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Complex Phosphoglycerides From Rat Liver Outer Mitochondrial Membranes

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The ethanolic extract from rat liver mitochondrial membranes contains a number of highly polar complex lipids, which are found in the aqueous layer when subjected to the usual chloroform-water partition procedures. Two of these lipids have been purified by thin-layer chromatography, and their structures partially elucidated. Apparently, both are derivatives of phosphatidylglycerol, with a bulky polar head containing sugars and aminoacids. They are specifically located in the outer membrane. Several structures are suggested, as well as a possible functional rôle for these compounds.

It is well known that most of the membrane lipids consist of the usual phospholipid classes (phosphatidylcholine, phosphatidylethanolamine, etc.). Minor components of the lipid bilayer are the so-called «neutral lipids» (glycerides, sterols, etc.). In addition, the presence of highly polar lipids in biological membranes is now well established. However, these polar lipids have not been extensively studied up to now. This could be due to a merely technical reason, namely that these lipids often exhibit a marked preference for the polar phase in the washing stage which usually follows the extraction procedure with organic solvent mixtures. Therefore, highly polar lipids might be lost when discarding the aqueous phase. In a search for this type of lipids in mitochondrial membranes, we have succeeded in isolating and purifying a series of fatty acid containing compounds which go preferentially to the aqueous phase using the washing procedure proposed by FOLCH *et al.* (3). These lipids can be readily extracted woth aqueous 80 % ethanol or with other highly polar solvents. This report is concerned with the isolation, purification, separation and chemical caracterization of two of these highly polar glycerophospholipids which also contain sugars and aminoacids. A previous paper (4) included the data corresponding to five highly polar, non-glycerol containing phospholipids.

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Materials and Methods

Rat liver mitochondria were isolated as described by HOGEBOOM (5). Outer and inner mitochondrial membranes were isolated and purified according to PARSONS *et al.* (12). Mitochondria, or mitochondrial membranes, were treated with 80 % aqueous ethanol (0.6-0.8 ml/mg of membrane protein) with continous stirring for one hour at room temperature. The suspension was centrifuged at 30,000 × *g* for 5 min. The pellet was re-extracted with an equal volume of aqueous 80 % ethanol for another hour and both extracts pooled. The combined extracts contained, besides 10 % of the original protein, 70 % of the lipid phosphorus, a certain fraction of the neutral lipids, and most of the highly polar lipids. The extract was evaporated to near dryness, and redissolved in approximately 0.2 ml per mg of original protein of chloroform-methanol-water (10:10:3). Ten parts of chloroform and three parts of water were then added, and phase separation took place. The aqueous phase was tren evaporated and redissolved in a small volume of chloroform-methanol-water (10:10:3). Chromatographic separation of this solution was carried out on plates of silicagel HF_{254} (Merck), by means of two consecutive runs with chloroform-methanol-acetic acid-water (100:100:10:15). The plates were developed with 0.2 % ninhydrin in acetone-lutidine (9:1) at room temperature.

Molecular weight determinations were carried out by gel-filtration chromatography. The molecules, purified by thin-layer chromatography were eluted with aqueous 80 % ethanol and chromatographed on Sephadex LH-20 with the same solvent as the mobile phase. The deacylated molecules (2) were chromatographed in aqueous solution on Sephadex G-10.

Lipid phosphorus was determined as described by BARTLETT (1). Total sugars were evaluated by the anthrone method (14). Glucose was measured by enzymatic analysis (16). Glycerol was determined by the chromotropic acid reaction (8). L-α-glycerophosphate was investigated by paper chromatography after acid hydrolysis (7). Fatty acids were determined as their methyl esters by gas-liquid chromatography with internal standards. The nature of the linkage between fatty acids and non-lipid moiety was studied according to DAWSON (2). Aminoacids were analyzed in a Hitachi KLA-5 autoanalyzer. Carbohydrate components were investigated, after hydrolisis with 1 N HCl in boiling water bath for 30 min, by descending paper chromatography, with butanol-pyridine-water (6:4:3) as solvent. After 40 h, the chromatograms were stained with an acid anilin phthalate reagent (13). The presence of purine and or pyrimidine bases was discarded after hydrolysis and chromatographic analysis (11).

Results

After ninhydrin staining of the chromatographic plates, a series of spots could be seen. Phosphatidylserine and phosphatidylethanolamine ran very close to the solvent front. Other spots with lower Rf values corresponded to unidentified highly polar lipids. The most prominent of these spots had an Rf value close to 0.70. It was a narrow, sharp band, showing a deep pink colour after staining with ninhydrin. Just below that band, a second one could be observed, with an Rf of about 0.63, broader and less sensitive to the ninhydrin stain, but readily visible under 254 ultraviolet light. The compounds giving rise to these two bands were designated, respectively, as compounds 1 and 2; their chemical nature is the object of this report.

Both compounds could be purified by elution and rechromatography. The investigation of these molecules in the different mitochondrial subfractions led to their

Table I. *Percent fatty acid composition of compounds 1 and 2.* Number of experiments: 6. The figures correspond to mean values ± standard deviation of the mean. See text for experimental details.

Compound	C12	C16	C18	C18:1	C18:2
1	4.2±1.07	36.4±2.86	30.2±3.20	21.0±4.21	13.2±2.60
2	3.2±0.98	25.6±1.08	32.2±1.59	21.2±3.72	18.2±1.62

Table II. *Molar proportions of the components present in compound 1 and 2.* Number of experiments: 3. Phosphorus is arbitraly taken as unit. For experimental details, see text.

Compound	Fatty acids	Glycerol	Phosphorus	Glucose	Galactose	Aminoacids
1	2.0	1.8	1.0	1.8	—	1.9
2	2.1	1.8	1.0	1.1	1.0	0.9

localization in the outer membrane. From gel-filtration studies, it was concluded that the molecular weights of both molecules were of approximately 1,000 to 1,300 daltons.

Table I shows the fatty acid composition of compounds 1 and 2. All the fatty acids were of the alkali labile type (2). The aminoacid analysis showed that compound 1 contained arginine, aspartate and ammonia in roughly equimolar amounts, as well as a small proportion of lysine. Compound 2 contained approximately equimolar amounts of aspartate, glutamate, serine, valine and ammonia. Both compounds contained glycerol and phosphorus. The presence of L-α-glycerophosphate in the acid hydrolizate was confirmed. In addition, compound 1 contained glucose, and compound 2, glucose and galactose.

Table II reflects the molar proportions of each component in these two lipids. Scheme I shows some tentative structures. Structures *a* or *c* could correspond to compound 1, and structure *b*, to compound 2.

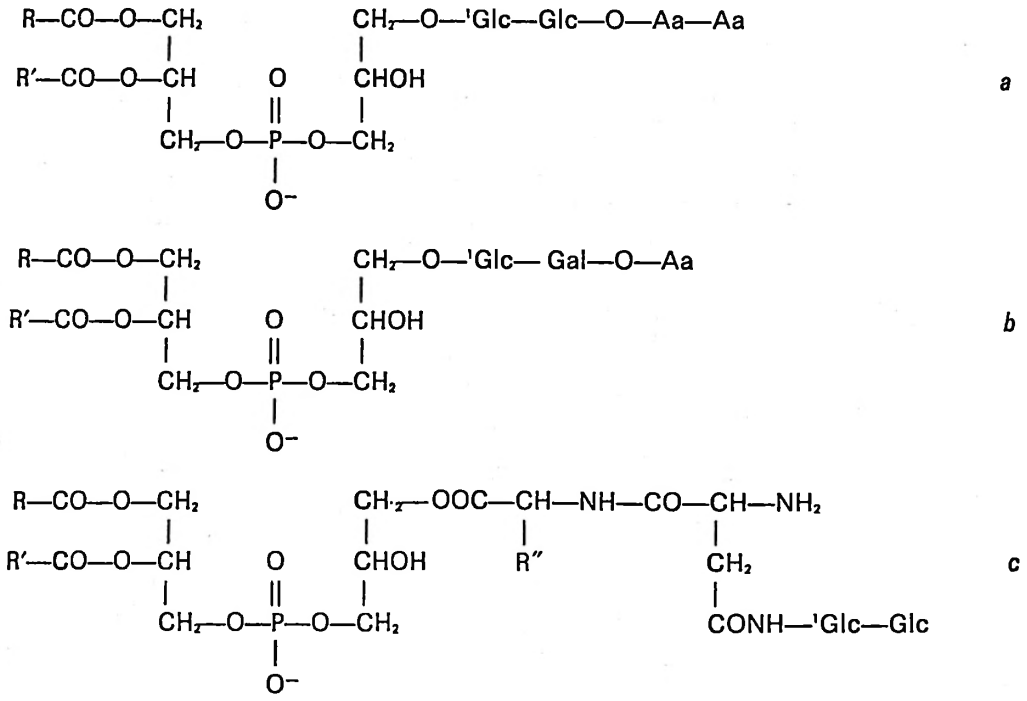
Discussion

Lipids are commonly found in nature as part of biological membranes. A great deal of the problems of lipid technology relies on lipid extraction techniques. As a rule, the extraction of lipids of different polarities requires the use of different solvents. Higher chaotropic effects are found with the use of more polar solvents, and it is thus advisable to use the different extraction solvents in decreasing order of polarity (15).

One of the more polar solvents in use for the study of membrane lipids is aqueous 80 % ethanol. It has been proposed by HUNTER and ROSE (6) for the extraction of microbial lipids, because of its ability to disrupt cell walls, which are usually resistant to the use of chloroform-methanol mixtures. The extraction of rat liver mitochondria with relatively high volumes of aqueous 80 % ethanol brings into solution, apart from a certain amount of the usual phospholipids, a series of highly polar lipids, up to now uncharacterized.

These polar lipids exhibit a marked preference for the «aqueous phase» (chloroform-methanol-water, 3:48:47) in the partition procedure described by FOLCH *et al.* (3). This rather unusual property might explain the fact that these lipids have not been more extensively studied previously. Nevertheless, this is not the first time that the existence of lipids in the «aqueous phase» is reported. Several workers have noticed this fact with respect to complex sphyngoglycolipids from the nervious system (17). It should be noticed, however, that sphyngoglycolipids are present only in negligible amounts in mitochondrial membranes (9).

The chemical composition of the lipids



Scheme 1. *Tentative structures for compounds 1 and 2.*
Structures *a* or *c* could correspond to compound 1, and structure *b*, to compound 2. R and R': fatty acid alkyl chains. R": Arg (or Lys) side chain. Glc: Glucose. Gal: Galactose. Aa: Aminoacid.

under study, (table II), is compatible with the structure of glycerophosphatides, bearing a bulky polar head. This hypothesis is confirmed by the alkali-labile character of the bound fatty acids (2) and the presente of L-α-glycerophosphate. Accordingly, scheme 1 shows some of the possible structures for compounds 1 and 2, on the basis of a glycerophosphatide type of lipid. Structure *c*, which could correspond to compound 1, includes an N-glycosidic linkage, which is widely found in glycoproteins (10).

The significance of these lipids in the outer membranes is not negligible from the quantitative point of view. The amount of compounds 1 and 2 in this membrane can be estimated as approximately 2.5 μmol of each par 100 mg protein. This corresponds to the same order of magnitude of other well known glycerophosphatides.

Increasing attention is being drawn to amphipathic molecules, such as the polyisoprenylsugars, acting as sugar carriers for protein glycosylation reactions, taking place within membranes. WHITE and WAECHTER (18) have recently shows the existence of an intermediate sugar acceptor, of lipid nature, soluble in chloroform-methanol-water (10:10:3) and insoluble in chloroform-methanol (2:1). The molecules here reported might correspond to substances of this type. This is supported by the fact that the outer membranes account for practically all the glycoproteins present in mitochondria.

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Resumen

El extracto etanólico de las membranas mitocondriales de hígado de rata contiene cierto número de lípidos complejos fuertemente polares, que se incorporan a la fase acuosa en los procedimientos usuales de partición cloroformo-agua. Dos de estos lípidos han sido purificados por cromatografía en capa delgada, y se han llevado a cabo estudios sobre su estructura. Ambos han resultado ser derivados del fosfatidilglicerol, con una cabeza polar muy voluminosa que contiene glícidos y aminoácidos. Están localizados específicamente en la membrana externa mitocondrial. Se proponen varias estructuras y un posible papel de estos compuestos.

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