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SYNTHESIS OF LIPIDS BY LIVER PLASMA MEMBRANES

INCORPORATION OF ACYL-COENZYME A DERIVATIVES INTO MEMBRANE LIPIDS *IN VITRO*

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SUMMARY

1. Plasma membranes were prepared from rat liver homogenates. The membrane preparation was characterized by its composition and the presence of particular enzymes which have been associated with membrane function. Lipid composition of the membrane was studied in detail and was compared with that reported for other membranes.

2. Palmityl-, stearyl-, oleyl- and linoleyl-CoA esters were prepared enzymatically from radiocarbon labeled acyl precursors. These substrates were incubated with the membranes and the incorporation of isotope into various lipids was investigated.

3. Under these conditions, small amounts of neutral glycerides were formed. In this process, unsaturated acyl-CoA derivatives were better utilized precursors than the saturated analogs. Fatty acids were principally incorporated into phosphatidic acid, phosphatidylethanolamine and phosphatidylcholine, presumably through a reacylation of their lyso derivatives.

4. The major portion of the fatty acids from [^{14}C]acyl-CoA esters was recovered as free fatty acid and in the form of *S*-acyl pantetheine.

INTRODUCTION

In a recent study of the utilization of free fatty acids for lipid synthesis in the liver *in vivo*, we observed that the phospholipid fraction of the hepatic plasma membrane had a higher specific activity than phospholipids isolated from other subcellular particles*.

So far, very little is known about lipid metabolism in isolated plasma membranes and the participation of the membrane in metabolic reactions has been inferred mostly from studies on isolated cells. However, several papers have appeared on the protein, enzymic and lipid content of isolated membranes[1–8]. The studies presented here and in the accompanying communication were undertaken in an effort to enlarge our knowledge of the composition, structure and function of plasma membranes.

* E. A. BROWN, W. L. STAHL and E. G. TRAMS, manuscript in preparation.

MATERIALS AND METHODS

Source of materials

[1-^{14}C]Palmitic, -oleic and -stearic acids: New England Nuclear; [1-^{14}C]linoleic acid: Applied Science Labs., State College, Pa.; sphingomyelin and phosphatidic acid: Pierce Chemical, Rockford, Ill. Other lipids: Applied Science Labs.; 2,6-dichlorophenolindophenol: Sigma Chemical; CoA: Pabst Laboratories; silica gel F_{254}, pre-coated thin-layer chromatographic plates, 250 μ (Merck, Darmstadt, Germany): Brinkmann Instruments; silica gel pre-coated thin-layer chromatographic plates, 250 μ (Uniplate): Analtech, Wilmington, Del.

Liver cell membranes

Male Sprague–Dawley rats weighing from 120–180 g were decapitated and the livers were removed, dissected free of vascular and adjacent tissue, and chilled in ice. The liver cell membranes were isolated from 40 g (or multiples thereof) of minced liver according to the method of NEVILLE[2].

Preparation of substrates

Rat liver microsomes were prepared by the method of SCHNEIDER AND HOGEBOOM[9]. The microsomal pellet derived from 20–21 g of liver was resuspended in 60 ml of cold, glass-distilled water and was centrifuged at 78000 $\times$ g (1 h). The washed microsomes were resuspended in distilled water and lyophilized to yield an average 900 mg of dry powder. This could be stored for several months at —20° without loss of fatty acid activating enzyme.

Long chain acyl-CoA derivates were prepared according to KORNBERG AND PRICER[10]. The incubation mixture contained: 2.5 mmoles Na_2ATP (pH 7.4 with 1 M NaOH), 5.0 mmoles cysteine·HCl (adjusted to pH 7.4 with 1 M NaOH), 0.1 mmole CoA (pH 7.4 with 1 M KH_2PO_4), 5.0 mmoles NaF, 5.0 mmoles $MgCl_2$, 5.0 mmoles KH_2PO_4 (pH 7.4 with 1 M NaOH), 350 mg microsomal powder and 0.1 mmole of [1-^{14}C]fatty acid in a final volume of 200 ml. The fatty acid was sonicated in 80 ml H_2O for 2–3 min utilizing a Branson Sonifier tuned for optimum sonication.

The final opalescent acyl-CoA was stored at —20°. Conversion of [^{14}C] fatty acid to [^{14}C]acyl-CoA, based on radioactivity, was usually 40–75 %. Lipid ester was estimated by the method of KORNBERG AND PRICER[10], and adenine by absorption at 260 mμ. The isolated acyl-CoA compounds had ester/adenine ratios of unity, and were chromatographed on Merck silica gel thin-layer chromatographic plates using chloroform–methanol–1 M sodium acetate (pH 3.55) (70:30:5, by vol.). They were found to be 95–99 % pure as judged by radioactivity scans.

Extraction and estimation of lipids

A suspension of membranes was extracted with 20 vol. of chloroform–methanol (2:1, by vol.) by the technique of FOLCH, LEES AND SLOANE-STANLEY[11]. After evaporation to dryness the lipid extract was dissolved in chloroform–methanol (2:1, by vol.) and was washed with 0.2 vol. of water. The upper phase washes were combined and dialyzed against cold distilled water and subsequently lyophilized. The gangliosides were estimated by the method of WARREN[12] using *N*-acetylneuraminic acid as standard. The lower phase lipids were initially separated on a silicic acid column as described in Fig. 1. Fraction A (neutral lipids) was further separated using thin-layer chromatographic System D whose composition is indicated in the next paragraph. Phospholipids were separated by thin-layer chromatographic System C. The lipids

were extracted from thin-layer chromatographic spots by extracting the appropriate areas of silica gel on a small sintered glass funnel with 1 ml $CHCl_3$, 2–5 ml $CHCl_3$–methanol (2:1, by vol.) and methanol containing 5 % concentrated NH_4OH to a final effluent volume of 10 ml. After evaporation under N_2, samples were analyzed for total lipid and phospholipid phosphorus by the methods of BRAGDON[13] and BARTLETT[14], respectively. Recovery of neutral lipids from thin-layer chromatographic plates averaged 95.7 % and recovery of phospholipids was 90 %.

Thin-layer-chromatographic systems

System A. Ethyl ether–glacial acetic acid (100:1, by vol.) (Merck silica gel pre-coated plates).

System B. Light petroleum (b.p. 30–60°)–ethyl ether (1:1, by vol.) (Merck silica gel pre-coated plates).

System C. Chloroform–methanol–1 M sodium acetate solution (pH 3.55) (50:50:4, by vol.) (Merck silica gel pre-coated plates).

System D. Thin-layer plates (20 cm × 34 cm) were prepared and lipids separated by the method of FREEMAN AND WEST[15] using diethyl ether–benzene–ethanol–acetic acid (40:50:2:0.2, by vol.) followed by diethyl ether–hexane (6:94, by vol.). These systems in combination give good separation of the lipid classes.

System E. Chloroform–methanol (9:1, by vol.) (Analtech. "Uniplate").

Plates were activated prior to application for 15 min at 105°. Lipids were visualized by one of the following methods: (1) exposure to iodine vapors; (2) spraying with rhodamine G; spraying with the reagent of DITTMER AND LESTER[16] for phosphates; spraying with orcinol reagent for glycolipids[17] and for hydroxamic acids by spraying with a $FeCl_3$ reagent[18]. Radioactivity was located with a Vanguard Model 880 scanner on strips 50 mm wide.

Amino acid analysis

Delipidated membranes were hydrolyzed in 6 M HCl for 24 h at 105° in an evacuated sealed tube. Analysis was performed as previously described[19] on a Phoenix amino acid analyzer. The data reported in Table III represent the average of two determinations.

Protein

This was determined by the method of LOWRY *et al.*[20] using human plasma albumin, Fraction V as standard.

Enzymic assays

Succinate dehydrogenase was measured by the method of DAVENPORT[21], which had been modified to use a 1.0-ml volume at 25° in a Cary Model 14 recording spectrophotometer. Glucose 6-phosphatase was assayed in 40 mM sodium glucose 6-phosphate, 7 mM histidine, 1 mM potassium EDTA (pH 6.5 (KOH)) in a final volume of 50 μl. After incubation at 37° for 10 min, the reaction was terminated with 25 μl of 15 % trichloroacetic acid and the phosphate liberated was measured using the procedure of LOWRY AND LOPEZ[22]. Phosphatidic acid phosphatase was assayed in a medium containing 3 mM sodium phosphatidate, 60 mM maleic acid (pH 6.5 (KOH)) in a final volume of 50 μl. The stock solution containing phosphatidic acid and maleic acid was initially sonicated for 2–3 min to disperse the substrate. Incubation was for 30 min at 37° and the reaction was stopped by the addition of 25 μl of 15 % trichloroacetic acid. Liberated phosphate was measured as indicated above. Cholinesterase was measured using acetylthiocholine iodide as substrate by a slight modifi-

cation of the method of ELLMAN *et al.*[23] which is described elsewhere[24]. Lipase was assayed using glyceryl tri[1-^{14}C]oleate as substrate. The reaction mixture consisted of 12 mμmoles of the triolein (370000 disint./min), 0.5 % sodium deoxycholate, 0.1 mM $CaCl_2$, 150 mM Tris–HCl (pH 7.4) and approx. 400 μg of membrane protein in a final volume of 200 μl. This was incubated at 37° for 30 min and the reaction was terminated with 50 μl of 2 M HCl. The mixture was extracted two times with isooctane and the combined extract was dried and chromatographed using thin-layer chromatographic System B. Free fatty acid and triglyceride were recovered and counted. The method was validated using crude pancreatic lipase (Sigma).

General conditions for incubation and analysis of membranes

Membranes (usually 150–180 μg of protein), [1-^{14}C] acyl-CoA or [1-^{14}C]palmitic acid, as indicated, and Tris–HCl (pH 7.4) (70–140 mM) in a final volume of 210–220 μl were incubated at 37° for 0.5 h in a 12-ml gradient conical centrifuge tube. When indicated, membranes were boiled for 5 min in buffer and before addition of acyl-CoA. After incubation the reaction was stopped by addition of 2 ml of chloroform–methanol (2:1, by vol.) and the mixture was stored at −20°. The mixture was washed with 1 ml of water and the upper phase was aspirated and discarded. Methanol was added to a volume of 2.0 ml. A 1.0-ml aliquot was taken to dryness under a stream of N_2 at 30° for thin-layer chromatography. The lipid extract in 100–200 μl chloroform–methanol (2:1, by vol.) was applied to the origin (O) of a 20 cm × 20 cm pre-coated Merck silica gel thin-layer chromatographic plate (as in Fig. 2) and was chromatographed with Systems A, B and C as indicated. Radioactivity was initially localized by scanning. The lipid-containing areas were transferred into counting vials; 1 ml of Hyamine 10-X and 10 ml of naphthalene–dioxane scintillation fluid[25] were added and the samples were counted in a Packard Tri-Carb scintillation spectrometer, Model 3003, equipped with automatic external standardization. Conversion of counts/min to disint./min was made by referring to a standard quench curve constructed for the solvent system with standard [^{14}C]benzoic acid (National Bureau of Standards).

RESULTS AND DISCUSSION

Composition of liver plasma membranes

Our data on the chemical and enzymic composition of the isolated liver plasma membranes are listed in Tables I–III. In our opinion, the current method for preparing rat liver plasma membranes[2] is an improvement of those previously used, since there was an improvement in yield, consistency and time of preparation. The significance of some of the differences in composition from those reported by others will have to be established. Approx. 0.5 mg of membrane protein was derived from 1 g of liver, a yield of about 0.2 % of the original protein, and very similar to the yield obtained by EMMELOT *et al.*[4]. The ratio of lipid protein (0.39:1) was rather low compared to that of SKIPSKI *et al.*[8] (0.7:1) and DOD AND GRAY[39] (0.6:1); however, the phospholipid content compares with that found by EMMELOT *et al.*[4] (0.39 μmole membrane P/mg membrane protein). The carbohydrate content, of about 28 %, appeared high, and could have been due to residual sucrose. The hexosamine and sialic acid contents appeared rather lower than those of EMMELOT *et al.*[4].

The results reported here for the lipid composition of the membranes are in general agreement with those of DOD AND GRAY[39] and SKIPSKI *et al.*[8]; although the

TABLE I

COMPOSITION OF LIVER PLASMA MEMBRANES

	*Mean ± S.E.***
Yield of protein* (mg/g wet wt. liver)	0.48 ± 0.13 (10)
Yield of dry delipidated membrane (mg/g wet wt. liver)	0.66 ± 0.13 (3)
Density (g/ml)	1.18
Lipid content (mg/mg membrane protein)*	0.39 ± 0.04 (3)
Phospholipid content (μmoles phospholipid phosphorus/mg membrane protein)*	0.33 ± 0.06 (2)
Hexose (μmoles/mg membrane protein)	0.55 ± 0.06 (4)
Hexosamine (μmoles/mg membrane protein)	0.030 ± 0.005 (2)
N-Acetylneuraminic acid content (μmoles/mg dried delipidated membrane)	0.01 (2)
Succinate dehydrogenase (mμmoles dichlorophenolindophenol reduced/mg protein per min)	4.6 (3)
Glucose 6-phosphatase (μmoles P_i formed/mg protein per h)	0.58 (2)
Cholinesterase (μmoles acetylthiocholine hydrolyzed/mg protein per h)	1.95 ± 0.25 (2)
Lipase (mμmoles fatty acid liberated/mg protein per h)	Nihil
Phosphatidic acid phosphatase (μmoles P_i formed/mg protein per h)	0.28 (1)

* Determined on membrane fraction before lipid extraction.

** The number in parenthesis represents the number of preparations analyzed, each in duplicate or triplicate.

TABLE II

LIPID COMPOSITION OF RAT LIVER CELL MEMBRANES

Lipids were extracted from a suspension of liver cell membranes (19 and 28 mg protein), and after partitioning with 0.2 vol. water to remove gangliosides; the lower phase lipids were initially separated on a silicic acid column (Fig. 1). Individual lipids were separated by thin-layer chromatography using Systems C and D. In one case phospholipids were separated by two-dimensional chromatography on Whatman SG-81 silica-gel-loaded paper as described by WUTHIER[38]: chloroform–methanol–diisobutyl ketone–acetic acid–water (23:10:45:25:4, by vol.) in the first dimension and chloroform–methanol–diisobutyl ketone–pyridine–0.5 M NH_4Cl (pH 10.4) (30:17.5:25:35:6, by vol.) in the second dimension. Neutral lipids were quantitated by the BRAGDON method[13] and phospholipids by phosphorus analysis[14]. The results are an average from two membrane preparations, chromatographed in duplicate.

Lipid	*% of total lipid extracted*
Free fatty acids	9.0
Monoglycerides	3.3
Diglycerides and triglycerides	8.8
Cholesterol	13.4
Cholesterol esters	0
Phosphatidylcholine* (*plus* lysophosphatidylethanolamine)**	25.8
Phosphatidylethanolamine** (*plus* phosphatidylglycerol)	17.8
Phosphatidylinositol	1.3
Phosphatidylserine	3.9
Sphingomyelin*	8.8
Polyglycerophosphatides	3.5
Uncharacterized cerebrosides and glycolipids	7.2
Gangliosides	0.1
Total recovery	102.9

* Total choline content, 0.68 μmole/mg lipid.

** Total ethanolamine content, 0.46 μmole/mg lipid.

latter authors were unable to account for 26 % of the total membrane lipids. The data reported in Table II, and that of DOD AND GRAY[39] and SKIPSKI *et al.*[8] all indicate that 34–35 % of the lipids of the plasma membrane are 'neutral lipids' *i.e.* cholesterol, cholesterol esters, free fatty acids, and glycerides, of which cholesterol comprises the major component (39–50 %). Table II shows that 61 % of the total lipid was recovered as phospholipid; DOD AND GRAY[39] found phospholipids comprised 59 % of the lipids of the membrane. Within the class of phospholipids the data for phosphatidylcholine and phosphatidylserine given in Table II agree well with that of DOD AND GRAY, but the percentage of phosphatidylethanolamine reported here is threefold higher, and the sphingomyelin value half that reported by DOD AND GRAY. These differences may have been due to differences in technique used for membrane isolation, strain of animal or diet, or in the chromatographic methods used. Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin comprised 86 % of the total phospholipid. This compares with rat red cell ghosts where the choline phosphatides and ethanolamine phosphatides comprise 99 % of the phospholipid fraction[26]; the bulk

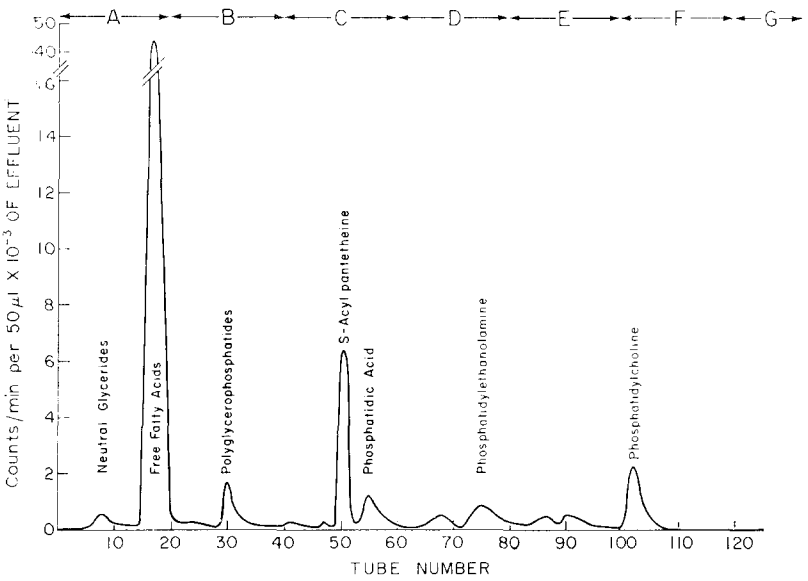

Fig. 1. Column chromatography of lipids from liver membranes. Membranes (27 mg) which had been frozen for 14 days at −20° were incubated with 0.42 µmole of [1-^{14}C]palmityl-CoA ($1.98 \cdot 10^7$ disint./min) and 300 µmoles of Tris–HCl (pH 7.4) in a final volume of 10.0 ml for 0.5 h at 37°. The reaction was halted by addition of 100 ml of chloroform–methanol (2:1, by vol.) and was stored for several hours at −20°. The mixture was washed with 1 vol. of water, and after brief centrifugation, the upper phase was aspirated off. Methanol was added to clarify the mixture which was then filtered through a sintered glass funnel and the filtrate was taken to dryness. The lipid extract was chromatographed on a 1 cm × 10 cm column of activated (30 min at 105°) Unisil (100–200 mesh). Fractions (5 ml) were collected during sequential elution by 50-ml portions of the following solvent systems: chloroform (Fraction A); chloroform–methanol (99:1, by vol.) (Fraction B); chloroform–methanol (96:4, by vol.) (Fraction C); chloroform–methanol (7:1, by vol.) (Fraction D); chloroform–methanol (2:1, by vol.) (Fraction E); chloroform–methanol (1:2, by vol.) (Fraction F); methanol (Fraction G). Radioactivity in the fractions was measured by liquid scintillation spectrometry. The major components of the column fractions were identified by thin-layer chromatography by comparison to authentic standards; radioactivity was localized using a Vanguard autochromatographic scanner and the major radioactive lipids were identified above the peaks.

of the neutral lipid in red cell ghosts is cholesterol. Likewise, in rat liver mitochondria and microsomes, the choline and ethanolamine phosphatides account for 80–90 % of the total phospholipid (*cf.* ref. 27).

The lipid content of these membranes contrasts sharply with the recently studied renal glomerular basement membranes which has a total lipid content of less than 1 % of its dry weight[28]. It was also observed that the ganglioside content of the plasma membrane fraction was very low while about 7 % of the lipid consisted of cerebrosides and glycolipids. These latter components appeared in Fraction C (Fig. 1) from the silicic acid column, and after thin-layer chromatography in System E, at least 7–10 discrete spots were observed with iodine vapor, anisaldehyde spray[29] and benzidine spray (for glycolipids)[30]. The identity of the neutral glycerides, free fatty acids and phospholipids was established by thin-layer chromatography with authentic reference compounds in several solvent systems, including the system of MARINETTI[31] with silicic acid impregnated paper. In certain cases phospholipids were isolated and P/ester ratios were determined. The presence of cardiolipin, which was not found by others in plasma membranes, may have been due to mitochondrial elements. Low but measurable succinate dehydrogenase activity may also be indicative of contamination with mitochondrial membranes, although no mitochondria were visible in the preparation either under phase contrast or in electron micrographs.

The amino acid analysis shown in Table III does not differ significantly from that of published values for most purified proteins, *e.g.* egg albumin[32] or from the glycoprotein of glomerular basement membrane[28], except that the latter has a significantly higher alanine content. However, the composition is unlike mitochondrial structural protein which appears to have a relatively higher content of amino acids with nonpolar side chains[33]. It is important to remember that the liver and other membrane preparations contain many proteins. NEVILLE[1] has shown that the alkaline-

TABLE III

AMINO ACID COMPOSITION OF LIVER PLASMA MEMBRANES

Amino acid	*Amino acid / Glutamic acid*
Cysteic acid	0.01
Asp	0.78
Thr	0.39
Ser	0.31
Pro	0.44
Glu*	1.00
Gly	0.59
Ala	0.69
Val	0.54
Cys	0.07
Met	0.19
Ile	0.39
Leu	0.88
Tyr	0.25
Phe	0.38
Orn	0.003
Lys	0.65
His	0.21
Arg	0.38

* 2.36 μmoles glutamic acid were derived from 2 mg, dry weight of delipidated membranes.

soluble fraction from liver membranes, containing 70 % of the total membrane protein, has at least 25 protein components and SCHNEIDERMAN[34] has demonstrated the heterogeneity of red cell stroma protein. The specific activity of glucose 6-phosphatase in the membrane preparation was seven times less than liver microsomal preparations (*cf.* Table II of accompanying communication) indicating a very low content of microsomal elements. Electron micrographs of the preparation indicated only very few fragments of endoplasmic reticulum. EMMELOT *et al.*[1] have reported that liver membranes, prepared by a slight modification of NEVILLE's original procedure, had glucose-6-phosphatase specific activities five times less than liver microsomes and were devoid of measurable succinate–cytochrome *c* reductase. The preparation under study here exhibited phosphatidic acid phosphatase activity which was nearly identical to both the original liver homogenate as well as to values reported by HOKIN AND HOKIN[35] for avian salt gland microsomes (assayed in the presence of detergent). The liver membranes had cholinesterase activity but were devoid of lipase using triolein as the substrate.

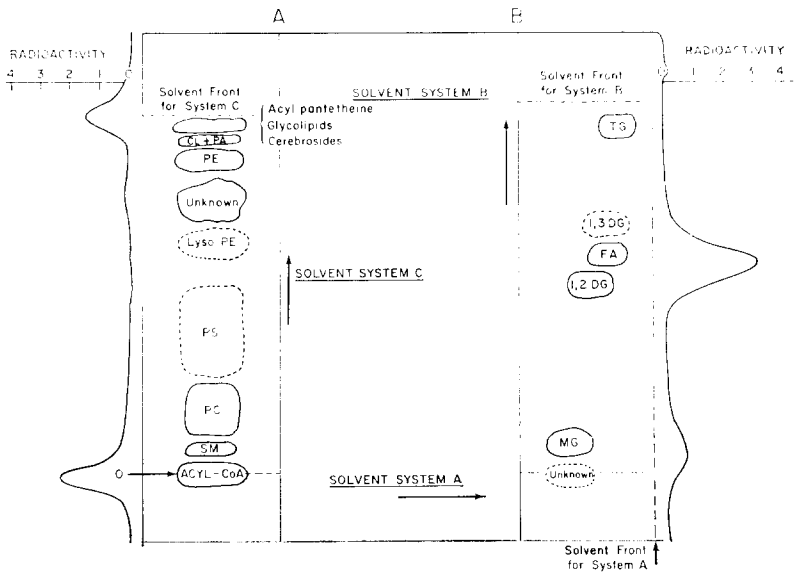

Fig. 2. Separation of membrane lipids by thin-layer chromatography. Liver plasma membranes (180 μg protein) were incubated with Tris–HCl (pH 7.4, 140 mM) and [1-^{14}C]palmityl-CoA (0.31 mM, $3.59 \cdot 10^6$ disint./min) in a final volume of 220 μl for 30 min at 37°. The reaction was halted with 2 ml of chloroform–methanol (2:1, by vol.) and the mixture was washed with 1 ml of water; the upper phase was aspirated and discarded. Methanol was added to clarify the washed extract and one half was taken to dryness under a stream of N_2 for chromatography. This lipid extract in 200 μl of chloroform–methanol (2:1, by vol.) was applied to the origin (O) of a 20 cm × 20 cm precoated Merck silica gel thin-layer chromatographic plate and was chromatographed with solvent System A to within a few mm of the top of the plate. A thin-layer plate was then scored and cracked along Lines A and B and the two resulting end plates (5 cm × 20 cm) were chromatographed as indicated in Systems B or C. Lipids were localized and radioactivity traces made as indicated in the text. The radioactivity scale is relative and the trace on the left was made at three times the sensitivity of that on the right. Lipids were recovered and radioactivity was as follows: Acyl-CoA, $3.25 \cdot 10^5$ disint./min; acyl pantetheine, $1.69 \cdot 10^5$ disint./min; MG, $3.73 \cdot 10^4$ disint./min; DG, $1.07 \cdot 10^4$ disint./min; TG, $1.8 \cdot 10^4$ disint./min; FA, $1.84 \cdot 10^6$ disint./min. Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; TG, triglyceride; DG, diglyceride; MG, monoglyceride; FA, fatty acid.

Incorporation of fatty acids into the plasma membrane neutral lipid fraction

Preliminary experiments indicated that free fatty acids were poorly incorporated into plasma membrane lipids, but were very effectively utilized as the CoA derivatives. With palmityl-CoA as substrate, the most prominent products were phospholipids, free fatty acid and *S*-palmityl pantetheine (Fig. 1).

For routine analysis after incubation of membranes utilizing [^{14}C]acyl-CoA as substrate, the protocol described in Fig. 2 was used. This procedure has the advantage that a reasonably large number of samples can be run daily, and these commercially available plates give excellent, consistent separation of the major lipid classes. In certain instances where rather large amounts of labelled fatty acid were present, it was desirable to utilize thin-layer chromatographic System D for separation of neutral lipids. Recovery of radioactivity applied to thin-layer chromatographic plates and separated by these thin-layer chromatographic systems was 95 % using the Hyamine scintillation fluid combination described in the text. Recovery of radioactivity using the scintillation fluid alone was inadequate.

Incorporation into neutral glycerides, as shown in Table IV, was in general of a low order of magnitude over a fairly wide range of substrate concentrations. With

TABLE IV

INCORPORATION OF FATTY ACIDS FROM [1-^{14}C]ACYL-CoA INTO NEUTRAL GLYCERIDES OF LIVER PLASMA MEMBRANES

Liver plasma membranes (180 μg protein) were incubated with Tris–HCl (pH 7.4) (140 mM) and [1-^{14}C]acyl-CoA as indicated, in a volume of 220 μl for 0.5 h at 37°. The specific activities of the acyl-CoA compounds were as follows: [1-^{14}C]palmityl-CoA, $5.29 \cdot 10^7$ disint./min per μmole; [1-^{14}C]stearyl-CoA, $4.34 \cdot 10^7$ disint./min per μmole; [1-^{14}C]oleyl-CoA, $1.02 \cdot 10^7$ disint./min per μmole; [1-^{14}C]linoleyl-CoA, $7.53 \cdot 10^6$ disint./min per μmole. The lipids were chromatographed and radioactivity determined as described in Fig. 2 and in the text.

Substrate	*Concn. acyl-CoA (mM)*	*Fatty acid incorporated (mμmoles per mg protein)*		
		Monoglyceride	*Diglyceride*	*Triglyceride*
[1-^{14}C]Palmityl-CoA	0.015	0.26	0.05	0.17
	0.031	0.44	0.09	0.34
	0.077	1.29	0.42	0.48
	0.155	1.85	0.58	1.47
	0.232	2.54	0.85	1.59
	0.310	3.92	1.12	1.80
[1-^{14}C]Stearyl-CoA	0.01	0.039	0.029	0.022
	0.02	0.090	0.048	0.043
	0.05	0.209	0.109	0.092
	0.10	0.363	0.185	0.082
	0.15	0.761	0.303	0.103
	0.20	1.036	0.375	0.205
[1-^{14}C]Oleyl-CoA	0.073	1.85	0.13	0.28
	0.147	4.61	0.36	0.64
	0.367	10.45	—	1.84
	0.734	21.39	0.69	2.49
	1.10	36.76	0.51	4.52
[1-^{14}C]Linoleyl-CoA	0.073	1.25	0.093	0.484
	0.147	2.50	0.224	0.336
	0.367	6.92	0.235	1.07
	0.734	15.10	0.621	1.97
	1.10	22.07	0.721	3.29

saturated acyl-CoA as substrate, conversion ranged from 2.9 to 1.2 % with increasing substrate concentration; with oleyl- and linoleyl-CoA, as much as 2.7–3.4 % of the substrate was converted to total neutral glyceride.

The formation of free fatty acid from acyl-CoA is due primarily to deacylation of the coenzyme derivative and of the acyl pantetheine. With saturated acyl-CoA compounds, increasing substrate concentration produces an apparent increase in velocity of the deacylase reaction (Fig. 3) whereas with the unsaturated acyl-CoA compounds, this activity presumably plateaus with an increase in acyl-CoA concentration. This possibly indicates two modes for the formation of free fatty acid. In the former case this may simply represent a deacylation of the saturated acyl-CoA, whereas in the latter case a more complex intermediate transacylation may be involved.

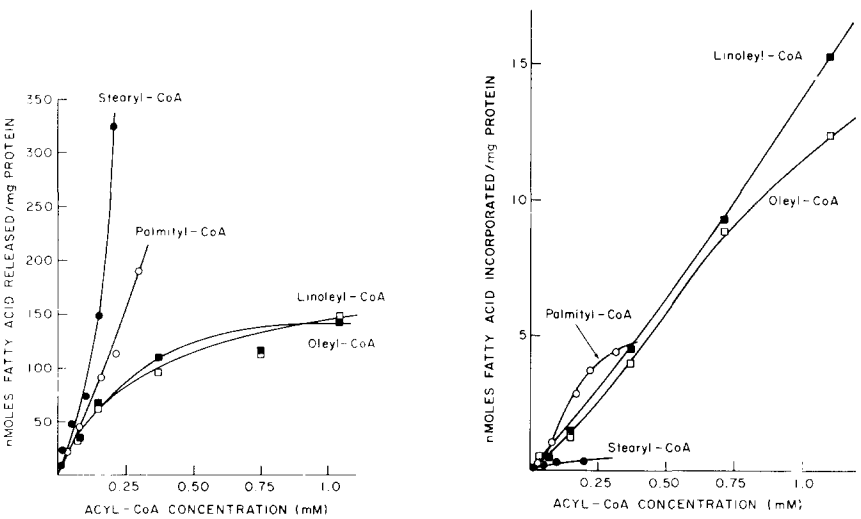

Fig. 3. Formation of free fatty acids on incubation of liver plasma membranes with [1-^{14}C]acyl-CoA. Incubation conditions and chromatographic procedures were as in Table IV.

Fig. 4. Incorporation of fatty acids from [1-^{14}C]acyl-CoA into phosphatidylcholine of liver plasma membranes. Incubation conditions are described in Table IV and chromatographic procedures as in Fig. 2.

Incorporation of fatty acids from acyl-CoA into phosphatidylcholine and phosphatidylethanolamine

The formation of two of the major phospholipids has been studied in a similar manner to the neutral glycerides. For synthesis of phosphatidylcholine, palmityl-CoA was a good substrate, even though the total incorporation with unsaturated acyl-CoA was greater (Fig. 4). This may have been due to formation of lysophosphatidylcholine. We would like to suggest that lysophosphatidylcholine is the substrate for the acylation reaction to form phosphatidylcholine, especially in view of the finding that phosphatidylcholine synthesis was greatest at pH 5.6. Such a mechanism has been demonstrated by SCHERPHOF AND VAN DEENEN[36]. Moreover, RESHEF AND SHAPIRO[37] have shown that at pH 5, incubation of liver subcellular particles induced phospholipase activity, thereby providing endogenous lysolecithin as an acceptor leading to

phosphatidylcholine synthesis. The data shown in Table V further substantiate this. Phosphatidylcholine synthesis was highest when no other cofactors were present in the system. [^{14}C]Palmitic acid alone was poorly utilized in phosphatidylcholine synthesis even in the presence of ATP, Mg^{2+} and CoA. Both NaF and deoxycholate strongly inhibited phosphatidylcholine synthesis.

TABLE V

INCORPORATION OF FATTY ACID FROM [1-^{14}C]PALMITYL-CoA AND [1-^{14}C]PALMITIC ACID INTO PHOSPHATIDYLCHOLINE OF LIVER PLASMA MEMBRANES

In Expt. 1 the control incubation mixture contained 70 mM Tris–HCl (pH 7.4), 0.34 mM [1-^{14}C]-palmityl-CoA ($4.4 \cdot 10^5$ disint./min), 174 μg of membrane protein in a final volume of 210 μl. When indicated the following were present: 2 mM CDP-choline, 30 mM α,β-diglyceride (diolein), 50 μM α-glycerophosphate, 15 mM NaF, or 0.5% sodium deoxycholate. In Expts. 2 and 3 the incubation mixtures contained 130 mM Tris–HCl (pH 7.4), 214 μg membrane protein and either 0.46 mM [1-^{14}C]palmityl-CoA ($4.94 \cdot 10^6$ disint./min) or 0.22 mM [1-^{14}C]palmitic acid ($1.27 \cdot 10^6$ disint./min) as indicated, in a final volume of 225 μl. When indicated 1 mM $MgCl_2$, 1 mM CoA, or 1 mM Na_2ATP were added. All incubations were carried out for 0.5 h at 37° and were halted and analyzed as described in Fig. 2 and in the text.

System	*Phosphatidylcholine (mμmoles fatty acid incorporated per mg protein)*
Expt. 1	
Control ([^{14}C]palmityl-CoA)	19.55
Membranes boiled	2.79
Control + CDP-choline	7.67
Control + CDP-choline + α,β-diglyceride + α-glycerophosphate	1.71
Control + NaF	0.71
Control + deoxycholate	0.05
Expt. 2	
Control ([^{14}C]palmityl-CoA)	7.01
Membranes boiled	0.54
Expt. 3	
Control ([^{14}C]palmitic acid)	0.12
Membranes boiled	0.11
Control + $MgCl_2$ + CoA + ATP	0.17
Control + $MgCl_2$	0.09
Control + CoA	0.09
Control + ATP	0.23
Control + CoA + ATP	0.39

In a series of experiments which will not be described in detail it was found that incorporation of ^{32}P from [^{32}P]ATP into phosphatidylcholine could not be demonstrated. Similarly [^{3}H]choline and α,β-[^{14}C]diglyceride were not incorporated into phosphatidylcholine. Using L-α-[^{14}C]glycerophosphate as a precursor, very small amounts of radioactive carbon were utilized for phosphatidylcholine synthesis, but this reaction was not studied in detail.

Synthesis of phosphatidylethanolamine was studied under conditions identical to those used in the study of phosphatidylcholine synthesis. The pattern and order of magnitude of phosphatidylethanolamine formation from acyl-CoA derivatives were similar to that of phosphatidylcholine except that fatty acid from palmityl-CoA was

not as well utilized (Fig. 5). In some experiments there was an apparent formation of a phospholipid which has been tentatively identified as lysophosphatidylethanolamine, but details of this reaction have not been worked out.

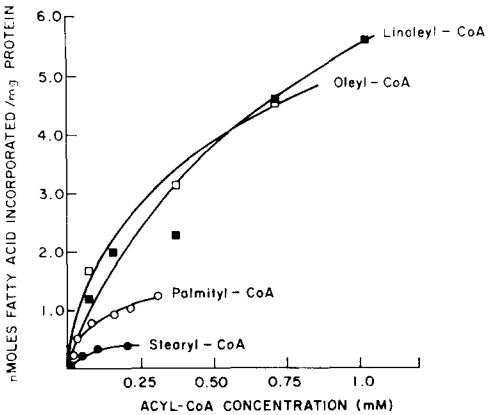

Fig. 5. Incorporation of fatty acids from [1-^{14}C]acyl-CoA into phosphatidylethanolamine of liver plasma membranes. Incubation conditions are described in Table IV and chromatographic procedures as in Fig. 2.

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