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LIPID COMPOSITION AND PROTEIN PROFILES OF OUTER AND INNER MEMBRANES FROM PIG HEART MITOCHONDRIA

COMPARISON WITH MICROSOMES

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SUMMARY

1 Mitochondria, inner and outer mitochondrial membranes and microsomes were isolated and purified from pig heart Their lipid composition and protein components were studied

2 The fatty acid distribution in the main phospholipids seemed specific rather of a given phospholipid and not of one type of membrane

3 Inner mitochondrial membranes were characterized by a high content in cardiolipin and a very low level of triglycerides together with a high degree of unsaturation and C_{18} acids Gel electrophoresis revealed 13 different polypeptide subunits of which 5 were major ranging in molecular weights from 10 000 to 215 000

4 In outer mitochondrial membranes, total lipid, phosphatidylcholine, phosphatidylinositol, plasmalogen and triglyceride contents were much higher than in inner membranes Fatty acids of phospholipids were mostly saturated and the polypeptide pattern showed 12 components, of which 4 were major of mol wt 75 000, 60 000, 20 000 and below 10 000

5 Compared to outer membrane, microsomes exhibited a much higher cholesterol content and markedly different protein profiles They contained significant amounts of cardiolipin and phosphatidylserine, this latter phospholipid being exclusively located in microsomes However odd similarities were observed in some lipid components of microsomes and inner mitochondrial membranes, but fatty acids were more saturated in microsomes and electrophoretic profiles of protein components appeared very different and revealed components of high mol wt

INTRODUCTION

Everybody agrees that membrane functions are determined by the nature of lipid and protein components involved and also by the spatial arrangements of these

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components within the membrane and their interactions with small ions and molecules In a previous work [1] inner and outer membranes from pig heart mitochondria were purified with a view to the study of thiol-bearing proteins possibly implicated in the energization of the inner membrane and energy conservation [2] and to elucidate the rôle of an isolated proteolipid in relation to glutamate transport [3] The present work is a detailed study of both lipid components and protein profiles of pig heart mitochondrial and microsomal membranes and their distribution Such a study has never been conducted in parallel on the same fractions even in rat liver mitochondria in spite of several investigations on proteins [4–7] or lipid fractions [8–14] Up to now, only partial results have been obtained with heart, either on lipids of whole mitochondria [15–21] or on outer membrane lipids or proteins [22–23] or on proteins of submitochondrial particles [24]

Great care was taken to control lysosomal and microsomal contaminants in mitochondrial fractions The results show marked differences in protein profiles and lipid composition of mitochondrial and microsomal membranes

METHODS

Pig hearts, obtained from the slaughter house and brought back to the laboratory packed in ice, were used 30 min after the electrocution of the animals Mitochondria were isolated by a procedure derived from that of Crane et al [25], and washed twice, tested for respiratory control ratios, protein concentration and ADP/O, as previously [26] Purified outer and inner membranes, and microsomes, were obtained as previously described [1]

Marker enzymes

The following markers were estimated as previously [1] to assess the purity of the membranes monoamine oxidase (EC 1 4 3 4) for outer membrane, rotenone-sensitive NADH-cytochrome *c* reductase (EC 1 6 99 3) and cytochrome oxidase EC 1 9 3 1) for inner membrane, NADPH-cytochrome *c* reductase (EC 1 6 2 4) was the only reputed microsomal marker to be used since pig heart does not contain glucose-6-phosphatase (EC 3 1 3 9)

Although our mitochondrial preparations did not appear to be contaminated by lysosomes [1], acid phosphatase activity (EC 3 1 3 2) was tested in the various fractions to evaluate possible lipid contaminations by lysosomal fragments The release of *p*-nitrophenol from *p*-nitrophenyl phosphate was measured spectrophotometrically at 420 nm after 5 and 10 min incubation of the fractions at pH 4 6, 0 1 M citrate (K) buffer, 37 °C according to Lindhardt and Walter [27]

Lipid extraction and analysis

Lyophilized pellets (mitochondria, inner or outer membranes, or microsomes) were successively extracted thrice with chloroform/methanol (2 1) under nitrogen (1 mg protein/5 ml solvent) Each extraction lasted 2 h After filtration of membrane residues on a Millipore glass-fiber filter, solvents were pooled and freed of non-lipid impurities by washing with 0 2 volume 0 9 % NaCl according to Folch et al [28] The lower phase was collected, dried over Na_2SO_4 and evaporated to dryness in a rotative vacuum device The total lipid obtained was dissolved in chloroform and

fractionated into neutral lipids and phospholipids by column chromatography on silicic acid (1 g silicic acid, Mallinckrodt, 100 mesh+0 5 g celite 545, Johns-Manville, for 30 mg total lipid, 1 mg lipid phosphorus) If present, glycolipids can be eluted from such a system by chloroform/acetone mixtures Neutral lipids were eluted by 20 ml chloroform, phospholipids by 20 ml methanol

Total glycerides were estimated in neutral lipid eluate after alkaline hydrolysis in 2% methanolic potassium hydroxide, 1 h, 80 °C, by enzymatic glycerol determination [29]

Neutral lipids were analyzed by thin layer chromatography on Silica Gel G plates For qualitative studies, five solvent systems were used I, hexane/ethyl ether/acetic acid (70 30 1) [30], II, hexane/ethyl ether/acetic acid (76 24 1) [15], III, hexane/ethyl ether/acetic acid (70 30 2) [31], IV, hexane/ethyl ether (80 20), V, hexane/ethyl ether (50 50) Free fatty acids, triglycerides, coenzyme Q, cholesterol, cholesteryl esters were detected as previously described [15]

For quantitative studies, solvent II was used, spots were revealed with iodine vapours, scraped off and eluted with chloroform/methanol (2 1) Cholesterol and cholesteryl esters were estimated colorimetrically according to Ferrand and Rieffel [32] Triglycerides were hydrolyzed in 2% methanolic-KOH, 1 h, at 80 °C, glycerol was determined enzymatically [29]

The polar lipids, obtained by elution of the silicic acid column with methanol, were separated on silica gel by thin layer chromatography, either by one migration in chloroform/methanol/water (75 25 4), or by two-dimensional migration, first in chloroform/methanol/water/28% aqueous ammonia (130 70 8 0 5), then in chloroform/acetone/methanol/acetic acid/water (100 40 20 20 10) according to Parsons and Patton [33] To estimate plasmalogens, an aliquot of the polar lipids was treated by two-dimensional thin layer chromatography after development in the first direction in chloroform/methanol/water (65 25 4) solvent a 5 mM $HgCl_2$ solution in 0 1 M acetic acid was sprayed on the lipid track After drying the plate in vacuo, the second direction migration was performed in butanol/acetic acid/water (60 20 20) Controls, identification of lipid spots were conducted as previously described [15]

Quantitative analysis of phospholipids was effected by phosphorus determination after two-dimensional thin layer chromatography according to Bartlett [34], spots being detected by iodine vapours, scraped off and eluted by three successive washings with chloroform/methanol (2 1)

The fatty acids from isolated phospholipids were analyzed by gas-liquid chromatography after direct transesterification into fatty acid methyl esters with 14% BF_3 in methanol (Merck reagent) according to Morrison and Smith [35] Analyses were carried out on an "Intersmat chromatograph" equipped with a flame ionization detector using a column packed with 10% diethyleneglycol/succinate polyester on chromosorb W-AW-DMCS, 80–100 mesh (2 m×0 125 inch), operated isothermally at 180 °C The structural assignments were based on comparisons with standards, by graphic plotting of logs of retention times as a function of carbon number, by modification of retention times by alternative passage through polar and non-polar columns (13% Apiezon impregnated Anakrom, 3m×0 125 inch, 196 °C) Peak areas were evaluated by the triangulation method or by planimetry

All standard errors of the mean have been calculated

Protein analysis

Protein estimations in fractions were determined according to Lowry et al [36] using bovine serum albumin as a standard

Electrophoresis was performed according to Maizel [37] in gels containing 7 5 % acrylamide, 0 2 % bisacrylamide, 0 1 % sodium dodecyl sulfate, 5 M urea, 0 01 % *N*, *N*, *N'*, *N'*-tetramethylene-diamine (TEMED), 0 1 % ammonium persulfate, 0 01 % EDTA, 0 19 M sodium phosphate buffer, pH 7 4 The gel solution was poured into glass tubes (6 mm × 150 mm) to a height of 130 mm and overlayered with water After overnight gelation, water was replaced by electrophoresis buffer 1 M sodium phosphate, 0 1 %, sodium dodecyl sulfate, pH 7 4, current (24 mA, 12 tubes) was run through the tubes for 1 h

Samples (50–100 μg protein/100 μl) were solubilized by 8 1 M urea/0 8 % sodium dodecylsulfate/16 mM β-mercaptoethanol, and dipped for 1 min into a water bath at 100 C Pyronin Y (10 μg/sample) was used as tracking dye Samples were layered below the buffer onto the gel surface A current of 96 mA (12 tubes) was administered until the tracking dye migrated to within 2–3 mm of the end of the gel (30 h) The use of 0 6 M phosphate buffer slowed migration time to 15 h Gels were fixed in 50 % trichloracetic acid for 8 h , rinsed in water and stained for protein with Coomassie blue 0 2 g/100 ml methanol water (1 1), 7 ml glacial acetic acid being added just before use Staining was performed 3–4 h at 45 °C Destaining was conducted in 75 ml acetic acid/250 ml ethanol/50 ml methanol, water to complete the volume to 1000 ml Gels were scanned with an ISCO scanning device, UA-5, at 580 nm The gel system was calibrated for molecular weight determinations by measuring the migration of several standard proteins of known molecular weight, solubilized in the same conditions as the samples (20 μg/100 μl) and kept at 55 C for 1 h cytochrome *c* (11 700, Boehringer), ovalbumin (43 000, Sigma), glutamate dehydrogenase monomer (53 000, Boehringer), bovine serum albumin (monomer 68 000, dimer 136 000, trimer 204 000, Koch-light), the purest grade

RESULTS

The distribution of protein in pig heart submitochondrial fractions was studied

The averages of three different experiments show that inner membrane + matrix (mitoplasts) contained 87 5 % of mitochondrial protein, purified inner membrane represented only 60 4 % after three centrifugations and two passages through two different sucrose gradients Only the purest layer, as tested by marker enzyme activities, was used for further analysis

In contrast, crude outer membrane amounted to only 6 4 % of mitochondrial protein, after purification and passage through a sucrose gradient, the purest outer membrane fraction represented only 1 % mitochondrial protein, as tested by monoamine oxidase activity, it was purified 13-fold and contained less than 8 % contamination by markers from the inner membrane, all the following determinations were conducted on this purest fraction It should be stressed that due to the very low content of the outer membrane as compared to the inner membrane, it cannot be completely recovered during the fractionation procedure, this means that the protein amount due to outer membrane, even in the crude fraction, is underestimated

Matrix represented 13 % and intermembrane space only 3 4 % of total protein Therefore, it can be assumed that the outer membrane contained about 9 % of total protein (100 % minus 87 5 % of mitoplasts = 12 5 % for outer membrane plus the intermembrane space) And as expected from electron micrography [1] inner membiane represented the major part of pig heart mitochondrial protein and the matrix appears as only a small compartment

1 Lipid composition of inner and outer membranes as compared to whole mitochondria and microsomes

Total lipid, lipid-P and cholesterol in pig heart mitochondrial and microsomal membranes Table I shows that lipid and cholesterol contents in outer membrane were much higher than in inner membrane, in fact the cholesterol content of inner membrane was not significant and diminished along the purification of the membrane The traces of cholesterol detected in inner membrane might be due to minor lysosomal contaminations No such contamination could be observed using electron microscopy Acid phosphatase is widely used to detect lysosomal contaminations, it was not possible to measure the specific activity of this enzyme in purified intact lysosomes since the first steps of the fractionation procedure of heart muscle, which gives intact mitochondria, disrupt a large part of the lysosomal fraction, thus solubilizing lysosomal enzymes [38–39] Indeed when the distribution of acid phosphatase activity was measured in the various subcellular fractions (several experiments), most of the activity was detected in the post-nuclear supernatant, the crude mitochondrial fraction contained only 8 %, the microsomes about 4 % of the total activity and the final post-microsomal supernatant contained most of the activity (66 %) The activity measured at pH 4 6 using *p*-nitrophenyl phosphate as substrate was insensitive to oligomycin, when tested at pH 8 3 (optimal pH for mitochondrial

TABLE I

TOTAL LIPID AND CHOLESTEROL CONTENT OF PIG HEART MITOCHONDRIAL AND MICROSOMAL MEMBRANES

In parenthesis number of different preparations Acid phosphatase activity expressed in nmol *p*-nitrophenol produced min^{-1} mg^{-1} protein, it was estimated to evaluate lysosomal contamination

	Mitochondria	Inner membrane	Outer membrane	Microsomes
mg lipid/mg protein	0 30±0 09 (10)	0 48±0 03 (3)	0 85±0 10 (3)	0 41±0 10 (3)
lipid-P/mg protein* (μmol P/mg protein)	0 26±0 07 (10)	0 40±0 08 (3)	0 66±0 10 (3)	0 36±0 10 (3)
cholesterol/mg protein (μmol/mg protein)	0 012±0 001 (4)	0 0047±0 001 (3)	0 026±0 001 (3)	0 048 (2)
cholesterol/lipid-P (molar ratio)	0 042±0 008 (4)	0 011±0 002 (3)	0 068±0 002 (3)	0 107 (2)
acid phosphatase	7 7±0 5 (16)	2 6±0 5 (6)	20 4±2 0 (5)	16 1±0 9 (9)

* Based on the average M_r of phospholipids, the weight of phospholipid in mg/mg protein is hardly different from the number of μmol lipid-P/mg protein Indeed the average M_r of a phospholipid = 30-fold the weight of phosphorus Therefore, 1 μmol lipid-P is equivalent to $1 \times 31 \times 30$ μg phospholipid, i e about 1 mg phospholipid

ATPase) mitochondria did not hydrolyse *p*-nitrophenyl phosphate, the soluble F_1-ATPase recently purified from pig heart [40] did not hydrolyze *p*-nitrophenyl phosphate at pH 4 6 or 8 3

The outer membrane appeared quite different from microsomes by its much higher lipid and phospholipid content and its much lower cholesterol content Its phosphatase activity reflects some contamination by lysosomal fragments (not detectable using electron microscopy [1]) and could be responsible for an overestimation of cholesterol in this membrane

The parallelism of total lipid and phospholipid content in each fraction indicates that practically the total lipid was constituted by phospholipids

No glycolipids were detected in mitochondrial fractions

Phospholipid distribution in pig heart mitochondrial and microsomal membranes Table II gives the distribution Inner membrane, like whole purified pig heart mitochondria, contained three major phospholipids phosphatidylcholine, phosphatidylethanolamine and cardiolipin Cardiolipin and phosphatidylethanolamine contents were higher in purified inner membrane, phosphatidylcholine and phosphatidylinositol levels were lower in inner membrane and higher in outer membrane than in mitochondria

No phosphatidylserine could be detected in either whole mitochondria or inner and outer membranes Lysoderivatives appeared as traces, the inner membrane containing minor amounts of lysophosphatidylethanolamine and lysophosphatidylcholine while outer membrane contained only the latter compound

No cardiolipin was detected in outer membrane

Mitochondria contained a minor amount of sphingomyelin which appeared

TABLE II

PHOSPHOLIPID DISTRIBUTION IN PIG HEART MITOCHONDRIAL AND MICROSOMAL MEMBRANES

In parenthesis number of different preparations, two assays for each preparation Phospholipid contents are expressed as percentages of total lipid-phosphorus Data represent mean values ± standard deviation

	Mitochondria (10)	Inner membrane (4)	Outer membrane (4)	Microsomes (7)
Lysophosphatidylcholine	0 5±0 2	0 6±0 2	1 3±0 7	0 2±0 1
Sphingomyelin	1 7±0 3	0 5±0 2	4 5±2 1	7 1±0 5
Phosphatidylcholine (plasmalogens* included)	42 4±0 9	26 5±2 3	56 3±2 1	32 ± 1 1
Phosphatidylinositol	4 5±0 2	3 4±0 1	9 3±3 1	8 3±0 4
Phosphatidylserine	0	0	0	1 5±0 5
Phosphatidylethanolamine (plasmalogens* included)	30 5±0 7	37 9±0 4	28 0±2 1	37 4±0 8
Lysophosphatidyl-ethanolamine	0 3	1 3±0 1	0	0
Cardiolipin (plus phosphatidic acid)	18 1±0 5	25 4±1 6	0 4±0 2	11 7 2 1

* See Table III for a detailed study of plasmogens

TABLE III

PLASMALOGEN CONTENT IN PIG HEART MITOCHONDRIAL AND MICROSOMAL MEMBRANES

In parenthesis number of different preparations, two assays for each preparation Phospholipid contents are expressed as percentages of total lipid-phosphorus Data represent mean values ± standard deviation

	Mitochondria (10)	Inner membrane (4)	Outer membrane (3)*	Microsomes (4)
Lecithin	42 4±0 9	26 5±2 3	56 3±2 1 (4)	31 9±1 1
Diacyl phosphatidylcholine	26 7±1 0	18 4±2 5	34 8±3 1	25 8±1 8
Choline plasmalogen	15 7±0 9	7 ±0 7	23 2±2 8	7 1±0 3
Ratio $\frac{\text{Diacyl phosphatidylcholine}}{\text{Choline plasmalogen}}$	1 7	2 6	1 5	3 6
Cephalin	30 5±0 7	37 9±0 4	28 ±2 1 (4)	37 4±0 8
Diacyl phosphatidylethanolamine	17 2±0 7	23 2±1 4	17 3±4 2	16 1±0 3
Ethanolamine plasmalogen	13 3±0 7	19 7±0 7	12 4±0 5	22 8±1 9
Ratio $\frac{\text{Diacyl phosphatidylethanolamine}}{\text{Ethanolamine plasmalogen}}$	1 3	1 2	1 4	0 7
Total plasmalogen content	29	26 7	35 6	29 9

* 3 different preparations unless stated otherwise

TABLE IV

FATTY ACID DISTRIBUTION IN THE MAIN PHOSPHOLIPIDS OF PIG HEART MITOCHONDRIAL AND MICROSOMAL MEMBRANES

Values are expressed as percentages of recording area of the sum of fatty acids in gas-liquid chromatography Fatty acids Shorthand designation Mito, whole mitochondria, IM, inner membrane, OM, outer membrane, Micro, microsomes Phospholipids were first separated by thin layer chromatography, the spots revealed with iodine vapours, scrapped, the iodine evaporated, transmethylation was directly conducted on the silica gel, then methylated fatty acids were extracted by pentane and passed through the chromatograph on a 10 % diethyleneglycol-succinate polyester column (for details see Methods) In parenthesis, number of experiments

Fatty acid	Phosphatidylethanolamine				Phosphatidylcholine				Cardiolipin		Phosphatidylinositol			
	Mito (2)	IM (4)	OM (2)	Micro (2)	Mito (2)	IM (4)	OM (2)	Micro (2)	Mito (2)	IM (4)	Mito (2)	IM (4)	OM (2)	Micro (2)
$C_{14\ 0}$	2 5	1 7	2 8	1 2	2 2	2 4	1 8	2 9	1 4	1 4	2 7	1 7	3 5	2 3
$C_{15\ 0}$	3 7	1 8	3 1	2 1	6 3	5 6	5 4	5 2	0 6	0	3 9	3 2	3 2	5 5
$C_{16\ br}$	3 3	3 2	5 7	4 0	1 4	1 0	0 7	3 2	0 5	1 7	1 0	1 2	1 1	2 3
$C_{16\ 0}$	6 9	9 1	10 8	7 9	23 5	27 6	29 9	26 3	10 7	9	22 3	23 2	26 3	26 2
$C_{16\ 1}$	7 7	3 8	1 4	1 6	1 7	1 6	1 0	2 5	1 7	2 4	2 0	2 2	2 9	2 1
$C_{17\ 0}$	1 6	2 0	1 1	2 9	2 2	0 8	2 0	2 2	0 9	1 6	0 8	1 5	2 9	1 1
$C_{18\ br}$	3 4	2 6	4 5	1 7	1 3	1 0	1 1	1 9	0	0	2 1	1 9	0 6	traces
$C_{18\ 0}$	20 3	19 7	25 1	15 1	8 7	9 4	8 2	8 9	10 3	6 3	36 3	32 3	30 5	34 6
$C_{18\ 1}$	5 1	8 6	9 3	5 4	15 0	16 4	14 1	14 6	16 1	11 5	6 1	9 3	8 9	7 2
$C_{18\ 2}$	19 4	22 1	18 1	27 9	33 0	29 0	29 7	27 3	55 4	65 2	16 2	13 0	14 6	13 9
$C_{18\ 3}$	5 6	2 3	2 8	3 4	0 6	1 3	1 4	0	0 3	0	0	4 3	0	0
$C_{20\ 1}$	0	2 1	0	3 3	0	0	0	0	0	0	2 1	2 2	1 1	0
$C_{20\ 4}$	20 0	20 5	14 5	22 5	4 2	1 8	4 1	4 8	1 8	0	4 2	2 8	4 2	4 5
Percent saturated	41 7	39 1	53 1	34 9	47 0	47 8	49 1	50 7	25 3	20	69 1	66 0	67 8	72 0
Percent unsaturated	57 8	59 4	46 1	64 1	52 5	52 1	50 3	49 2	75 3	79 1	30 6	33 8	31 7	27 7
Ratio $\frac{\text{saturated}}{\text{unsaturated}}$	0 72	0 66	1 15	0 54	0 89	0 91	0 97	1 03	0 33	0 25	2 6	1 93	2 1	2 6
Σ C18	53 8	55 3	59 8	53 5	58 6	59 1	54 5	52 7	82 1	83	62 8	64 0	55 7	55 7

to be located in outer membrane, indicating, like acid phosphatase activity (Table I), some contamination by lysosomal debris

Microsomes contained no lysoderivatives, some phosphatidylserine, significant amounts of sphingomyelin, phosphatidylinositol and cardiolipin (but they did not exhibit cytochrome oxidase activity), their major components were phosphatidylethanolamine and phosphatidylcholine

Table III gives the distribution of plasmalogens in the various fractions

The inner membrane appeared to be very poor in choline plasmalogen as compared to the outer membrane and whole mitochondria, but has the same content as microsomes

Outer membrane was very rich in lecithin and cephalin derivatives, diacyl- and plasmalogen-derivatives, its plasmalogen content is very high compared to the other fractions

Microsomes appeared very poor in choline plasmalogen and rich in ethanolamine plasmalogen containing a third of the content of outer membrane in choline plasmalogen, and twice as much ethanolamine plasmalogen as the outer membrane

Coenzyme Q content and glyceride distribution in pig heart mitochondrial and microsomal membranes Only minor glyceride amounts were detected, as expressed in nmol mg^{-1} protein mitochondria 14, inner membrane 6 4, outer membrane 12 7, microsomes 6 4, they were essentially triglycerides

Coenzyme Q could be detected only in whole mitochondria and in inner membranes (absorbance at 275 nm)

Fatty acid distribution in the main phospholipids of pig heart mitochondrial and microsomal membranes We see in Table IV that the fatty acids from phosphatidylcholine and phosphatidylinositol appeared to be evenly distributed in mitochondria, inner and outer membranes, and in microsomes Their distribution depended mainly on the nature of the phospholipid and not of that of the membrane This was not the case for phosphatidyl ethanolamine mitochondria and the inner membrane always presented the same pattern and contained more palmitoleic acid (16 1) than did outer membrane and microsomes, the outer membrane was rich in stearic acid (18 0) and poor in arachidonic (20 4), microsome content in oleic acid (18 1) was higher than mitochondrial content while stearic acid (18 0) was lower

The distribution of fatty acids in each phospholipid appeared different from one another

The degree of unsaturation was high 80 % in cardiolipin, 60 % in phosphatidylethanolamine (except in outer membrane, 46 %) 50 % in phosphatidylcholine In contrast, phosphatidyl-inositol was very saturated (70 % saturated acids)

A high C_{18} content was observed 60 % in phosphatidylethanolamine, -choline or -inositol, while it reached 83 % in cardiolipin Very high values of linoleic acid ($C_{18\ 2}$) were observed in cardiolipin

Arachidonic acid ($C_{20\ 4}$) seemed almost exclusively located in phosphatidylethanolamine which contained also much stearate ($C_{18\ 0}$) and little palmitate ($C_{16\ 0}$) On the contrary phosphatidylcholine was rich in palmitate and poor in stearate, and contained more C_{18} unsaturated acids (48 %) than did phosphatidylethanolamine (24 %)

Phosphatidylinositol was the richest in stearate Low levels of branched or

odd numbered acids were observed except in cardiolipin, pentadecanoic acid being the most important Dimethylacetals derived from plasmalogens were searched for and could not be detected

Inner membrane appeared very unsaturated and outer membrane more saturated Striking differences were observed between microsomes and outer membrane at the level of phosphatidyl ethanolamine ($C_{18\ 0}$, $C_{18\ 2}$ and $C_{20\ 4}$)

Protein gel electrophoresis of pig heart mitochondrial and microsomal membranes

Fig 1 shows the polypeptide patterns obtained in urea-sodium dodecyl

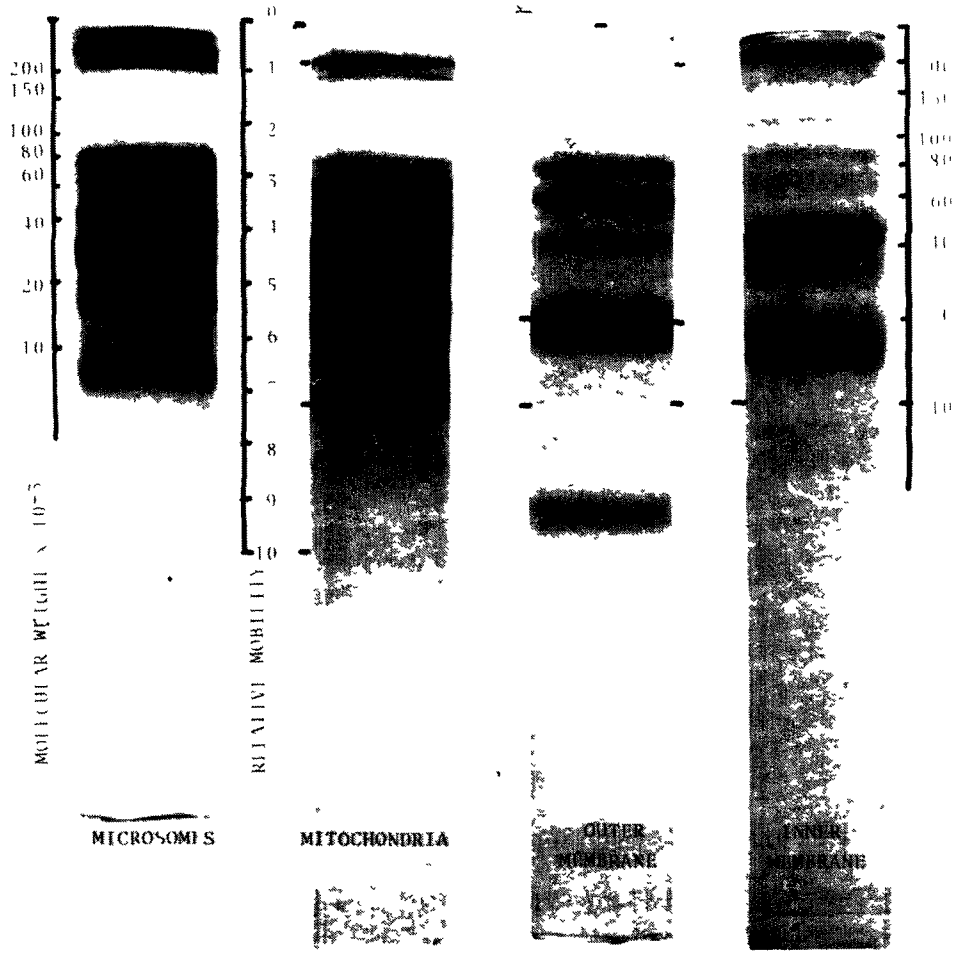

Fig 1 Compared polypeptide patterns of pig heart mitochondrial and microsomal membranes in urea/sodium dodecyl sufate/polyacrylamide gel electrophoresis Procedure and calibration of molecular weight (left, for microsomes, and right scale for mitochondrial fractions) as described in Methods Staining with Coomassie blue Densitometry scanning at 580 nm revealed the following bands of molecular weight microsomes, 300 000, 245 000, 200 000, 70 000, 63 000, 56 000, 50 000 42 000, 36 000, 27 000, 15 000, 10 000 inner membrane, 215 000, 170 000, 125 000, 90 000 72 000, 48 000, 36 000, 25 000, 20 000, 16 600, and three 10 000, outer membrane, 170 000 75 000, 60 000, 50 000, 44 000, 38 000, 32 000, 26 000, 20 000, 10 000

sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining after solubilizing whole mitochondria, purified inner and outer mitochondrial membranes and microsomes in 8 M urea, 0 8 % sodium dodecyl sulfate, 16 mM β-mercaptoethanol With whole mitochondria, 16 distinct polypeptide bands were separated, 12 with outer membrane, 13 with inner membrane and 13 with microsomes

The number of different bands revealed is probably not the most important result since it has been shown that this number may vary as a function of the electrophoresis system [41–42] But densitometry scanning at 580 nm (Fig 1) shows that the four fractions had different profiles which can be summarized as follows Inner membrane contained 5 major peaks (mol wt 215000, 48000, 36000, 20000, 16 600) and 8 minor among which 3 < 10 000 Outer membrane exhibited 4 major peaks (mol wt 75000, 60000, 20000 by the most important < and 10000) and 8 minor, among which one of mol wt 170000 Microsomes were mainly characterized by 3 components of high mol wt (300000, 245000 and 200000) and 1 < 10000, which were not detected in outer membrane

DISCUSSION

The cholesterol content of pig heart membranes appeared to be very low as compared to liver mitochondrial [12, 43–46] or microsomal [44–48] fractions The fact that pig heart outer membrane contained only half the cholesterol content of rat liver outer membrane might explain that digitonin was not efficient to isolate this outer membrane [49, and unpublished results by ourselves], however digitonin treatments lowered the molar ratio cholesterol/phospholipid, from 0 025 to 0 011 in pig heart mitochondria Our values are in good agreement with already published data on heart whole organelles beef [17, 18, 22], pig [16]

As expected, outer membranes contained high total lipid levels Most lipids in whole mitochondria as well as in the membranes were phospholipids The distribution of phospholipids in purified pig heart mitochondria was previously compared to those of mitochondria from other sources [15]; it is noteworthy that at the same time, Awasthi et al [21] found a similar distribution for beef heart mitochondria.

Outer membranes contained two major phospholipids (85 %) phosphatidylethanolamine and phosphatidylcholine, they appeared very different from microsomes by their protein profiles and by their lipid composition and distribution phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, cardiolipin, plasmalogens, cholesterol, triglycerides, the high degree of unsaturation of phosphatidylethanolamine This confirms the lack of microsomal contaminants in our outer membrane preparations and agrees with the marker estimations and electron micrographies [1] The absence of cardiolipin is striking and agrees with the very low activity of inner membrane markers and the very different protein profiles As compared to beef heart outer membranes [22–23], pig heart outer membranes contained 12 polypeptides instead of 14, but their relative importance in densitometry appeared to be very different

Inner membranes contained essentially three phospholipids phosphatidylcholine, phosphatidylethanolamine and cardiolipin as widely observed in mitochondria from other sources [8, 9, 11, 12, 50, 51] but cardiolipin content was higher than in any other mitochondria, these phospholipids were dominant in whole mitochondria

The fact that the content in phosphatidylcholine and phosphatidylinositol of whole mitochondria did not reflect exactly that of inner membrane in spite of the high percentage of inner membrane might be explained as follows As stated above, outer membrane contribution was probably underestimated, its content in phospholipids is much higher than in inner membrane (mg lipid/mg protein = 0 85 in outer membrane against 0 48 in inner membrane) and its levels of phosphatidylcholine and phosphatidylinositol, respectively, were twice and thrice those of inner membrane Moreover, the phospholipid distribution is universally expressed as a percentage of total phospholipid for each fraction and some phospholipids are absent in some fractions (e g absence of cardiolipin in outer membrane) Finally we cannot exclude the fact that during the five additional steps of the purification procedure some phosphatidylcholine leaks out of inner membrane especially if it is located on an external part of the membrane Inner membranes did not seem to contain any significant amount of cholesterol and very little triglycerides Gel electrophoresis revealed 13 different polypeptide sub-units of which 5 are major these polypeptides can be compared to those described for rat liver mitochondria [4, 5, 7] with the exception of the 215 000 one which was not described before The polypeptide profiles are quite different from those of outer membrane and they are consistent with the presence of ATPase since they reveal the characteristic protein subunits of the F_1-ATPase recently purified in our laboratory from pig heart mitochondria [40] Hare and Crane [24] detected more polypeptide subunits in a membrane fraction obtained from submitochondrial particles after sonication and centrifugation of beef heart mitochondria, recently Albracht and Heidrich [52] clearly demonstrated by EPR studies that such submitochondrial preparations are a mixture of inner- and outermembrane fragments

Microsomes were analyzed mainly to control the purity of our mitochondrial fractions since outer membranes exhibited after repeated purifications a significant NADPH-cytochrome *c* reductase activity [1] As stated above, microsomes differed in many ways from mitochondrial outer membranes Their major lipids were phosphatidylcholine, phosphatidylethanolamine, cardiolipin, contrarily to mitochondrial membranes they contained phosphatidylserine The cardiolipin content (12 % of phospholipids) was strikingly higher than in rat liver [12] but quite comparable to that of heart microsomes from beef [17, 53] man [54], rat [55]

Such a high level in cardiolipin is not indicative of inner membrane contamination since no cytochrome oxidase or significant rotenone-sensitive NADH-cytochrome *c* reductase activities could be detected in our microsomal preparations

Although the polypeptide profiles of microsomes were very different from those of inner membranes, striking similarities were observed in the lipids of both these membranes same main phospholipids, triglyceride contents, total plasmalogens, total lipid and phospholipid, however, differences concerned the absence of cholesterol and phosphatidylserine in inner membrane, and the relative distribution of lecithin and cephalin plasmalogens

The fatty acid distribution seemed specific of a given phospholipid and not of one type of membrane, with the exception of phosphatidylethanolamine $C_{18\ 0}$ and $C_{18\ 2}$ acids However, owing to the specific phospholipids involved, the three types of membranes appeared very different, for example, inner membrane was

very rich in C_{18} acids and highly unsaturated while outer membrane was more saturated and contained less C_{18}

The next steps of our work will be to determine the rôle of a given phospholipid, acidic or neutral, highly unsaturated or saturated, in the organization of the membrane, and to correlate the presence of polypeptides with specific lipids and special enzymatic or transport functions

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