Enzyme activity	*Specific activity (nmoles/min per mg)*	
	Control	*Phenobarbital treated*
N-Demethylation of ethyl morphine	4.0	9.3
Glucose-6-phosphatase	51	24
Alkenyl glycerylphosphoryl choline hydrolase	34	36
Acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase		
with 20:4 acyl-CoA	29	28
with 18:1 acyl-CoA	17	17
Acyl-CoA hydrolase		
with 20:4 acyl-CoA	17	17
with 18:1 acyl-CoA	8	8

activity for N-demethylation of ethyl morphine increased 2-fold in phenobarbital-treated rats, and the specific activity of glucose-6-phosphatase decreased. Treatment of rats with phenobarbital had no effect, however, upon the specific activities of alkenyl glycerylphosphoryl choline hydrolyase, acyl-CoA hydrolyases and acyltransferases, or the fatty acid composition of microsomal lipids (as shown in Table II).

Phospholipase A treatment of microsomes

To consider the possibility of a functional role for phosphoglycerides in acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase activity, rat-liver microsomes were treated with phospholipase A which hydrolyzes the 2-acyl ester bond of phosphoglycerides. Fig. 1 shows the effect of phospholipase A treatment upon the acyltransferase activity of microsomes and the hydrolysis of diacyl glycerylphosphoryl choline and diacyl glycerylphosphoryl ethanolamine. During the first 30 min of treatment with phospholipase A, 85 % of diacyl glycerylphosphoryl choline and 80 % of diacyl glycerylphosphoryl ethanolamine were converted to the corresponding monoacyl derivatives; however, no significant loss of acyltransferase activity was observed during this period of time. Maximal hydrolysis (90 %) of the microsomal phosphoglycerides occurred within 60 min at which time 85–90 % of the acyltransferase activity remained. Approx. 80 % of the combined diacyl glycerylphosphoryl serine and diacyl glycerylphosphoryl inositol was also converted to more polar derivatives

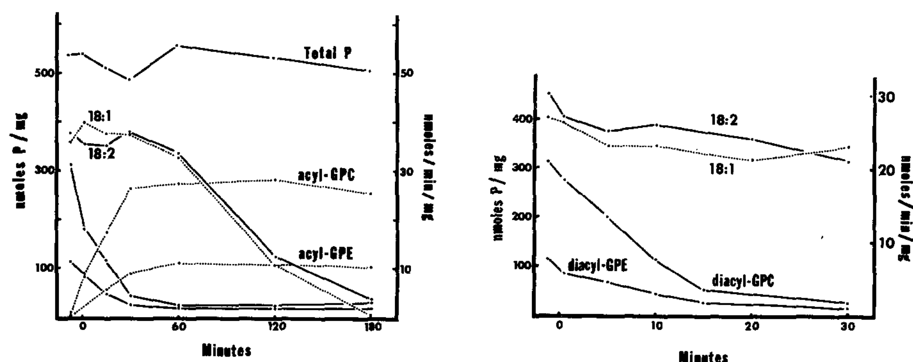

Fig. 1. Treatment of rat-liver microsomes with phospholipase A. Rat-liver microsomes (Method B) were treated with phospholipase A as described in the text. Aliquots of the reaction mixture were taken at indicated intervals for analysis of lipid phosphorus and acyltransferase activity. A total lipid extract was prepared according to FOLCH *et al.*[19], and resolution of phosphoglycerides was achieved on thin-layer chromatography as described in the text. Apparent R_F's of 0.12, 0.20, 0.33, 0.43 and 0.74 were calculated respectively for acyl glycerylphosphoryl choline, sphingomyelin, diacyl glycerylphosphoryl choline, acyl glycerylphosphoryl ethanolamine and diacyl glycerylphosphoryl ethanolamine. Estimation of phosphorus (left ordinate) is described in the text. The two solid, unlabeled lines at the bottom of the graph represent the remaining amount of diacyl glycerylphosphoryl choline (top) and diacyl glycerylphosphoryl ethanolamine (bottom). Acyltransferase activity (right ordinate) was assayed by following the increase in absorbance at 412 nm which was produced by the reaction of 5,5'-dithio-bis-(2-nitrobenzoic acid) with sulfhydryl groups. The reaction mixture contained 1 μmole 5,5'-dithio-bis-(2-nitrobenzoic acid), 25 nmoles of acyl-CoA, 175 nmoles acyl glycerylphosphoryl choline, 90 μmoles of Tris–HCl buffer (pH 7.4) and 0.20 mg of microsomal protein in a total volume of 1.0 ml (ref. 5). Abbreviations: acyl-GPC, acyl glycerylphosphoryl choline; acyl-GPE, acyl glycerylphosphoryl ethanolamine.

Fig. 2. Treatment of microsomes with phospholipase C. Rat-liver microsomes were treated with phospholipase C as described in the text. Analysis of lipid phosphorus (left ordinate) and of acyltransferase activity (right ordinate) are described in Fig. 1. Abbreviations: see Fig. 1.

at this time. Further exposure of the membranes to the action of phospholipase A led to a rapid and extensive loss of acyltransferase activity although further hydrolysis of diacyl phosphoglycerides was not observed. The acyltransferase activity of the treated microsomes was similar for oleoyl-CoA and linoleoyl-CoA.

Phospholipase C treatment of microsomes

Although the above results did not show a dependency of acyltransferase activity upon the presence of phosphoglycerides, it remained possible that the resultant monoacyl phosphoglycerides maintained the acyltransferase activity while remaining adsorbed to the membranes. This possibility was further considered by treating microsomes with phospholipase C which cleaves the phosphodiester bond of phosphoglycerides forming 1,2-diacyl glycerols. Fig. 2 shows the effect of phospholipase C treatment of microsomes upon the acyl-CoA-1-acyl glycerylphosphoryl choline acyltransferase activity and the disappearance of phosphoglycerides with time. 90 % of the microsomal diacyl glycerylphosphoryl ethanolamine and diacyl glycerylphosphoryl choline were converted to diacyl glycerols after 30 min of incubation, but only 10–20 % of the acyltransferase activity had been lost. No significant hydrolysis of diacyl glycerylphosphoryl inositol, diacyl glycerylphosphoryl serine or sphingomyelin was observed.

Storage of phospholipase C-treated microsomes led to extensive loss of acyltransferase activity. This suggested the possibility that exogenous phosphoglycerides might stabilize the loss of activity. This possibility was tested using synthetic phos-

TABLE V

EFFECT OF SYNTHETIC DIACYL GLYCERYLPHOSPHORYL CHOLINES ON STABILIZING ACYL-CoA:1-ACYL GLYCERYLPHOSPHORYL CHOLINE ACYLTRANSFERASE ACTIVITY OF PHOSPHOLIPASE C-TREATED MICROSOMES

Rat-liver microsomes (Method B) were treated with phospholipase C for 30 min as described in the text. Sonicated solutions of diacyl glycerylphosphoryl cholines (800 nmoles of lipid P per mg microsomal protein) in 0.1 M Tris–HCl buffer alone were added to aliquots of the treated microsomes and the samples were stored at 1–3°. Acyltransferase activity was determined as described in Fig. 1.

Additions	*Acyltransferase activity (nmoles/min per mg)*					
Hours after treatment	*0.5*	*2*	*5*	*8*	*13*	*27*
Linoleoyl-CoA						
Buffer	32	23	21	17	18	2
Distearoyl glycerylphosphoryl choline	44	37	33	24	16	6
Dipalmitoyl glycerylphosphoryl choline	31	26	27	20	18	11
Dioleoyl glycerylphosphoryl choline	27	26	25	18	14	4
Dimyristoyl glycerylphosphoryl choline	26	21	13	15	9	4
Untreated	44	36	42	46	28	23
Oleoyl-CoA						
Buffer	27	18	14	11	6	1
Distearoyl glycerylphosphoryl choline	39	19	18	16	11	6
Dipalmitoyl glycerylphosphoryl choline	24	19	19	14	13	6
Dioleoyl glycerylphosphoryl choline	22	19	18	12	12	2
Dimyristoyl glycerylphosphoryl choline	14	16	13	14	8	4
Untreated	40	33	34	36	26	20

phatidyl cholines. For this purpose sonicated solutions of dimyristoyl glycerylphosphoryl choline, dipalmitoyl glycerylphosphoryl choline, distearoyl glycerylphosphoryl choline and dioleoyl glycerylphosphoryl choline were added to phospholipase C-treated microsomes (800 nmoles of lipid phosphorus per mg of microsomal protein). Table V shows the effect of exogenous lecithins upon the acyltransferase activity of phospholipase C-treated microsomes stored at 1–3°. The acyltransferase activity of phospholipase C-treated microsomes was almost completely lost during 27 h of storage in an ice bath and approximately one-half the activity was lost in the untreated microsomes. None of the synthetic diacyl glycerylphosphoryl cholines was completely effective in preventing loss of activity during storage, but distearoyl glycerylphosphoryl choline, dipalmitoyl glycerylphosphoryl choline and dioleoyl glycerylphosphoryl choline afforded some protection against loss of activity. The degree of pro-

TABLE VI

EFFECT OF EXOGENOUS DIPALMITOYL GLYCERYLPHOSPHORYL CHOLINE UPON ACYL-CoA:1-ACYL-GLYCERYLPHOSPHORYL CHOLINE ACYLTRANSFERASE ACTIVITY OF RAT-LIVER MICROSOMES

Sonicated dipalmitoyl glycerylphosphoryl choline in 0.1 M Tris–HCl buffer (pH 7.4) was added to microsomes as indicated (nmoles of diacyl glycerylphosphoryl choline per mg of microsomal protein). Enzymic assay is described in Fig. 1.

Dipalmitoyl glycerylphosphoryl choline (nmoles)	*Oleoyl-CoA (nmoles/min per mg)*	*Linoleoyl-CoA (nmoles/min per mg)*
None	45	41
480	43	42
960	45	42
1440	43	41

TABLE VII

RATES OF FORMATION OF SATURATED AND UNSATURATED PHOSPHATIDYL CHOLINES

Acyltransfer rates, expressed as nmoles/min per mg protein, were measured spectrophotometrically in 1-ml cuvettes as described earlier[5]. Each reaction mixture was assayed for 5 min in the absence of added acceptor, 1-acyl glycerylphosphoryl choline, then with the acyl glycerylphosphoryl choline and finally with a second thiol ester added. The order of addition was varied for the saturated and unsaturated thiol esters as indicated. The unsaturated thiol ester had fully reacted by the end of the 5-min period.

Addition		*Transfer rate*		
First	*Second*	*Control*	*First*	*Second*
18:0	20:4	3.4	4.3	20.8
20:4	18:0	4.7	21.9	4.8
16:0	20:4	3.3	7.5	22.2
20:4	16:0	3.7	22.5	4.5
20:0	20:4	5.3	0.9	22.3
20:4	20:0	5.3	21.0	0.9
18:0	18:2	4.9	5.0	25.4
18:2	18:0	4.9	20.8	2.8
16:0	18:2	2.3	5.8	19.5
18:2	16:0	3.0	16.8	4.0
20:0	18:2	4.3	0.6	23.6
18:2	20:0	4.3	21.0	0.9

tection was more pronounced when acyltransferase activity was assayed with linoleoyl-CoA than with oleoyl-CoA. That the addition of exogenous diacyl glycerylphosphoryl choline did not alter the activity of the acyltransferase is indicated in Table VI. This table shows the effect of exogenous diacyl glycerylphosphoryl choline upon the acyltransferase activity of microsomes. No change in activity with either oleoyl- or linoleoyl-CoA was observed.

Although added exogenous saturated phosphatide did not inhibit acyltransferase activity, the possibility remained that saturated phosphatides formed *in situ* on the membranes would alter the enzymic activity. This concept was tested with a sequential addition of saturated or unsaturated acids to 1-acyl glycerylphosphoryl choline as indicated in Table VII. The low rate of transfer noted during preincubation with the long-chain saturated thiol esters did not appear to produce an inhibitor since linoleate and arachidonate were transferred at similar rates regardless of the preincubation conditions.

DISCUSSION

When this work was initiated in 1965, considerable uncertainty existed about the nature of membrane formation in terms of whether or not it was a two- or three-dimensional array of discrete subunits, each containing all the components of the macroscopic membrane. Membrane synthesis then could be regarded as simply producing more or less of these subunits. If so, the lipid, protein and enzymes would be incorporated into these subunits in proportions characteristic of the membrane being produced, and no mechanism is provided for deleting or altering one component without altering the other components of the subunit. On the other hand, the membrane could be a mosaic of materials with the ability to undergo large changes in one component without any change in another. The latter concept has gained increasing support recently and is supported by our findings.

Phosphoglycerides are always present in mammalian membranes and are generally regarded as essential components of these structures. Certain membrane-bound enzymes, which are exceedingly difficult to purify, seem to require phospholipid in order to carry out their catalytic functions. These enzymes could be regarded as true components of membranes, in contrast to the more easily removed materials adsorbed or included in vesicular pockets. The incorporation of such enzymes into the membranes would seem to require a corresponding incorporation of phosphatide. Therefore, if the membrane were an assembly of subunits, changes in amount or in composition of phosphoglyceride would be reflected by changes in other membrane components. Our results, however, indicate that the species of phosphoglycerides can be altered over a wide range without concomitant changes in the enzymes characteristic of the membranes. Also the amount of an enzymic activity can be altered greatly without a corresponding change in phosphoglycerides or other enzymes. This type of result was also found by OMURA *et al.*[27] who concluded that the pattern of a specific membrane is stable, while its individual molecular components are not. The more recent studies of ARIAS *et al.*[28] also show that the endoplasmic reticulum is a dynamic system consistent with a mosaic model for membrane biogenesis. The difficult questions remain in regard to how the different turnovers of components in the mosaic model can be regulated to produce a characteristic structural and functional

pattern recognized as the "integrity" of the membrane. Our experiments were designed to attempt to answer the four questions presented in INTRODUCTION regarding the role of phosphoglycerides in the functional integrity of membranes. In a sense similar to that for membranes, the different moieties of a phosphoglyceride have different rates of turnover[29], but the molecule is recognized by a characteristic pattern. We have, however, come to realize various species of a glyceride molecule whose physical properties can differ greatly due to the nature of the different acyl groups attached. This allows the separate recognition of a structural and a functional aspect of integrity for the molecule. Now it is possible to describe conditions *in vivo* in which not only the enzymic composition of the endoplasmic reticulum is altered, but also the fatty acid composition of the constituent phosphoglycerides. Such conditions afforded us the opportunity to experimentally consider the interrelationship between enzymic activity and the membrane environment under both normal and altered conditions.

The preferential esterification of essential fatty acids, or their derivatives, to Position 2 of the phosphoglycerides suggests the possibility that specific fatty acids such as arachidonic acid, may endow membranes with certain structural properties which are essential to the overall function and integrity of the membrane[30]. Alterations in the permeability and fragility of erythrocytes[31] and mitochondria[32] from animals deficient in essential fatty acids support the suggestion that those acids or their derivatives are a requisite moiety in the structure of phosphoglycerides in some membranes. Data now available on liquid crystals and membranes led one laboratory[33,34] to deduce that poikilothermic organisms and bacteria appear to have a feedback mechanism linked to the environmental temperature enabling the fatty acid residue of the phospholipids to be altered so as to keep the hydrocarbon chain fluidity fairly constant.

Various studies from this laboratory have been concerned with the process by which a phosphoglyceride acquires its characteristic fatty acid composition. Our studies have indicated that various factors such as substrate availability, enzyme specificity and alternate pathways of introduction of fatty acids into phosphoglycerides are involved in determining the fatty acid composition of a specific phosphoglyceride. The enzymes, acyl-CoA-monoacyl phosphoglyceride acyltransferases, are of special interest in that they afford the cell with a means of retailoring the preexisting fatty acid composition of the phosphoglycerides. Such retailoring makes possible a mechanism whereby certain physical properties of lecithins could be modulated, and these would then affect the functional integrity of the membrane. The activity of the acyltransferases could, in turn, be modulated by changes in the physical properties of its microenvironment in the membrane[8,35].

Alterations in the enzymic composition of microsomes from animals of different dietary conditions were monitored by measuring the glucose-6-phosphatase activity which is regarded to be characteristic of endoplasmic reticulum[24]. The specific activity of glucose-6-phosphatase in fasted animals increased about 2 times that of control animals which agrees with the changes observed by WEBER *et al.*[10] and ARION AND NORDLIE[11]. The total units of glucose-6-phosphatase in the homogenates, however, increased slightly in the rats fed different diets. The abundance of this marker for endoplasmic reticulum thus suggests that the amount of those membranes was slightly elevated. Total units of the alkenyl glycerylphosphoryl choline hydrolase, however, were less in the homogenates from the fasted animals and normal again in the refed

rats. In agreement with the studies of ELOVSON[12], the conversion of stearic acid to oleic was negligible in fasted animals, but was markedly increased in fasted, refed animals. The pattern of total units of enzymic activity noted for the different homogenates was also observed in the isolated microsomes, indicating that the isolated fraction was representative of the total endoplasmic reticulum in the tissue homogenates. In other words, the isolation procedure did not select for specific subfractions of the microsomes in any of the preparations from the rats fed different diets.

Since the microsomes utilized in this study were not highly contaminated with either acid phosphatase from lysosomes or with succinate–cytochrome *c* reductase from mitochondria, enzymic changes observed were not due to the presence of contaminating cell components. Thus, the observed changes in the enzyme specific activities and in fatty acid composition were due to changes in the total material present with the endoplasmic reticulum. The various functional enzymes, proteins and phospholipids clearly do not increase or decrease in a concerted fashion.

The loss of stearic acid, arachidonic and linoleic acids accompanied by an increase of eicosatrienoic and oleic acids from liver phosphoglycerides, may reflect alterations in either the composition of the acyl-CoA pools or in the specificity of acyltransferases. In our studies, as in the studies of ALLMAN *et al.*[7], significant changes in the fatty acid composition of microsomal phosphoglycerides were observed after only 2 days of fasting and 2 days of refeeding. In a similar sense, JOHNSON *et al.*[36] have observed increases in the linoleic and arachidonic acid content of liver phosphoglycerides from essential fatty acid-deficient rats within 7–15 h after feeding of safflower oil. These rapid changes in the fatty acid composition of phosphoglycerides underscore the question of whether such changes are due only to the available acyl-CoA or also to changes in the specificity of acyltransferases for certain acyl-CoA's.

These changes in fatty acid composition of phosphoglycerides were accompanied by an increase in the specific activity of acyltransferase(s) catalyzing the transesterification of acyl-CoA's to Position 2 of diacyl glycerylphosphoryl choline, but the increase in specific activities for different acyl-CoA's was proportionally similar. Acyl-CoA hydrolyase activity was essentially unchanged. Acyltransferase specificities for the synthesis of diacylphosphoglycerides from monoacylphosphoglycerides have shown an excellent correlation with the observed distribution of fatty acids in natural lecithins[8] and with the distribution *in vivo* of unnatural isomers of fatty acids[37] in liver lecithins. Further studies by WAKU AND LANDS[38] showed that the acyltransferase specificity and the availability of the various acyl groups must both be considered in predicting the fatty acid composition at Position 2 of erythrocyte lecithins. In the present study, the difference in fatty acid composition cannot be attributed to altered specificities of acyltransferases or acyl-CoA hydrolyases, but rather are probably attributable to changes in the availability of precursors for acyl-CoA synthesis. This rationale is especially appropriate for the content of oleate which is normally produced within the cell by a desaturation of stearate. ELOVSON[12], found that conversion of stearic acid to oleic acid was negligible in rats fasted for 48 h, but was considerable in similar rats refed a carbohydrate diet. The increased levels of this enzyme during carbohydrate refeeding is probably responsible for the increased oleic acid and decreased stearic acid content in the lipids from livers of carbohydrate-refed rats.

The fact that stearoyl-CoA desaturase activity in the endoplasmic reticulum was altered by orders of magnitude under different dietary conditions without con-

comitant changes in the activity of other enzymes indicates that not all microsomal enzymes are constitutive components even though characteristically located in those membranes. Various enzymic constituents of the endoplasmic reticulum, NADPH–cytochrome *c* reductase[39], cytochrome P_{450} (ref. 40), and enzymes involved in the metabolism of drugs[26], are increased by the administration of phenobarbital. Under these conditions, the character of the membrane was presumably altered by the addition and deletion of various proteins or subunits while the composition of the lipid remained essentially constant as did the relative specific activities of the various acyltransferases. Perhaps a subunit containing the desaturase and its activating phosphoglycerides can be added to or removed from the membranes in accord with a mosaic model of composition. Less dramatic changes were produced with the rise in the specific activity of the N-demethylation accompanying a decrease for the glucose-6-phosphatase. Apparently more protein relative to glucose-6-phosphatase was added to the membranes and these added proteins contained much more N-demethylase than found in the average normal endoplasmic reticulum. Fasting produced an opposite effect in that the specific activity of glucose-6-phosphatase rose as if the subunit containing it was incorporated in relatively greater amounts under these conditions. The acyltransferase activities, already shown to have a distribution in membranes similar to glucose-6-phosphatase[6], also increased in proportion to that enzyme. On the other hand, the specific activity of the acyltransferase(s) remained constant while glucose-6-phosphatase decreased and N-demethylase increased following phenobarbital treatment. Such constancy resembles that of a common denominator within the membrane to which other adaptive functional units can be added.

In this regard, no adaptive behavior was discerned for 1-alkenyl glycerylphosphoryl choline hydrolyase, whose specific activity remained constant after all treatments used (see Tables I and IV). Such findings suggest that as the bulk of the proteins is added to the endoplasmic reticulum there is a corresponding stoichiometric increase in alkenyl glycerylphosphoryl choline hydrolyase activity. Perhaps 1-alkenyl glycerylphosphoryl choline is truly a constitutive enzymic activity of the endoplasmic reticulum membrane, as compared to glucose-6-phosphatase, and it may offer a better index to a base unit of the membrane than do the other enzymes under consideration.

The relationship of microsomal acyltransferases to microsomal phosphoglycerides was examined after partial removal of phosphoglycerides. The two major phospholipids of rat-liver microsomes are diacyl glycerylphosphoryl choline and diacyl glycerylphosphoryl ethanolamine which comprise approx. 80 % of the lipid phosphorus present in microsomes. Approx. 90 % of the microsomal diacyl glycerylphosphoryl choline and diacyl glycerylphosphoryl ethanolamine was hydrolyzed by phospholipases without an appreciable loss of acyltransferase activity. The failure to correlate hydrolysis of diacyl phosphoglycerides with the loss of enzymic activity suggests that they are not required for the activity of acyl-CoA: 1-acyl glycerylphosphoryl choline acyltransferase.

Most of the enzymes involved in the synthesis of glycerolipids are localized in the particulate fraction of the cell, especially the endoplasmic reticulum[6,41,42]. Although no systematic study of the requirement of lipid for the activity of these enzymes is available, phosphoglycerides have been indicated to stimulate the activity

of stearoyl desaturase[43], acyl-CoA:glycerol-3-phosphate acyltransferase[44], CTP: cholinephosphate cytidyltransferase[45], phosphatidate phosphohydrolyase[46] and 1-alkenyl glycerylphosphoryl choline hydrolyase[16].

Although a functional role for phospholipid in acyl-CoA:1-acyl glycerylphosphoryl choline acyltransferase activity was not demonstrated, the acyltransferase activity of phospholipase-treated microsomes was more labile than that of untreated microsomes. Such findings suggest that phosphoglycerides are essential for the integrity of the membrane, but not for the activity of the acyltransferase; and it appears that loss of phosphoglyceride from the membrane alters the microenvironment in such a way that the integrity of the membrane and its constituent enzymes is slowly lost. The addition of exogenous diacyl glycerylphosphoryl choline did not prevent this loss of activity.

Other studies have indicated that acyltransferase activity is sensitive to environmental changes which presumably reflect changes in the integrity of the membrane. JEZYK AND LANDS[35] have demonstrated that the rate of esterification of 9-octadecenoyl-CoA to 1-acyl glycerylphosphoryl choline is more sensitive to inhibition by short chain alcohols or *N*-alkylureas than the esterification of 9,12-octadecadienoyl-CoA. In a similar sense, REITZ *et al.*[47] showed that the acyltransferase activity of deoxycholate-treated microsomes was lost more rapidly for 9-octadecenoyl-CoA than for 12-octadecenoyl-CoA. It is, thus, readily apparent that the specificity for esterification of fatty acids to acyl glycerylphosphoryl choline may be altered by changes in the environment or by various manipulations of the enzyme. Alterations of the microsomal phosphoglycerides by phospholipases produced neither an immediate nor a disproportionate loss in activity with the two tested acyl thioesters. Likewise the decay of acyltransferase activity in the phosphoglyceride-depleted microsomes was approximately the same for either acyl-CoA.

Because of the highly condensed nature of surface films of saturated lipids, it was anticipated that the additions of a disaturated lecithin to the phosphoglyceride-poor microsomes could alter the physical environment and activity of the acyltransferases. The effect of the presence of a disaturated lecithin would be most efficiently overcome if the specificity of acyltransferases were enhanced for unsaturated acids. This led us to consider a decreasing polarity in the microenvironment as a possible agent in a feedback control process which regulates the rate of unsaturated ester formation. The present studies using 9-octadecenoyl-CoA and 9,12-octadecadienoyl-CoA gave no indication that such effects could be modulated through lipid–protein interactions, but they cannot be excluded since the micellar form of lipid may be inappropriate for this effect. In a similar sense, no changes in specificity of the acyltransferases were observed in microsomes containing markedly different fatty acid patterns developed *in vivo* as a result of different dietary conditions. In that case the lipids could be regarded as being made available in the correct physical form, but the total content of saturated acid was not as high as obtained *in vitro*.

An additional attempt to show an effect of saturated lipid upon acyltransferase rates was related to some concepts of atherogenesis. The possible deleterious effect of atherogenic diets may be related to high transitory levels of saturated fatty acids in the blood. Conceivebly, those higher levels could lead to an increased probability of disaturated lecithins being formed which could, in turn, alter the membrane's fluidity and its ability to bind cholesterol. Such a rationale is hindered by the

lack of evidence for the accumulation of appreciable levels of disaturated lipids. Recent results of BRANDT AND LANDS[23], however, reopened this consideration since the formation of long-chain saturated lipids was very poor. Although the acyltransfer rates seemed relatively independent of the acyl group already present in the 1-acyl glycerylphosphoryl choline, the rate of synthesis of saturated lecithins decreased from 8.6 tot 0 as the total acyl carbon atoms in the product increased from 26 to 38. The results raised the possibility that removal of the relatively insoluble products from the enzymic site may be a rate-limiting process. If that were the case, the enzymes could be markedly inhibited by extremely small amounts of newly formed saturated products. Such an inhibition might then hinder entrance of the normal amounts of polyunsaturated acids into the phosphoglycerides. This hypothesis differs from the previous one by suggesting that undetectable amounts of saturated lipid would hinder polyunsaturated lipid synthesis in contrast to larger amounts providing a physical environment that allows the polyunsaturated acids to serve as preferred substrates. The results (Table VII) do not support this hypothesis. Thus, the low rates of synthesis do not seem due to a limited removal of the saturated product from the active site, but rather to an intrinsic specificity of the acyltransferase system.

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