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Highly Polar Non-Glycerol Containing Lipids Present in Outer Mitochondrial Membranes

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Aqueous 80 % ethanol extracts from the outer mitochondrial membranes contained a series of compounds, insoluble in chloroform-methanol (2:1) and soluble in chloroform-methanol-water (10:10:3). These compounds contain fatty acids, amino acids and hexoses. Procedures are described for the extraction, purification and cromatographic separation of these compounds. Five of them have been partially characterised. Some structural data are given. It is suggested that some relationship could exist with the glycosylation of the outer membrane proteins.

From the chemical point of view no clear-cut definition of the term «lipid» is available. In current laboratory practice the term «lipid» is applied to substances which can be extracted from biological material by certain organic solvents and will incorporate preferentially into the organic phase in a partition procedure, such as the well known chloroform-methanol-water (2:1:0.6) proposed by FOLCH *et al.* some years ago (3). Better yields are usually obtained by substituting the water by salt solutions, such as 0.88 % KCl. However, several workers have noticed the presence of fatty-acid containing material in the water phase, especially when working with polar lipids such as the complex sphingoglycolipids of the central nervous system (16). Moreover, in the field of microbial chemistry, there are many claims of complex lipopolysaccharides and lipopeptides partitioning preferentially into the water phase. Some of them may be found in both phases. Unpublished results from our laboratory show that the highly polar, ninhydrin positive phospholipid found in the yeast *Candida lipolytica* and referred to as «unidentified» by KATES and BAXTER (5) goes mainly into the water phase. As with many other lipids, the amount of these very polar lipids which can be extracted depends largely on the nature of the sol-

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vent employed. The aqueous 80 % ethanol solution is perhaps the most polar of the solvents currently in use in procedures of lipid extraction. It permits quantitative extraction of the more polar phospholipids and glycolipids. Preliminary work showed that ethanol extracts of rat liver mitochondria contained several «lipids» that went mainly, or only, into the aqueous phase using the FOLCH partition method (3). Two of those lipids were characterized as glycerol phosphatides, and their tentative structures are published separately (4). This report is concerned with the isolation, purification, separation and chemical characterization of five highly polar, non-glycerol containing phospholipids.

Materials and Methods

Wistar rats weighing approximately 200 g were used in all the experiments. Livers were homogenized in 0.175 M KCl and mitochondria isolated according to the method of HOGEBOOM (5). Inner and outer mitochondrial membranes were prepared according to the method of PARSONS *et al.* (11). Ascorbate-induced peroxidation was carried out as described by SANTIAGO *et al.* (14), except that 0.25 M sucrose was substituted by 0.175 M KCl. Proteins were determined by the method of LOWRY *et al.* (10). Lipid phosphorus was determined as described by BARTLETT (1) after wet ashing. Total sugars were determined by a micromodification of the anthrone method (13). Glucose was measured by enzymatic analysis (16). Fatty acids were determined as their methyl esters as described by LÓPEZ *et al.* (9). Aminoacids were quantified by means of the ninhydrin reaction, and analyzed in a Hitachi KLA-5 autoanalyzer. Carbohydrate components were investigated, after hydrolisis with 1 N HCl in boiling water baht 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 (12).

Polar lipids were extracted as follows: rat liver mitochondria were treated with 80 % aqueous ethanol (0.6-0.8 ml/mg of mitochondrial protein) with magnetic stirring for one hour. The suspension was centrifuged for 5 min at 30,000 × *g*, and the pellet reextracted and centrifuged in the same way. The supernatants were pooled to constitute the «ethanolic extract».

The ethanolic extract was evaporated to near dryness at 37° C, and redissolved in 23 ml of chloroform-methanol-water (10:10:3). The solution was then poured into a separation funnel, and 10 ml of chloroform and 3 ml of water added. The solution was agitated and the funnel kept at 4° C until phase separation was complete. The organic phase was discarded, and the aqueous layer evaporated to dryness under reduced pressure. The dry residue was then redissolved in a small volume of chloroform-methanol-water (10:10:3) and transferred onto the chromatographic plate. Alternatively, proteins fractions belonging to a second, well marked peak, were pooled, evaporated and fractionating the sample in a small Sephadex LH-20 column, with 80 % ethanol as eluent. In this case the eluate was monitored by measuring the absorption at 215 nm. A first peak, corresponding to the void volume, was discarded, and the fractions belonging to a second, well marked peak, were pooled, evaporated and redissolved in chloroform-methanol-water (10:10:3) for the chromatographic separation, as indicated.

Separation was carried out on thin layer chromatography plates of Silicagel HF 254 (Merck), activated at 120° C for 30 minutes. Polar lipids corresponding to some 30 mg of mitochondrial proteins were applied in the form of a narrow strip 3 cm long and under cold air. The plates were developed in two consecutive runs of chloroform-methanol-acetic acid-

water (100:100:10:15), thoroughly drying the plate between them. After the second run, the plates were dried. One part of each plate was stained with a ninhydrin reagent at room temperature in order to locate each spot. The non-stained spots were scraped off with the additional aid of the U.V. light, and eluted with chloroform-methanol-water (10:10:3).

Results and Discussion

The ethanolic extract contained 10.5 ± 0.26 % of the original protein, and 70 ± 3.4 % of the original lipid phosphorus. Practically all the protein, and the phosphorus corresponding to the main glycero phosphatides, were eliminated by means of the chloroform-water partition and Sephadex chromatography.

The ninhydrin stained chromatogram showed, besides several pink-red spots of higher Rf, five purple spots of Rf ranging from approximately 0.6 to 0.1. These five spots could be purified by elution and rechromatography, as well as by two-dimensional chromatography, using diisobutylketone-acetic acid-water (40:25:5) as the second solvent. Figure 1 shows the recromatography of these five spots, which will be referred to respectively as A, B, C, D and E. The presence of fatty acids, phosphorus, carbohydrate and aminoacids was evidenced in all these five spots. The presence of purine and/or pirimydine bases, glyceroy, sugar alcohols, aminosugars and sialic acids was discarded by several procedures.

These polar lipids were investigated in different mitochondrial subfractions, with the result that they were located predominantly, or exclusively in the outer mitochondrial membrane.

Mitochondria were subjected to ascorbate-induced peroxidation (14). After mitochondrial lysis, ethanol was added up to a 50 % concentration, and the suspension centrifuged at 100,000 × g for 1 hour at 4° C. The polar lipids could be found in the pellet of mitochondria incubated without ascorbate, but not in that obtained from mitochondria whose lipids had been peroxidized. This result suggests that either these lipids have been destroyed by peroxidation, or they have been solubilized following the destruction of the outer membrane.

Table I shows the fatty acid composition of each band, as derived from gas-liquid chromatography. It can be seen that each spot shows a characteristic pattern of fatty acids. Bands A and B were the only ones containing 20:4, but band B contained less 18:1. Band C was characterized by its high 16:0 and low 18:2 content. Band D had a high proportion of 18:2. Finally, band E contained a high

Fig. 1. *Rechromatography of the five compounds described in the text.*
Chromatographic conditions and other details are described in the text. The chromatogram was stained by charring at 200° C for 20 min after having sprayed 5 % H_2SO_4 in absolute ethanol.

Table I. *Percent fatty acid composition of the five compounds described in the text.* Number of experiments: 6. The figures correspond to mean values ± standard deviation of the mean.

Compound	C 12	C 16	C 18	C 18:1	C 18:2	C 20:4
A	5.2±0.58	22.0±2.61	22.6±1.17	17.6±2.73	14.4±1.50	18.2±1.02
B	1.8±0.32	26.0±1.52	30.2±1.77	9.0±1.00	13.2±1.11	18.8±1.50
C	4.0±0.32	40.8±0.37	25.6±2.11	20.4±1.21	7.0±1.30	—
D	4.0±0.32	31.2±2.4	23.6±1.69	20.2±1.69	21.4±1.99	—
E	3.4±0.24	39.2±2.4	28.4±1.50	17.8±1.69	11.8±0.73	—

percentage of 16:0. When subjected to alkaline hydrolisis under very mild conditions (2) all the fatty acids were liberated. This result leads us to think that all the fatty acids are probably linked through ester bonds. The only sugars present were glucose and/or galactose (table II). Table III shows the amino acid pattern of each of the five bands. The presence of aspartic acid and ammonia in equimolar amounts may indicate the presence of asparagine.

Attempts were made to determine the molecular weight of these compounds by chromatography on Sephadex LH-20. The lipids, deacylated according to DAWSON (2), were also chromatographed on Sephadex G-10. Molecular weights of 1,000-1,300 daltons were assigned to the polar lipids under study.

Quantitative determination of the different components of each band was carried out, in order to establish the molar proportions of each component (table IV). The data presented here are in agreement with the given molecular weights.

These experiments permit also an estimation of the amount of polar lipids, and of their significance in the molecular architecture of the mitochondrion. Some 0.1-0.2 μmoles of each of the five compounds were present per 100 mg of mitochondrial protein. However, when these polar lipids are considered only in relation with the lipid content of the outer membrane, where they are located, a relationship of approximately 2.5 μmoles per 100 mg protein has been found. The amounts of other well known lipid components of this membrane are approximately of the same order.

Although a definite evidence for the complete molecular structure of these liquids is not yet available, some reasonable assumptions could be made, as a working hypothesis. Probably, the backbone of the molecule is, in every case, the sugar moiety, having —OH groups sterified by the fatty acids and phosphate. We have already mentioned that the fatty acids were linked through alkali-labile bonds. Moreover, the original lipids were not susceptible to periodate oxidation thus indicating the absence of free vicinal hydroxyl groups. On the other hand, the deacylated products gave a positive perio-

Table II. *Presence of carbohydrates in the five compounds mentioned in the text.*

	A	B	C	D	E
Glucose	+	—	—	+	+
Galactose	+	+	+	+	—

Table III. *Aminoacids present in the five compounds mentioned in the text.*

Compounds	Lys	NH_3	Arg	Asp	Ser	Cys
A	+	+	+	+	+	—
B	+	+	—	+	+	+
C	+	+	—	+	+	—
D	+	+	—	+	+	—
E	+	+	—	+	+	+

Table IV. *Molar proportion of the different components of lapids described in the text.*

Compounds	Fatty acids	Phosphorus	Glucose	Galactose	Amino acids
A	3	1	1	1	1
B	3	2	—	2	1
C	2	2	—	3	1
D	3	1	1	2	1
E	2	3	1	—	2

date-bencidine test. Ester bonds between fatty acids and sugars have already been described by other authors (7). Acid hydrolysis under mild conditions (1 N HCl, 30 min in boiling water bath) easily removed the amino acids. This may indicate again an ester bond between the carboxyl group of the amino acid and the sugar. In the case of the presence of asparagine, an N-glycosidic bond could exist between the anomeric carbon of the sugar and the ε-amide group of the amino acid. Both types of bonds have been described in several glycoproteins (8).

With respect to the possible function of the polar lipids described, no data are available at present. However, some relation could exist with the glycosilation of glycoproteins. It must be remembered that nearly all mitochondrial glycoproteins are located in the outer membrane. Similarly polar lipids are found in microsomal membranes, that are rich in glycoproteins (unpublished). Recently it has been shown (18) that there is a lipid intermediate between the polyisoprenylsugar and the protein in the glycosylation reaction. This lipid, whose structure is not given, is, as those here described, insoluble in chloroform-methanol (2:1) and soluble in chloroform-methanol-water (10:10:3).

Another possible function could be related to the permeability of the outer membrane that, quite surprisingly, is much higher than that of the inner membrane, although its lipid content is three times higher, and contains most of the mitochondrial cholesterol.

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Resumen

La disolución acuosa de etanol al 80 % extrae de las membranas externas mitocondriales unos compuestos insolubles en cloroformo-metanol (2:1) y solubles en cloroformo-metanol-agua (10:10:3). Estos compuestos contienen ácidos grasos, aminoácidos y hexosas. Se describe un procedimiento de extracción, purificación y separación cromatográfica de estos compuestos, y se estudia la composición química de cinco de ellos. Se proponen datos estructurales y se sugiere su relación con las reacciones de glicosilación de las proteínas de la membrana externa.

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