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Biosynthesis and Composition of Phosphatides in Outer and Inner Mitochondrial Membranes*

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Summary: A comparative study of five enzymes involved in phospholipid biosynthesis was carried out with outer and inner mitochondrial membranes, whole mitochondria, and the microsomal and the cytoplasmic fractions. In general, the highest activities of phospholipid synthesizing enzymes are found in the microsomal fraction. Phosphatidic acid phosphohydrolase activity resides predominantly in the lysosomal fraction. The five enzymes responsible for the synthesis of complex lipids are also present in the mitochondria; they are localized exclusively in the outer mitochondrial membrane. These lipid synthesizing properties of the outer mitochondrial membrane point to the close relationship between the outer mitochondrial membrane and the endoplasmic reticulum.

The composition of the phospholipids of whole mitochondria, of its outer and inner membranes and matrix, and of the microsomal fraction was determined quantitatively. The data are presented. Diphosphatidylglycerol (cardiolipin) is only present in the inner mitochondrial membrane. The fatty acid residues of phosphatidylcholine and -ethanolamine of the cell and submitochondrial fractions are rather similar.

Abbreviations:

lysophosphatidic acid = 1-acyl-3-glycerophosphate;
phosphatidic acid = 1,2-diacyl-3-glycerophosphate;
1,2-diglyceride = 1,2-diacyl-glycerol;
lysolecithin = 1-acyl-3-glycerophosphorylcholine.

Enzymes:

Acyl-CoA dehydrogenase, Acyl-CoA:(acceptor) oxidoreductase (EC 1.3.99.3)
Cholinephosphotransferase, CDPcholine:1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2)
Enoyl-CoA hydratase, L-3-Hydroxyacyl-CoA hydro-lyase (= crotonase) (EC 4.2.1.17)
Glucose oxidase, β-D-Glucose:oxygen oxidoreductase (EC 1.1.3.4)
Glucose-6-phosphatase, D-Glucose-6-phosphate phosphohydrolase (EC 3.1.3.9)
Glycerolphosphate acyltransferase, Acyl-CoA:L-glycerol-3-phosphate *O*-acyltransferase (EC 2.3.1.15)
Lysolecithin acyltransferase, Acyl-CoA:lysolecithin *O*-acyltransferase (EC 2.3.1.?)
Lysophosphatidate acyltransferase, Acyl-CoA:lysophosphatidic acid *O*-acyltransferase (EC 2.3.1.?)
Monoamine oxidase, Monoamine:oxygen oxidoreductase (deaminating) (EC 1.4.3.4)
Phosphatidate phosphatase, L-α-Phosphatidate phosphohydrolase (EC 3.1.3.4)
Phospholipase A, Phosphatide acyl-hydrolase (EC 3.1.1.4)
Phospholipase C, Phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3)
Phospholipase D, Phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4)
Succinate dehydrogenase, succinate (acceptor) oxidoreductase (EC 1.3.99.1)

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Zusammenfassung: *Biosynthese und Zusammensetzung der Phosphatide in der äußeren und inneren Mitochondrienmembran.* Die subzelluläre und submitochondriale Aktivitätsverteilung von fünf Enzymen der Lecithinbiosynthese wurde untersucht. Die Mikrosomenfraktion besitzt die höchste Aktivität an Acyl-CoA:3-Glycerophosphat-*O*-Acyltransferase, CDP-Cholin:1.2-Diglycerid-Cholinphosphotransferase und Acyl-CoA:Lysolecithin-*O*-Acyltransferase. Die Hauptaktivität der Phosphatidsäure-Phosphohydrolase ist in den Lysosomen lokalisiert. Alle Enzyme für die Biosynthese des Lecithins sind in den Mitochondrien nachweisbar und dort fast ausschließlich in der äußeren Mitochondrienmembran lokalisiert. Dieser Befund unterstreicht die enge Beziehung zwischen äußerer Mitochondrienmembran und endoplasmatischem Reticulum. Die Zusammensetzung der Phosphatide der Gesamtmitochondrien, der äußeren und inneren Mitochondrienmembranen, der Matrixfraktion und der Mikrosomen wurde untersucht. Die Analysendaten sind angegeben. Cardiolipin ist fast ausschließlich in der inneren Mitochondrienmembran lokalisiert. Die Analyse der Fettsäurebesetzung des Phosphatidyläthanolamins und -cholins aus Gesamtmitochondrien, inneren und äußeren Mitochondrienmembranen, Matrixfraktion und Mikrosomen zeigt keine signifikanten Unterschiede zwischen den einzelnen Membranen und Kompartementen der Zelle und der Mitochondrien.

The ultrastructure of the mitochondria has been extensively studied by PALADE[1] and SJÖSTRAND[2] and recently by the technique of negative staining by FERNÁNDEZ-MORÁN[3]. Their studies revealed a structure composed of an outer membrane and an inner membrane with continuous invaginations, the cristae mitochondriales, and the intercristal space, the mitochondrial matrix. The subfractionation of mitochondria and the isolation of reasonably pure inner and outer mitochondrial membranes have been performed in various laboratories[4–8].

Whole mitochondria contain about 50—60 enzymes which catalyze the tricarboxylic acid cycle, fatty acid oxidation, electron transport and oxidative phosphorylation. Furthermore it has been claimed that besides the enzyme proteins almost 40% of the total mitochondrial protein is represented by the "structural" protein[9]. Varying amounts of lipids have been reported to be present in whole mitochondria: 27% of dry weight of beef heart mitochondria[10], 29% of rat liver mitochondria[11] and 50% of brain mitochondria[12]. The mitochondrial phospholipids which comprise almost 90% of the total lipids are rich in diphosphatidylglycerol (cardiolipin)[13]. The fatty acid residues of the mitochondrial phosphatides are said to resemble those of the endoplasmic reticulum[14]. Experiments carried out in GREEN's laboratory[15,16] indicate that the enzymic activity of the four par-

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ticulate complexes of electron transport depends on the presence of lipids. A hypothesis has been put forth according to which phospholipids coating the protein subunits guarantee the membranous arrangement. β-Hydroxybutyrate dehydrogenase isolated from mitochondria is a lipoprotein. It can be reversibly inactivated and activated by the depletion and re-addition of lipids[17–20]. In general and in detail, the function of the different phospholipids in these membranes is unknown.

Studies of either the enzymic properties, or the chemical structure of the whole mitochondria permit no conclusions with regard to the structure or biochemical function of the outer or inner mitochondrial membrane.

Protein synthesis in mitochondria has been studied *in vivo*[21] and *in vitro*[22,23]. The inner mitochondrial membrane is supposed to incorporate most of the radioactive amino acids[24,25]. Since mitochondria also possess DNA, RNA and ribosomes, it has been speculated that a relationship may exist between this independent mitochondrial protein synthesizing system and the inner membrane and between the outer membrane and matrix enzymes and the cytoplasmic ribosomal system, controlled by nuclear DNA[26].

So far nothing is known about the capability for phosphatide biosynthesis in the outer and inner mitochondrial membranes, about the origin of their phospholipids and their exact composition. It is known that phospholipid components such as 3-glycerolphosphate[27], long chain fatty acids[28] and choline[29] are incorporated by isolated whole mitochondria. However, the predominant activity of all enzymes of phospholipid synthesis except phosphatidic acid phosphohydrolase has been found in the microsomal fraction of rat liver[30–32].

In order to obtain further information about the biosynthesis of the mitochondrial membranes, we studied the biosynthetic enzyme activities involved in phospholipid synthesis of the outer and inner mitochondrial membranes. These activities were compared with the corresponding activities of the microsomal and cytoplasmic fractions. Furthermore a quantitative analysis of the phospholipid mixture involved in the structure of the isolated membrane fractions was performed.

Results

The cell fractions and mitochondrial membranes were prepared by the procedure of SOTTOCASA[6]. The purity of the isolated fractions was controlled by electron microscopy and determinations of marker enzyme activities. Succinate dehydrogenase was chosen as the marker enzyme for intact mitochondria and inner mitochondrial membranes[6], glucose-6-phosphatase of the microsomal fraction, and monoamine oxidase of outer mitochondrial membranes[7]. The data summarized in Table I indicate that the microsomal fraction is free of mitochondria and their fragments. The maximal microsomal contamination of the whole mitochondrial fraction is 4—6%. The outer membranes contain fragments of inner membranes and the inner membrane fraction 6—10% of the outer mitochondrial membrane monoamine oxidase activity.

I. Enzymic Studies

Our enzymic studies were concerned with five enzymes involved in the biosynthesis of phosphatidylcholine[33], the main component of the phospholipid mixture: acyl-CoA : L-glycerol-3-phosphate *O*-

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Table 1. Relative distribution of marker enzymes in cellular and mitochondrial fractions.

The data summarize the specific enzymic activities (units/mg protein).

Definitions:

One unit of glucose-6-phosphatase (Glc-6-*P*ase) liberates 1 μg glucose per 30 min.

One unit of succinate dehydrogenase (SDH) produces a decrease in optical density at 600 mμ of 0.001 per 5 min.

One unit of monoamine oxidase (MAO) produces an initial rate of increase in optical density at 250 mμ of 0.001 per min.

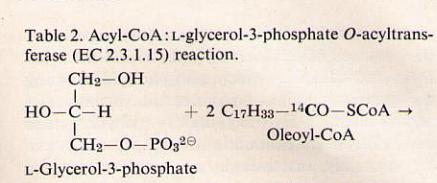
	Glc-6-*P*ase	SDH	SDH	MAO
Mitochondria	4	155	175	33
Microsomes	91	0	1	38
Mitochondria				
inner membrane			318	27
outer membrane			210	278
"soluble" (= matrix)			4	1

acyltransferase (EC 2.3.1.15), acyl-CoA:lysophosphatidic acid *O*-acyltransferase (EC 2.3.1.?), L-α-phosphatidate phosphohydrolase (EC 3.1.3.4), CDPcholine:1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2), and acyl-CoA:lysolecithin *O*-acyltransferase (EC 2.3.1.?). All these activities were studied using labeled substrates which permitted a reliable and accurate quantitative analysis of the reaction products.

The acyl-CoA:L-glycerol-3-phosphate *O*-acyltransferase reaction, which was studied with 3-glycerolphosphate and [1-^{14}C]oleoyl-CoA as substrates, yielded radioactive phosphatidic acid. The results are described in Table 2. The data of this and the following tables have been obtained in repeated experiments with striking reproducibility. The enzymic activity of the microsomal fraction is four times higher than that of the whole mitochondrial fraction. Only the outer membrane of the mitochondria carries the acyltransferase responsible for the transfer of acyl-groups to L-glycerol-3-phosphate.

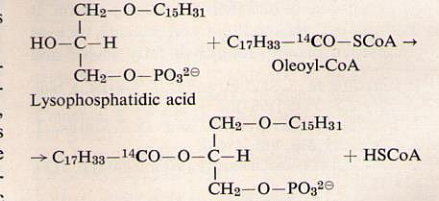
On the other hand, the rate of transfer of the fatty acid residue from [1-^{14}C]oleoyl-CoA to lysophosphatidic acid, catalyzed by the acyl-CoA:lysophosphatidate *O*-acyltransferase, is four times higher with the mitochondrial than with the microsomal fraction (Table 3). The outer mitochondrial membrane possesses a thirty times higher transferase activity than the inner membrane.

Table 2. Acyl-CoA:L-glycerol-3-phosphate *O*-acyltransferase (EC 2.3.1.15) reaction.



Fractions	Phosphatidic acid synthesized [dpm · mg protein^{-1} · 30 min^{-1}]	[nmoles · mg protein^{-1} · 30 min^{-1}]
Mitochondria		
inner membrane	230	0.7
outer membrane	8600	25.2
Homogenate	190	0.6
Whole mitochondria	1360	4.0
Intermediate fraction	2150	6.3
Microsomes	4900	14.4
105000×*g* supernatant	—	—

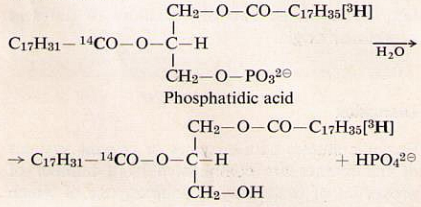
Table 3: Acyl-CoA:lysophosphatidic acid *O*-acyltransferase (EC 2.3.1.?) reaction.



Fractions	Phosphatidic acid synthesized [dpm · mg protein^{-1} · 30 min^{-1}]	[nmoles · mg protein^{-1} · 30 min^{-1}]
Mitochondria		
inner membrane	2444	14.4
outer membrane	66000	388
Homogenate	400	2.4
Whole mitochondria	2460	14.5
Intermediate fraction	285	1.7
Microsomes	655	3.9
105000×*g* supernatant	—	—

The phosphatidate phosphohydrolase activity could be measured conveniently by following the hydrolysis of radioactive phosphatidic acid to radioactive 1,2-diglyceride. The enzymic activity present in the microsomal fraction and whole mitochondria is comparable, in the mitochondria the hydrolase predominantly resides in the outer membrane. Table 4 clearly indicates that the intermediate fraction, which is essentially a lysosomal fraction, contains the main phosphatidate phosphohydrolase activity. This finding confirms the observation of WILGRAM and KENNEDY[30] on the localization of this enzyme.

Table 4. L-α-Phosphatidate phosphohydrolase (EC 3.1.3.4) reaction.

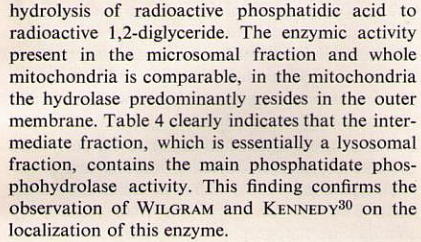


Fractions	1,2-diglyceride synthesized [dpm · mg protein^{-1} · 30 min^{-1}]	[nmoles · mg protein^{-1} · 30 min^{-1}]
Mitochondria		
inner membrane	480	4.3
outer membrane	4400	40
Homogenate	1600	14.5
Whole mitochondria	5150	47
Intermediate fraction	7300	67
Microsomes	6900	63
105000 × *g* supernatant	550	5

The incorporation of labeled 1,2-diglyceride into lecithin in the CDP-choline: 1,2-diglyceride cholinephosphotransferase reaction by all fractions is rather low. One reason might be the serious difficulty in solubilizing the 1-stearoyl-2-linoloylglycerol. Despite this drawback the data (Table 5) indicate again that the microsomal fraction is more active than the mitochondrial fraction. The outer mitochondrial membrane has low activity compared to no activity at all in the inner membrane*.

* See note added in proof on p. 1026.

Table 5. CDP-choline: 1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2) reaction.

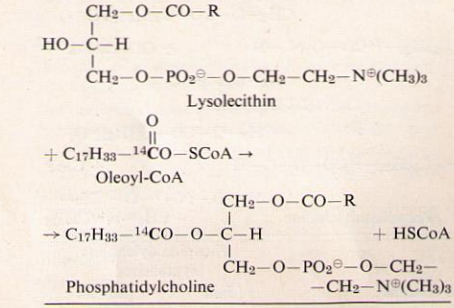


Fractions	Phosphatidylcholine synthesized [dpm · mg protein^{-1} · 30 min^{-1}]	[nmoles · mg protein^{-1} · 30 min^{-1}]
Mitochondria		
inner membrane	—	—
outer membrane	15	0.14
Homogenate	17	0.16
Whole mitochondria	55	0.50
Intermediate fraction	47	0.43
Microsomes	134	1.2
105000 × *g* supernatant	—	—

An enzyme not responsible for the *de novo* synthesis of phospholipids, but able to change molecular species of phospholipids in a deacylation-reacylation cycle, is the acyl-CoA: lysolecithin *O*-acyltransferase. We studied this reaction with lysolecithin as acceptor and [1-^{14}C]oleoyl-CoA as substrate. The radioactivity of the isolated lecithin formed in this reaction was determined (Table 6). It has been shown previously that the microsomal fraction has a very high enzymic activity[31,32]. In these experiments again it was found to be four times higher than the mitochondrial activity. In the mitochondria only the outer membrane showed transferase activity.

It should be mentioned that in all acyl-transfer reactions studied with [1-^{14}C]oleoyl-CoA as substrate and the inner mitochondrial membrane as enzyme source, a highly labeled by-product has been isolated. The chromatographic behaviour, co-chromatography on thin layer plates with β-hydroxymyristic acid (solvent system: ethyl ether/acetic acid 99:1), thin layer chromatography of the acetylated derivative on silver nitrate impregnated silicagel H plates (solvent system: 1,2-dichloroethane/acetic acid 99:1) indicates that this by-pro-

Table 6. Acyl-CoA: lysolecithin *O*-acyltransferase (EC 2.3.1.?) reaction.



Fractions	Phosphatidylcholine synthesized [dpm · mg protein^{-1} · 30 min^{-1}]	[nmoles · mg protein^{-1} · 30 min^{-1}]
Mitochondria		
inner membrane	630	3.7
outer membrane	9650	57
Homogenate	4030	23.7
Whole mitochondria	4700	27.6
Intermediate fraction	9200	54
Microsomes	17300	102
105000 × *g* supernatant	160	1

duct is β-hydroxyoleic acid. Apparently the inner mitochondrial membranes still possess acyl-CoA dehydrogenase and enoyl-CoA hydratase (crotonase) activities, but the procedure of membrane preparation causes the loss of the coenzymes required for further steps in the β-oxidation.

II. Composition of the phospholipids of cell fractions and inner and outer mitochondrial membranes

The lipid extracts of whole mitochondria, outer and inner mitochondrial membranes, mitochondrial matrix fraction, and the microsomal fraction were separated by two-dimensional thin layer chromatography[34] (Fig. 1), and the single phospholipids were determined by quantitative phosphorus analysis. The data are summarized in Table 7. The inner mitochondrial membrane has a lipid content similar to that of whole mitochondria and is characterized by its high cardiolipin concentration (0.02 μmole/mg protein) whereas the phosphatidylserine and -inositol content is low. The outer membrane, with a lipid content (0.38 μmole/mg protein) twice that of whole mitochondria (0.16 μmole/mg protein), is rich in phosphatidylserine and -inositol with only traces of cardiolipin.

It was of great interest to compare the fatty acid patterns of phosphatidylethanolamine and phosphatidylcholine in the different membrane fractions. The two phospholipids were purified by column chromatography[35] and their fatty acids as methyl esters quantitated by gas liquid chromatography. Table 8 indicates clearly that there is no appreciable difference in the composition of the fatty acids of the two phosphatides of different subcellular origin.

Discussion

Enzymic studies with enzymes or enzyme systems of membranes are complicated by a number of properties of these enzyme sources, one of which is the diffusion process of substrates within a particulate (solid) phase to the active site of the enzymes. Furthermore in our experiments the substrates and (or) products are hydrophobic, barely soluble compounds, some of them with amphipathic structure (acyl-CoA ester, lyso-compounds). The influence of these physico-chemical properties on the enzymatic reaction is completely unknown. We have avoided the use of detergents except in the CDP-choline:1,2-diglyceride-cholinephosphotransferase reaction, but instead applied mild sonication for the dispersion of the particulate enzyme fractions and of the substrates. Under the conditions described in detail in the experimental section, we were able to show that the mitochondrial outer membrane is equipped with all enzymes necessary for phosphatidylcholine synthesis. The inner mitochondrial membrane lacks almost all enzymes for the biosynthesis of phosphatides; the activity found can easily be explained by contaminating outer membranes. However, the predominant enzymic activity for phospholipid synthesis is present in the microsomal fraction. Further experiments are intended to elucidate the role of these phospho-

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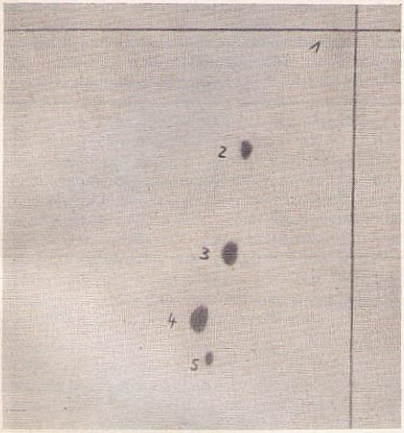
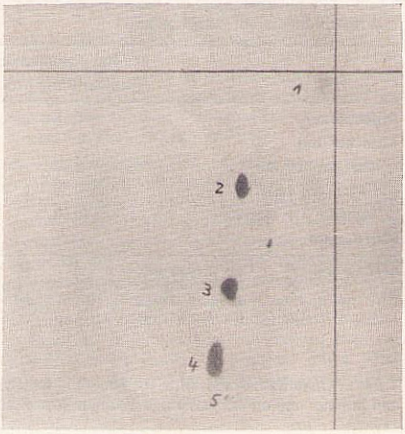
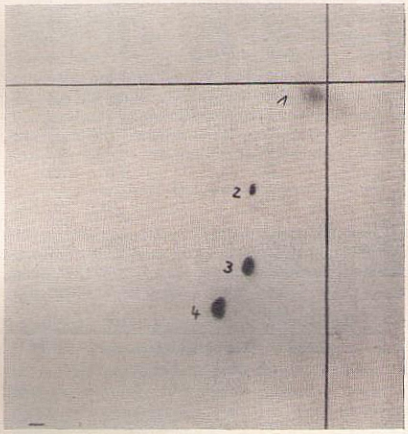


Whole mitochondria



Inner membrane

Outer membrane



Matrix fraction

Fig. 1. Two-dimensional thin layer chromatograms of the total lipid extracts from whole mitochondria and mitochondrial subfractions.

Solvent systems: vertical direction: chloroform/methanol/water 65:25:4; horizontal direction: n-butanol/acetic acid/water 60:20:20.

The substances are: (1) neutral lipids, (2) cardiolipin, (3) phosphatidylethanolamine, (4) phosphatidylcholine, (5) phosphatidylinositol, phosphatidylserine.

Table 7. Quantitative analysis of the composition of phosphatides in cell fractions and in inner and outer membranes and matrix fraction of mitochondria.

	Total phosphatide [μmoles P/mg prot.]	PC	PE	PI/PS	Sph	Card	PG
		[% of total phosphatide]					
Mitochondria	0.16	47	26	13	—	14	—
Mitochondria							
inner membrane	0.20	41	35	2	—	21	1
outer membrane	0.38	49	31	17	—	3	—
"soluble" (= matrix)	0.02	64	28	—	—	8	—
Microsomes	0.46	60	21	14	5	—	—

Abbreviations: PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; Sph = sphingomyelin; Card = cardiolipin; PG = phosphatidylglycerol

Table 8. Quantitative analysis of the fatty acid patterns of phosphatidylethanolamine and -choline in cell fractions and in inner and outer membranes and matrix of mitochondria (area percentages).

	Whole mitochondria		Inner membrane		Outer membrane		"Soluble" (= matrix)		Microsomes	
	PE	PC	PE	PC	PE	PC	PE	PC	PE	PC
C_{12}	0.5	< 0.5	< 0.5	< 0.5	< 0.5	0.5	0.5	2.0	< 0.5	< 0.5
C_{14}	< 0.5	< 0.5	< 0.5	< 0.5	1.0	< 0.5	1.5	1.0	< 0.5	< 0.5
C_{16}	18.0	17.0	13.5	18.0	12.0	22.0	15.0	18.5	13.0	18.0
$C_{16:1}$	< 0.5	< 0.5	< 0.5	0.5	< 0.5	< 0.5	< 0.5	3.0	< 0.5	0.5
C_{17}	0.5	0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	1.0	< 0.5	< 0.5
C_{18}	27.0	31.0	32.5	25.5	31.0	25.5	27.5	29.0	33.5	26.0
$C_{18:1}$	2.0	5.0	2.5	7.0	5.5	8.0	5.5	12.0	4.5	6.5
$C_{18:2}$	4.5	6.5	4.0	7.0	5.5	5.0	4.5	6.0	5.0	9.5
$C_{18:3}$	3.0	2.5	—	—	—	—	—	—	1.5	1.0
$C_{20:3}$	—	—	—	0.5	—	—	—	—	—	—
$C_{20:4}$	31.0	32.0	35.5	34.0	31.0	30.5	28.0	23.0	31.0	32.0
$C_{20:5}$	< 0.5	—	—	—	1.5	0.5	2.5	1.5	—	—
$C_{22:5}$	1.5	—	—	0.5	2.5	< 0.5	1.5	—	1.5	0.5
$C_{22:6}$	10.5	5.0	11.0	6.0	10.0	6.5	12.5	4.0	9.0	5.0

Abbreviations: PE = phosphatidylethanolamine PC = phosphatidylcholine

lipid synthesizing enzymes of the outer mitochondrial membrane in the biogenesis and growth of mitochondria.

Experimental

All coenzymes were purchased from C. F. Boehringer, Mannheim. Chromatographically pure soya lecithin was kindly provided by Fa. Nattermann, Köln, and diisopropylfluorophosphate by Fa. Bayer, Leverkusen.

The *coenzyme A ester* of [1-^{14}C]oleic acid[36] was prepared according to KORNBERG and PRICER[37]. 1-palmitoyl-3-glycerolphosphate *(lysophosphatidic acid)* was obtained by total synthesis[38]. Enzymic hydrolysis of

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1 - [^{3}H]stearoyl - 2 - [^{14}C]linoloyl - 3 - glycerophosphoryl-ethanolamine[39] with phospholipase C from *B. cereus*[40] yielded 1-[^{3}H]stearoyl-2-[^{14}C]linoloyl-glycerol (1,2-diglyceride), and with phospholipase D from cabbage[41] 1 - [^{3}H]stearoyl - 2 - [^{14}C]linoloyl - 3 - glycerolphosphate *(phosphatidic acid)*. The two reaction products were chemically and radiochemically pure as judged by radio thin layer chromatography (solvent systems for 1,2-diglyceride: petroleum ether/ethyl ether/water 70:30:2; 1,2-dichloroethane/methanol 98:2; solvent systems for phosphatidic acid: chloroform/methanol/30% aqueous methylamine 65:25:8[42]; chloroform/methanol/HCl 87:13:0.5 on silicagel G plates prepared in 0.5M oxalic acid[43]). *Lysolecithin* was prepared from lecithin by phospholipase A hydrolysis (*Crotalus adamanteus* venom, Fa. Celo, Zweibrücken) and purified by several precipitations from chloroform with ether.

Rat liver was homogenized in 20 vol. of 0.25M sucrose. *Cell fractions* and *mitochondrial membranes* were prepared following the procedure described by SOTTOCASA et al.[6] with swelling, shrinking, sonication and centrifugation through a discontinous density gradient (45 ml of 1.18M sucrose, rotor SW 25.2 of a Beckman Ultracentrifuge L-2). The purity of the isolated cell fractions and mitochondrial membranes was controlled by electronmicroscopy and by determination of the activity of marker enzymes.

Succinate dehydrogenase was assayed by following the reduction of 2,6-dichlorophenolindophenol[44]. *Glucose-6-phosphate phosphohydrolase* was estimated by incubating 0.10 μmoles glucose-6-phosphate with the cell fractions in 0.1M citrate buffer, pH 6.5, and, after denaturation, determination of the glucose in the supernatant with glucose oxidase (test combination, C. F. Boehringer, Mannheim). *Monoamine oxidase* was analyzed according to TABOR[45]. *Protein* was determined by the biuret method.

Total lipids were extracted from the lyophilized cell fractions and from mitochondrial membranes with chloroform/methanol 2:1. The extracts were washed with water, concentrated in an atmosphere of nitrogen and dissolved in chloroform/methanol 2:1. Equal aliquots were separated and identified by two-dimensional thin layer chromatography[34] (plates coated with silica gel H + 10% magnesium silicate; solvent systems: vertical direction: chloroform/methanol/water 65:25:4; horizontal direction: n-butanol / acetic acid / water 60:20:20). The spots were detected with iodine vapour. Phosphate was determined from phospholipid containing spots of three chromatoplates according to a modified BARTLETT procedure[46, 47].

Phosphatidylethanolamine and *-choline* were separated from the lipid mixture by silicic acid chromatography[35] and their fatty acid methyl esters obtained by interesterification with 5% HCl in methanol (w/w). *Fatty acid methyl esters* were analyzed by quantitative gas liquid chromatography on a 15% diethylene glycol succinate column using a Packard Gas Chromatograph Series 7820 equipped with a flame ionization detector (Model 7821). The peaks were evaluated by triangulation. The quantitative results with a standard of purified fatty acids agreed with the known composition with a relative error of less than 5% for major components and less than 10% for minor components.

Enzymic assays

Homogenate ($750\times g$ supernatant), mitochondria ($750-6500\times g$ sediment), intermediate fraction (6500 to $15000\times g$ sediment), microsomes ($15000-105000\times g$ sediment) and $105000\times g$ supernatant were obtained by differential centrifugation. The particles were suspended in water and sonicated for 15 seconds with a Mullard sonifier at maximal tune. Prior to the addition of acyl-CoA as substrate, cell and mitochondrial membrane fractions were incubated for 60 min at room temperature with DFP (c = 5mM) in order to inhibit the active acyl-CoA-hydrolase[48]. The incubation mixtures contained:

Acyl-CoA: L-glycerol-3-phosphate O-acyltransferase (EC 2.3.1.15):

0.2 μmoles oleoyl-CoA (170000 dpm), 2.0 μmoles L-glycerol-3-phosphate, 100 μmoles potassium phosphate buffer, pH 7.4, enzyme protein 5—10 mg (pretreated with DFP). Total volume 2 ml.

Acyl-CoA: lysophosphatidate O-acyltransferase (EC 2.3.1.?):

0.2 μmoles oleoyl-CoA (170000 dpm), 1.0 μmole lysophosphatidic acid, 100 μmoles potassium phosphate buffer, pH 7.4, enzyme protein 5—10 mg (pretreated with DFP). Total volume 2 ml.

Phosphatidate phosphohydrolase (EC 3.1.3.4):

1—2 μmoles phosphatidic acid (110000 or 220000 dpm), 100 μmoles maleate buffer, pH 6.3, enzyme protein 5—10 mg. Total volume 2 ml.

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CDPcholine:1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2):

1—2 μmoles 1,2-diglyceride (110000 or 220000 dpm) in 1% aqueous Tween 20-solution, 10 μmoles $MgCl_2$, 100 μmoles Tris-HCl buffer, pH 8.0, 2 μmoles CDP-choline, enzyme protein 5—10 mg. Total volume 2 ml.

Acyl-CoA:lysolecithin O-acyltransferase (EC 2.3.1.?):

0.2 μmoles oleoyl-CoA (170000 dpm), 1 μmole lysolecithin, 100 μmoles potassium phosphate buffer, pH 7.4, enzyme protein 5—10 mg (pretreated with DFP). Total volume 2 ml.

The *incubations* were stopped after 30 min at 30°C by freezing and lyophilized. The residue was repeatedly extracted with chloroform/methanol 2:1. The washed lipid extracts were separated on thin layer plates coated with silicagel H.

Solvent systems for thin layer chromatography:

I. ethyl ether/acetic acid 99:1

II. chloroform/methanol/water 65:25:4

III. chloroform/methanol/30% aqueous methylamine 65:25:8

IV. petroleum ether/ethyl ether/acetic acid 70:30:2

In solvent system I neutral lipids and free fatty acids migrate with the solvent front. This band was eluted from the silicagel with chloroform/methanol 2:1 and rechromatographed in solvent system IV which separates fatty acids, mono-, di-, and triglycerides (Fig. 2). In solvent system I the phosphatides remain at the start. They are subsequently separated in solvent system II or III. System II separates phosphatidylethanolamine and -choline, solvent system III phosphatidic acid, phosphatidylcholine and -ethanolamine (Fig. 3). The chromatoplates were scanned in a Packard Radiochromatogram scanner Model 7201. The radioactive compounds were identified by cochromatography of test substances. After the quantitative transfer of the silicagel spot area into BRAY's solution[49], the radioactivities of the spots were measured with a Packard Tricarb Liquid Scintillation Spectrometer Model 3214. The counting efficiency was determined for each sample by the addition of an internal standard.

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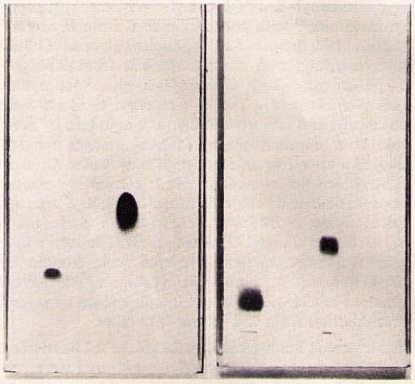

Fig. 2. Fig. 3.

Fig. 2. Thin layer chromatogram demonstrating the separation of 1,2-diglyceride (left) and fatty acid (right). Solvent system: petroleum ether/ethyl ether/acetic acid 70:30:2.

Figure 3. Thin layer chromatogram demonstrating the separation of phosphatidic acid (left) and phosphatidylcholine (right). Solvent system: chloroform/methanol/30% aqueous methylamine 65:25:8[42].

Note added in proof: Lecithin synthesis increased about three times when sodium 1-stearoyl-2-linoloylglycerol-phosphate was used as substrate instead of the diglyceride underlining the solubility problem in the study of this reaction.

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