BBA 75384

THE COMPOSITION OF PHOSPHOLIPIDS IN OUTER AND INNER MITOCHONDRIAL MEMBRANES FROM GUINEA-PIG LIVER

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(Received August 25th, 1969)

SUMMARY

1. Mitochondria, outer and inner mitochondrial membranes and microsomes were prepared from guinea-pig liver.

2. The inner mitochondrial membrane exclusively contained succinate:cytochrome *c* reductase while the outer membrane was characterized by a high level of monoamine oxidase. Low levels of microsomal contamination of the outer membrane were indicated by assays of glucose-6-phosphatase and NADPH:cytochrome *c* reductase.

3. The phospholipids were separated by thin-layer chromatography and the fatty acids analysed by gas–liquid chromatography. In all the membranes lecithin had a relatively high content of stearate and linoleate. Phosphatidylethanolamine contained less linoleate than lecithin but had a significantly ligher level of arachidonate. Phosphatidylinositol was the most highly saturated lipid, containing large amounts of stearate, while cardiolipin was over 90 % unsaturated, due mostly to linoleic acid. It is concluded that in guinea-pig liver each phospholipid has a rather distinctive fatty acid pattern which is approximately the same in all membranes.

4. Some implications of the results are discussed.

INTRODUCTION

Precise roles for phospholipids in mitochondrial structure and function remain to be established. Experiments demonstrating the restoration by phospholipids of enzymatic activity in lipid-depleted mitochondria[1] and in purified enzyme preparations[2] emphasize that normal membrane function must be critically dependent on as yet unspecified lipid–protein interactions, probably involving both hydrophobic and polar groups.

Whole mitochondria were shown to have a unique pattern of phospholipids[3,4] and to have some capacity to synthesize phospholipids[5,6]. Methods developed recently for the separation of outer and inner mitochondrial membranes in relatively clean form provide a means of examining in greater detail to what extent mitochondria may be autonomous with respect to phospholipid synthesis and of defining further the relation between lipid requirement and membrane function. As a preliminary aspect of a study of these questions mitochondrial and microsomal membranes from guinea-pig liver have been isolated and the phospholipids analysed by thin-layer and gas–liquid chromatography. The general conclusion can be made that each membrane

contains the major phospholipids in proportions highly characteristic of the membrane. However, each phospholipid has a distinctive spectrum of fatty acids that is similar in all membranes. A preliminary account of this work has been given elsewhere[7].

METHODS

Preparation of mitochondria, mitochondrial subfractions and microsomes

Male guinea-pigs of body weight 350–450 g were used. The animals were fed guinea-pig chow (Purina) but were deprived of food 18 h before the experiment (water *ad libitum*). Sub-cellular organelles were prepared from liver homogenates as described by PARSONS *et al.*[8], with the following modifications. The mitochondria were washed 4 times instead of twice in buffered sucrose and spun down in a Servall SS-34 rotor at 4000 rev./min for 8 min and then at 9000 rev./min for 2 min. In our hands the increased washing of mitochondria was necessary to reduce microsomal contamination. The fraction designated low-speed pellet (LSP) by PARSONS *et al.*[8], obtained after swelling the mitochondria in hypotonic buffer and centrifuging at 4000 rev./min for 15 min, was taken to represent inner mitochondrial membrane. Purified outer membrane was collected at the interface between 23.2 and 37.7 % sucrose after the gradient-centrifugation step. The membranes were examined in the electron microscope after negative staining and were assayed for various enzymes. Protein was measured by the method of LOWRY *et al.*[9].

Enzyme assays

Monoamine oxidase. The oxidation of tyramine was monitored with a Clark oxygen electrode. Tissue suspension was incubated in 2.5 ml of 0.05 M phosphate buffer (pH 7.6) at 37° for 10 min, then 0.2 ml of 5 % tyramine in phosphate buffer was added.

Succinate:, NADH: and NADPH: cytochrome c reductases. Tissue suspension was incubated with 2.5 ml of 0.1 M phosphate buffer (pH 7.0), 0.3 ml of 0.005 M KCN and 0.05 ml of oxidised cytochrome *c* (15 mg/ml). The reactions were initiated by adding 0.02 ml of 1.0 M disodium succinate or 0.02 ml of 0.02 M NADH or 0.02 ml of 0.02 M NADPH and the change in absorbance at 550 nm was followed in a recording spectrophotometer.

Glucose-6-phosphatase. A modification of the method of SWANSON[10] was employed. 0.1 ml of 0.04 M glucose-6-phosphate was incubated with 0.2 ml of 0.1 M citrate buffer (pH 6.2) and tissue suspension (final volume 0.4 ml) for 30 min at 30°. The reaction was terminated by cooling the tubes in ice and adding 0.1 ml of 5 % bovine serum albumin and 2 ml of 10 % $HClO_4$ (w/v). After centrifuging and filtering the filtrate was analysed for inorganic phosphate by the method of FISKE AND SUBBAROW[11]. Enzyme and substrate blanks were carried through simultaneously.

Phospholipid analysis

Tissue suspensions were pipetted into 20 vol. of chloroform–methanol (2:1, by vol.) and the extracts filtered through pre-washed glass wool. The filtrates were taken to dryness under reduced pressure and the lipids were redissolved in 20 ml of

chloroform–methanol (2:1, by vol.) and purified by the method of FOLCH *et al.*[12]. The extracts in chloroform solution were stored under N_2 at −15°. In experiments in which fatty acid compositions were determined, phospholipids and neutral lipids were separated on short columns of silicic acid–celite (2:1, by wt.). Neutral lipids were eluted with chloroform and phospholipids with methanol. Phospholipids were separated on thin layers (0.5 mm) of silica gel HR in the solvent, chloroform–methanol–glacial acetic acid–water (65:43:1:3, by vol.). Spots were visualized with I_2 vapour or by spraying the plates with 2′,7′-dichlorofluorescein and viewing under ultraviolet light. Lipids were identified by comparing their mobilities to authentic standards. Identification was further confirmed with the Dragendorff test for choline-containing lipids, the ammoniacal $AgNO_3$ spray for inositol lipids and the ninhydrin test for serine and ethanolamine phosphatides. For determination of phospholipid concentrations silica-gel scrapings from the thin-layer plates were transferred to boiling tubes and the lipids were oxidised with 72 % $HClO_4$ (w/v). The inorganic phosphate was measured by the method of BARTLETT[13]. The values were corrected for gel blanks which were treated similarly. Recovery of phospholipid phosphorus from the chromatograms averaged 90 %.

The methyl esters of fatty acids from the phosphatides were prepared according to the method of FELDMAN AND ROUSER[14]. Silica-gel scrapings from the thin-layer chromatograms were mixed with 2.0 ml of 6 % H_2SO_4 in methanol in pyrex tubes. The tubes were sealed and heated at 90° for 16 h. The methyl esters were extracted into *n*-hexane, taken to dryness under N_2 and redissolved in CS_2. Chromatographic analyses were made on a Varian Aerograph gas chromatograph, Model 2100. The chromatographic separations were performed isothermally at 180° on glass columns packed with 10 % EGSS-X on 100–120 mesh Gas-ChromP (Applied Science Laboratories, State College, Pa., U.S.A.) and the fatty acids were identified by comparison with retention data of authentic standards and with published values.

RESULTS AND DISCUSSION

Several methods have been described for the separation of outer and inner mitochondrial membranes. Since there are difficulties, however, in establishing acceptable criteria for assessing the identities of the isolated membranes, there is, as yet, no complete agreement on their chemical composition and catalytic properties. In the present work the membrane fragments had the morphological appearance in the electron microscope as described by PARSONS *et al.*[8]. While the low-speed pellet after negative staining showed mostly inner membrane with the characteristic 90-Å sub-units some adhering fragments of outer mitochondrial membrane were also evident. The fraction is therefore considered to be enriched in inner mitochondrial membrane and not completely pure, but the terms low-speed pellet and inner membrane are herein used synonymously.

Enzyme distribution

The activities of a number of enzymes distributed among the different membrane fractions are shown in Table I. Confirming previous reports[21], succinate: cytochrome *c* reductase was located exclusively in the inner mitochondrial membrane. The activity associated with the outer membrane suggested very low contamination

by inner membrane, in the order of 1 % or less. Monoamine oxidase, with benzylamine as substrate, has been described as a marker for outer mitochondrial membrane from rat liver. With guinea-pig liver preparations benzylamine was found to be a poor substrate compared with tyramine. As shown in Table I the ability to oxidize tyramine was highly concentrated in the outer membrane fraction. This conclusion is in agreement with the findings of several groups[15–18] although RAGLAND AND MITCHELL[19] and GREEN *et al.*[20] have claimed that monoamine oxidase is located in the inner membrane.

TABLE I

DISTRIBUTION OF MARKER ENZYMES BETWEEN MITOCHONDRIAL AND MICROSOMAL MEMBRANES FROM GUINEA-PIG LIVER

Values are expressed as mean ± S. D. from a minimum of three separate determinations.

Fraction	*Succinate: cytochrome c reductase**	*Monoamine oxidase***	*Glucose-6-phosphatase****	*NADPH: cytochrome c reductase**
Mitochondria	78.7 ± 22.3	0.5 ± 0.3	0.07 ± 0.11	0.9 ± 0.6
Inner membrane	206.4 ± 56.1	0.4 ± 0.4	—	Not detected
Outer membrane	1.4 ± 2.0	11.1 ± 6.3	0.02 ± 0.02	5.4 ± 1.7
Microsomes	0.6 ± 0.7	0.04 ± 0.04	5.19 ± 2.66	39.1 ± 18.5

* nmoles of cytochrome *c* reduced per min per mg protein.
** μl of O_2 consumed per min per mg protein.
*** μg of inorganic phosphorus released per min per mg protein.

The extent of microsomal contamination of the outer mitochondrial membrane was assessed by measuring NADPH:cytochrome *c* reductase and glucose-6-phosphatase as enzyme markers for microsomes (Table I). The outer membrane from well-washed mitochondria showed a very low level of phosphatase, corresponding to less than 1 % contamination by microsomes. However, based on the assay of NADPH: cytochrome *c* reductase, microsomal contamination was in the order of 15 %. PARSONS *et al.*[8] and SOTTOCASA *et al.*[21] found virtually no NADPH:cytochrome *c* reductase in outer mitochondrial membranes. MCMURRAY AND DAWSON[22], however, found a persistent level of enzyme in the outer membrane from rat-liver mitochondria. In a recent paper BRUNNER AND BYGRAVE[23] found that significant levels of glucose-6-phosphatase and NADPH:cytochrome *c* reductase were retained in preparations of outer mitochondrial membranes after repeated washings that completely removed microsomal cytochrome P450. They have suggested that this residual activity is actually associated with outer membrane rather than microsomal contamination. Their results, however, and those reported in Table I do not exclude the possibility of limited contamination of outer membrane by a heterogeneous population of microsomes.

Phospholipid analysis

The phospholipid composition of the mitochondrial membranes, as determined by thin-layer chromatography was essentially similar to the results of PARSONS *et al.*[8]. Lecithin and phosphatidylethanolamine were the major components in both outer

and inner membrane. Cardiolipin was concentrated in the inner membrane while phosphatidylinositol was enriched in the outer membrane. The phospholipid pattern of outer membrane was rather similar to that of microsomes. Other minor lipids, phosphatidic acid and phosphatidylglycerol, reported to be present in mitochondria, were not detected and probably co-chromatographed with the major phospholipids.

Fatty acid analysis of the phosphatides was undertaken to determine whether further definition of the mitochondrial membranes could be obtained. The composition of the lecithins is shown in Table II. The species was characterized by an appreciable

TABLE II

FATTY ACID COMPOSITION OF LECITHIN

Figures in parentheses are the numbers of separate determinations. Values (area %) are expressed as mean ± S. D.

Fatty acid	*Mitochondria* (7)	*Inner membrane* (4)	*Outer membrane* (4)	*Microsomes* (5)
16:0	18.0 ± 2.6	18.1 ± 1.6	16.6 ± 0.7	17.5 ± 1.4
16:1	0.6 ± 0.6	0.6 ± 0.4	0.6 ± 0.2	0.7 ± 0.1
17:0	1.8 ± 2.6	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
18:0	28.4 ± 1.0	26.7 ± 1.7	29.5 ± 2.3	28.3 ± 1.5
18:1	9.4 ± 1.2	9.2 ± 2.1	8.6 ± 1.5	9.2 ± 0.8
18:2	34.1 ± 2.3	35.9 ± 3.0	34.5 ± 5.4	35.5 ± 1.8
18:3	1.3 ± 0.9	2.5 ± 0.8	1.5 ± 0.8	1.0 ± 0.5
20:2	0.9 ± 0.7	0.6 ± 0.4	1.2 ± 1.3	0.6 ± 0.6
20:3	2.0 ± 0.8	1.6 ± 1.2	2.5 ± 2.1	2.5 ± 1.7
20:4	3.1 ± 0.9	3.1 ± 1.1	3.0 ± 1.2	3.0 ± 0.9
> 20:4	0.4 ± 0.6	0.6 ± 0.8	0.8 ± 0.6	0.6 ± 0.6
Percent saturated	48.2	46.0	47.3	47.0

TABLE III

FATTY ACID COMPOSITION OF PHOSPHATIDYLETHANOLAMINE

Figures in parentheses are the numbers of separate determinations. Values (area %) are expressed as mean ± S. D.

Fatty acid	*Mitochondria* (5)	*Inner membrane* (3)	*Outer membrane* (4)	*Microsomes* (4)
16:0	5.6 ± 1.3	5.7 ± 1.0	6.7 ± 0.7	7.4 ± 0.3
16:1	0.2 ± 0.2	0.3 ± 0.4	0.3 ± 0.0	0.4 ± 0.4
17:0	1.4 ± 0.5	2.5 ± 1.2	1.2 ± 0.5	0.9 ± 0.7
18:0	41.1 ± 3.2	38.6 ± 3.5	38.0 ± 1.4	37.2 ± 3.8
18:1	3.9 ± 1.3	4.9 ± 4.7	5.6 ± 0.7	5.0 ± 1.8
18:2	20.7 ± 2.0	18.7 ± 5.0	23.2 ± 2.2	26.0 ± 2.6
18:3	1.5 ± 0.5	2.1 ± 0.5	1.4 ± 0.7	1.0 ± 0.3
20:2	0.5 ± 0.7	0.4 ± 0.4	1.2 ± 1.1	0.6 ± 0.4
20:3	1.0 ± 1.5	0.9 ± 0.7	2.2 ± 2.2	1.7 ± 1.6
20:4	20.5 ± 1.7	20.2 ± 2.2	16.5 ± 3.0	14.6 ± 1.4
> 20:4	3.6 ± 2.0	5.7 ± 3.5	3.7 ± 0.9	5.2 ± 1.3
Percent saturated	48.1	46.8	45.9	45.5

content of stearate followed by palmitate as saturated components and by linoleate and oleate as major unsaturated acids. There appeared to be no significant differences between the lecithins of microsomes, whole mitochondria and the sub-mitochondrial fragments. Phosphatidylethanolamines, shown in Table III, had a higher content of stearate and a lower level of linoleate as compared with lecithin. Phosphatidylethanolamine had the highest content of arachidonate, 15–20% compared with

TABLE IV

FATTY ACID COMPOSITION OF PHOSPHATIDYLINOSITOL

Figures in parentheses are the numbers of separate determinations. Values (area %) are expressed as mean ± S.D.

Fatty acid	*Mitochondria* (4)	*Inner membrane* (2)	*Outer membrane* (3)	*Microsomes* (4)
16:0	3.3 ± 1.7	4.4	2.5 ± 0.5	2.5 ± 0.7
16:1	0.3 ± 0.3	0.6	0.4 ± 0.3	0.7 ± 0.3
17:0	0.5 ± 0.3	0.7	0.4 ± 0.3	0.8 ± 0.1
18:0	55.2 ± 5.7	57.6	53.6 ± 4.6	49.7 ± 0.8
18:1	9.7 ± 2.3	13.6	12.1 ± 2.3	9.0 ± 1.6
18:2	14.1 ± 2.9	6.0	12.3 ± 1.0	17.0 ± 1.3
18:3	0.6 ± 0.6	0.9	0.5 ± 0.3	0.7 ± 0.4
20:2	3.0 ± 1.3	2.0	3.1 ± 1.0	3.6 ± 0.8
20:3	3.3 ± 1.3	1.3	4.0 ± 2.0	2.8 ± 1.2
20:4	10.0 ± 2.1	10.4	9.7 ± 3.1	11.8 ± 2.3
> 20:4	Trace	2.5	1.5 ± 0.4	1.5 ± 1.0
Percent saturated	59.0	62.7	56.5	53.0

TABLE V

FATTY ACID COMPOSITION OF CARDIOLIPIN*

Figures in parentheses are the numbers of separate determinations. Values (area %) are expressed as mean ± S. D.

Fatty acid	*Mitochondria* (4)	*Inner membrane* (4)	*Outer membrane* (3)
16:0	4.0 ± 2.1	4.6 ± 2.1	7.5 ± 0.8
16:1	1.3 ± 1.1	0.6 ± 0.5	1.4 ± 0.8
17:0	0.1 ± 0.1	0.5 ± 0.4	0.2 ± 0.2
18:0	2.4 ± 1.2	1.6 ± 0.7	9.2 ± 2.5
18:1	6.9 ± 1.7	6.8 ± 1.7	12.4 ± 1.0
18:2	77.1 ± 2.4	75.4 ± 2.5	58.9 ± 4.7
18:3	2.2 ± 1.3	3.0 ± 1.5	1.1 ± 0.3
20:2	2.3 ± 0.8	2.6 ± 0.8	2.9 ± 2.9
20:3	2.8 ± 2.5	4.2 ± 1.2	2.2 ± 2.5
20:4	0.8 ± 0.1	0.7 ± 0.6	1.2 ± 1.2
> 20:4	0.1 ± 0.1	Trace	3.1 ± 3.2
Percent saturated	6.5	6.7	16.9

* Includes phosphatidic acid.

only 3 % in lecithin. The fatty acid composition of phosphatidylethanolamine in all membranes was relatively constant.

The composition of the phosphatidylinositols is shown in Table IV. This was the most highly saturated lipid (approx. 60 %) containing a high content of stearic acid. Phosphatidylinositol showed no major unsaturated fatty acid, the level of unsaturation being made up mostly by oleate, linoleate and arachidonate in roughly similar quantities. In contrast to phosphatidylinositol, the fatty acids of mitochondrial cardiolipin were over 90 % unsaturated (Table V). The level of unsaturation in cardiolipin was due chiefly to the presence of linoleate; only very low levels of arachidonate could be detected. The cardiolipin of the outer mitochondrial membrane had a lower content of linoleate and more stearate than that of the inner membrane. However, the cardiolipin content of outer membrane was very low and the apparent differences may be due to problems in analysing minute amounts by gas–liquid chromatography. The cardiolipin spot may also have contained phosphatidic acid in different proportions in outer and inner membrane, contributing to the fatty acid patterns.

The findings lead to the conclusion that, although the proportions of the major phospholipids are markedly different in outer and inner mitochondrial membranes and microsomes of guinea-pig liver, each phospholipid has a characteristic spectrum of fatty acids which is more or less reproduced in all membranes. Only marginal differences are evident at this level of analysis. Similar results have been reported for whole mitochondria and microsomes from rat liver by MACFARLANE *et al.*[25] and GETZ *et al.*[3]. The composition of cardiolipin in guinea-pig liver is similar to that found in rat liver[3]. However, phosphatidylinositol and lecithin in guinea-pig liver appear to be considerably lower in arachidonate and higher in linoleate and stearate than the corresponding lipids in rat liver. Differences in composition may be expected because of dietary and species variations. STOFFEL AND SCHIEFER[24] also found that the fatty acids of phosphatidylcholine and phosphatidylethanolamine were essentially the same in different sub-mitochondrial fractions from rat liver. HUET *et al.*[26], however, found a higher ratio of saturated to unsaturated fatty acids in the phospholipids in outer mitochondrial membrane from rat liver. In guinea-pig liver, with the possible exception of cardiolipin, none of the lipids showed significant differences in the level of saturation between the two membranes.

The question of the origin of mitochondrial phospholipids has been examined in a number of papers. Evidence has been obtained for mitochondrial synthesis of the phospholipids present in low concentrations, phosphatidic acid[18], phosphatidylglycerol[6] and diphosphoinositide[5]. However, synthesis of lecithin occurs mostly in the endoplasmic reticulum[27] and recently, an extensive exchange *in vitro* of mitochondrial and microsomal phospholipids was demonstrated by WIRTZ AND ZILVERSMIT[28] and by MCMURRAY AND DAWSON[22]. The report that several enzymes concerned with phospholipid synthesis are present in the outer mitochondrial membrane[24] contrasts with the conclusion[22] that the synthetic activity associated with isolated mitochondria can be explained by microsomal contamination. Mitochondrial phospholipid may be derived both as a result of endogenous synthesis and from the endoplasmic reticulum. The close similarities between the major phospholipids of microsomes and mitochondrial membranes, shown in the present work, suggest that they could be derived from a common metabolic pool. However, the final selection

of the required pattern of fatty acids for each of the phospholipids could, in part, be carried out independently by both organelles by the acyl transferase reactions first described for microsomes[29] since phospholipase A and acyl-CoA:phospholipid acyltransferase also appear to be present in mitochondria[24,30,31].

ACKNOWLEDGEMENTS

We thank Mr. L. PINTERIC for examining the membrane preparations under the electron microscope. The investigation was supported by a grant from the Medical Research Council of Canada.

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