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ENZYMIC CHARACTERIZATION AND LIPID COMPOSITION OF RAT LIVER SUBCELLULAR MEMBRANES

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SUMMARY

1. Mitochondria, inner and outer mitochondrial membranes, microsomes, smooth and rough endoplasmic reticulum membranes and plasma membranes were isolated from rat liver.

2. Different purification methods, *i.e.* centrifugation in sucrose density gradients, gentle digitonin treatment and use of Triton WR-1339, were used to decrease the mutual contamination of subcellular membrane preparations obtained by current methods. The results of such treatments are discussed.

3. Marker enzymes were used to characterize the different types of membranes. Their specificity and their use in evaluating the degree of purity of a membrane preparation are discussed.

4. The cholesterol content of each type of membrane preparation was determined and compared to the phospholipid content and to the activities of marker enzymes. Plasma membrane is by far the richest in cholesterol (molar ratio cholesterol/phospholipid-P = 0.76). Outer mitochondrial membrane and endoplasmic reticulum membranes have a relatively low cholesterol content, (molar ratio cholesterol/phospholipid-P = 0.12 for outer membrane and microsomes, 0.06 for rough endoplasmic reticulum). A value of 0.24 found for smooth endoplasmic reticulum includes also non "structural" cholesterol. The inner mitochondrial membrane appears to contain no cholesterol.

5. The phospholipids were separated by thin-layer chromatography and their fatty acids analyzed by gas-liquid chromatography. The inner mitochondrial membrane contains about 40 % of phosphatidylethanolamine and 40 % of phosphatidyl choline whereas microsomes and endoplasmic reticulum membranes (either smooth or rough) contain no more than 25 % phosphatidylethanolamine and about 60 % phosphatidylcholine. In the outer mitochondrial membranes the proportions are: phosphatidylethanolamine 35 %, phosphatidylcholine 50 %. Phosphatidylinositol is practically absent from the inner mitochondrial membrane; it represents about 10 % of the total phospholipids of outer mitochondrial membrane and smooth and rough endoplasmic reticulum. Cardiolipin is localized in the inner membrane and represents 20 % of the phospholipid phosphorus of that membrane.

6. The fatty acid distribution in phosphatidylcholine and phosphatidylethanolamine is roughly similar in the inner and outer mitochondrial membranes and in microsomal membranes (smooth and rough); arachidonic acid represents 23 % of the

fatty acids in phosphatidylethanolamine and 16 % in phosphatidylcholine, whereas palmitic acid and stearic acid are in about equal proportions (about 25 % each). Phosphatidylinositol is the more highly saturated phospholipid (60–65 % of saturated fatty acids) and contains up to 45 % of stearic acid. On the other hand cardiolipin contains 90 % of unsaturated fatty acids, the main one being linoleic acid. It is concluded that the fatty acid pattern is more characteristic of the phospholipid species than of the membrane.

7. Inner mitochondrial membrane and plasma membrane are the two which differ most among the cytomembranes analyzed. Inner mitochondrial membrane is characterized by a high degree of unsaturation, the presence of cardiolipin (20 % of total lipid-P) and the absence of cholesterol whereas plasma membrane is highly saturated, contains more than 20 % of sphingomyelin and has a molar ratio cholesterol/phospholipid-P higher than 0.7.

INTRODUCTION

The structure of biological membranes is still a matter of considerable discussion and speculation. In particular the contribution of lipids is not yet understood. Analysis of the molecular composition of membrane preparations whose purification had been carefully assessed should lead to a fuller understanding of the molecular organization and the specific behaviour of those membranes.

During the last few years, we have focussed our attention on a critical appraisal of the efficiency of some methods currently used to fractionate endocellular membranes from rat liver and have tried to correlate the enzymatic characteristics of membrane fractions with their lipid composition. Such work is underway in a number of laboratories (*cf.* for review refs. 1–11), but the data so far published are generally restricted to one type of cellular organelle isolated according to a single type of procedure; furthermore, in earlier publications, the estimate of the contamination by other cellular organelles or membranes, in particular by plasma membrane, was not evaluated.

The results presented in this paper include: (1) a discussion of the possibilities and limitations of the fractionation and purification methods used to isolate whole organelles and in particular mitochondria and endomembranes, *i.e.* inner and outer mitochondrial membranes and smooth and rough endoplasmic reticulum membranes; (2) an analysis of the main lipid components of rat liver subcellular membrane fractions, namely cholesterol, the main phospholipid classes and the fatty acid distribution.

MATERIALS

D-Glucose 6-phosphate, cytochrome *c* (type VI) and adenosine monophosphate (AMP) were obtained from Sigma (St Louis, Mo., U.S.A.), NADPH from Boehringer (Mannheim, Germany), benzylamine sulfate, and *p*-nitrophenyl phosphate from Prolabo, Silicagel G from Merck (Darmstadt, Germany), and Triton X-100 and Triton WR-1339 from Rohm and Haas (Philadelphia, Pa., U.S.A.). Digitonin (A grade) from Calbiochem (Los Angeles, Calif., U.S.A.). Authentic samples of methyl-

esters of fatty acids were obtained from Applied Science Laboratories (State College, Pa., U.S.A.). All other reagents were reagent grade and the solvents were redistilled before use.

METHODS

Subcellular membrane fractions

Albino adult rats, 300 g in weight, were used.

Mitochondria were isolated by the classical differential centrifugation method in 0.27 M sucrose buffered by 2 mM Tris – HCl, pH 7.6 from a 10 % (w/v) homogenate. Nuclei, red blood cells and cell debris were removed by centrifugation for 10 min at 600 × g_{av} in an SS1-Sorvall centrifuge. Mitochondria were spun at 5000 × g_{av} for 10 min in 60 ml polyethylene centrifuge tubes (full) and washed three times with half of the starting volume of 0.27 M sucrose–Tris at 6000 × g_{av} for 10 min. In the latest experiments, in order to remove the contaminating red cells and nuclei more completely, a supplementary washing followed by centrifugation at 500 × g_{av} for 3 min was performed before the three washings and centrifugation at 6000 × g_{av} for 10 min. These washings were designed to remove, in particular, pieces of rough endoplasmic reticulum membrane. The low speed of the second centrifugation although causing some loss of mitochondria allows the contamination by lysosomes and microbodies and also by plasma membrane to be minimized. By this procedure starting from 115 g wet weight, *i.e.* from about 20 g of proteins in the whole homogenate, about 1.4 g of mitochondrial proteins were recovered.

In order to decrease the contamination by lysosomes, a preliminary step of purification of the mitochondrial preparation was used at the beginning of these studies[12,13]; it is based on the observation of WATTIAUX *et al.*[14] that lysosomes, when filled with Triton WR-1339, become lighter than mitochondria. Rats were injected intraperitoneously with 170 mg of Triton WR-1339 (in 0.9 % NaCl) 4 days before the experiment. The mitochondria isolated as usual and resuspended in buffered 0.27 M sucrose, pH 7.6, were then layered on a 2-layer sucrose gradient formed by successively layering 22 ml of 41.4 % (w/v) and 22 ml of 39.3 % (w/v) buffered sucrose and the suspension was centrifuged in a Spinco SW-25.2 bucket rotor at 23 500 rev./min (55 000 × *g*) for 2 h. Mitochondria were collected as a pellet (E-fraction) and the lysosomes filled with Triton WR-1339 (H-fraction or tritosomes) remained on top of the gradient.

The mitochondrial pellet E was then treated as described by PARSONS *et al.*[15] for the isolation of inner and outer membranes. After swelling in 20 mM phosphate buffer, pH 7.4, for 20 min at 0°, the membranes were gathered by centrifugation at $7 \cdot 10^5 \times g \cdot$min and then separated by differential centrifugation (Spinco Rotor 30). A crude "inner membrane matrix" fraction was obtained by low speed centrifugation (19000 rev./min for 10 min) (45000 × *g*) (low speed pellet) and then purified by centrifugation through a discontinuous, 3-layer sucrose density gradient (51.3 %, 37.7 %, 25.2 %) in a SW-25 rotor of a Spinco centrifuge (23 500 rev/min for 90 min). The purified inner membrane + matrix fraction was collected at the bottom of the tube. The crude outer membrane contained in the supernatant fluid was collected as a high speed pellet at $7 \cdot 10^5 \times g \cdot$min and then recentrifuged in a SW-39 Spinco-rotor. The re-run gradient consisted of 1.2 ml of 51.3 % (w/v) sucrose, 1.2 ml of 37.7 %,

and 2.0 ml of 23.2 % (w/v) sucrose and 1 ml of the input. It was centrifuged at 38 000 rev./min for 45 min. All of the sucrose solutions used for the density gradients were made in 20 mM phosphate buffer, pH 7.4[15]. At the end of the centrifugation the outer membrane fraction was collected between the 37.7 % and the 25.2 % layer. The mitochondrial inner membrane fraction was obtained from the "inner membrane + matrix fraction" by further extraction of the matrix proteins by phosphate treatment and centrifugation through a sucrose gradient (see text below).

Digitonin inner membrane plus matrix particles and digitonin outer membrane were prepared by the method of SCHNAITMAN AND GREENAWALT[16]. Before isolating the *microsomes*, an intermediate fraction, the L-fraction, was first sedimented by centrifugation of the mitochondrial supernatant at 21 000 rev./min for 5 min, and then the microsomes were sedimented at 30 000 rev./min for 60 min (78 000 × *g*) in a rotor 30 of a L-Spinco centrifuge. Microsomes were isolated from a sucrose solution containing either 1 mM or 5 mM $MgCl_2$ (*cf. Results*) and from rats that had not been injected with Triton WR-1339. The smooth and rough endoplasmic reticulum membranes were first isolated according to the procedure of MOLNAR[17], slightly modified, by layering the crude microsome suspension on 20 ml of 1.3 M sucrose containing 1 mM $MgCl_2$, and centrifuging it at 25 000 rev./min for 3 h in SW-25 swinging buckets. (Spinco centrifuge). The smooth membranes were heterogeneous and formed two layers, a white one above the 1.3 M sucrose boundary and a yellow one just below. The two layers were taken up together with a Pasteur pipette and rediluted. The rough endoplasmic reticulum which sedimented at the bottom of the tube was homogenized and rediluted in 0.27 M sucrose. The two endoplasmic reticulum fractions were resedimented separately at 78 000 × *g* for 45 min and eventually purified through the 3-layer sucrose gradient of PARSONS *et al.*[15, 18].

Plasma membrane was isolated by the method of NEVILLE[19].

Marker enzymes

The marker enzymes used to assess the purity of the fractions were: glutamate dehydrogenase and malate dehydrogenase[20], cytochrome oxidase[21], and monoamine oxidase[22] for, respectively, the matrix, the inner membrane and the outer membrane of mitochondria, acid phosphatase for lysosomes[21], D-glucose-6-phosphatase[23] and NADPH cytochrome *c* reductase for microsomes[24] and 5'-nucleotidase for plasma membranes[25].

To measure the activity of membrane-bound enzymes, the membranes were lysed first with Triton X-100 (final concentration between 0.01 and 0.05 %, depending on the enzyme tested), except in the case of D-glucose-6-phosphatase, which was inactivated; besides, since the glucose-6-phosphatase activity fell off upon incubation at 37° for periods longer than 30 min[23] (and unpublished results) the incubation time was never allowed to exceed 20 min. Except for NADPH cytochrome *c* reductase and monoamine oxidase, which were sometimes measured the day after the isolation of the membranes, all of the marker-enzyme activities were determined on membrane preparations freshly isolated.

Acid phosphatase was measured by the increase of $A_{400\ nm}$ due to the release of *p*-nitrophenol from *p*-nitrophenyl phosphate during incubation for 5 min at 37°, pH 4.8[26]. D-glucose-6-phosphatase and 5'-nucleotidase were determined after a 20 min incubation period at 37° by the amount of inorganic phosphate released at pH 6.5

for D-glucose-6-phosphatase[27] and at pH 7.5 for 5'-nucleotidase[25], inorganic phosphate being estimated by the method of FISKE AND SUBBAROW[28]. Monoamine oxidase was measured at pH 7.5 and at 25° by measuring the increase of $A_{250\ nm}$ due to the formation of benzaldehyde from benzylamine[29]. Cytochrome oxidase was determined at 25° by measuring, at pH 7.4, the decrease in $A_{550\ nm}$ of cytochrome *c* that had been previously reduced by dithionite[21]. Since the oxidation of reduced cytochrome *c* is first order with respect to its concentration, the rate constant *k* was deduced from a logarithmic plot of the reaction. The rate of oxidation of the reduced cytochrome was calculated from the rate constant. Glutamate dehydrogenase and malate dehydrogenase were measured spectrophotometrically at 25° by the method of BEAUFAY *et al.*[20] and ENGLARD AND SIEGEL[30] respectively, and NADPH cytochrome *c* reductase by the method of OMURA *et al.*[24] at pH 7.8. Unless specified all enzymic activities are expressed as nanomoles of substrate used per min. per mg of protein. Protein was determined by the biuret method[31] with bovine serum albumin as standard.

Lipid extraction and analysis

Extraction of membrane lipids was effected according to DAWSON *et al.*[32] as follows: the membrane suspension (1 – 2 ml, 20 – 30 mg of protein per ml) is homogenized with 7 vol. of methanol and then 14 vol. of chloroform are added to the mixture, which is rehomogenized in a Potter–Elvejhem homogenizer, and the extract filtered. After rinsing the tissue residue with chloroform–methanol (2:1, v/v), the lipid extract is washed with 0.2 vol. of 0.9 % NaCl[33] and kept overnight at 2°. The lower phase is collected, dried over Na_2SO_4, and evaporated to dryness in a vacuum at room temperature. The residue is then made to volume with chloroform and eventually stored under nitrogen at –20°. The lipids dissolved in chloroform were fractionated into neutral lipids and phospholipids by column chromatography on silicic acid (1 g of silicic acid (Mallinckrodt, 100 mesh) + 0.5 g of Celite 545 (Johns-Mansville). 1 ml of concentrated lipid extract containing 0.2 – 0.5 mg of lipid phosphorus was applied to the column, and the neutral lipids were removed by 15 ml of chloroform; the phospholipids were then eluted by 25 ml of methanol. The eluates were concentrated under a stream of nitrogen and made to volume with chloroform.

The neutral lipid fraction obtained from the silicic acid column was analyzed for its glyceride and cholesterol content. Total glycerides were estimated after alkaline hydrolysis[34] (in 2.5 % methanolic potassium hydroxide, 1 h at 80°) by enzymic determination of free glycerol[35]. Neutral lipids were resolved into mono-, di- and triglycerides, cholesterol and cholesterol esters by thin layer chromatography on 0.2 mm layers of Silica gel G by using light petroleum (b.p. 30–60°)–diethyl ether–acetic acid (70:30:2, by vol.)[36]. After detection by iodine vapor and evaporation of the I_2, the spots were scraped off and eluted with chloroform–methanol (2:1, v/v) and the esters were hydrolyzed (in 2.5 % methanolic potassium hydroxide, 1 h at 80°), glycerol was determined enzymatically[35], and cholesterol colorimetrically with the ferric sulfuric reagent[37].

The polar lipids obtained by elution of the silicic acid column with methanol were separated on silica gel by thin-layer chromatography, either by two successive migrations in the following systems[38]: (a) chloroform–light petroleum–acetic acid

(65:35:2, by vol.), and then, after evaporation of the solvent from the plate *in vacuo*, (b) chloroform–methanol–water (65:35:4, by vol.), or by two-dimensional migrations as described by PARSONS AND PATTON[39], initially with chloroform–methanol–water–28 % aqueous ammonia (130:70:8:0.5, by vol.) and then in the second dimension with chloroform–acetone–methanol–acetic acid–water(100:40:20:20:10, by vol.). Identification of lipid spots was accomplished by staining reactions (with ninhydrin, then with molybdate) and by comparison with the R_f values of known lipids. For quantitation of phospholipids, the lipid spots were detected either by iodine vapor or by spraying slightly with water. The excised components were then transferred in a stoppered tube and eluted by three successive washings with chloroform–methanol (2:1, v/v). After alkaline hydrolysis of the phospholipids[40] or acid hydrolysis of sphingomyelin[41], the fatty acids were methylated with diazomethane and the esters analyzed on a 2 m × 0.125 inch column packed with 20 % diethylene glycol succinate on Chromosorb W-AW (100 mesh) operated at either 180° or between 140 and 180°, carrier gas N_2, by using an F and M Scientific Hewlett-Packard Model 5750 gas chromatograph. The instrument was equipped with flame-ionization detectors and a Hewlett-Packard Integrator, Model 3370. The structural assignments were based on comparisons with authentic samples of various fatty acids, by graphic plotting of the gas-chromatographic retention times in comparison with those of known methyl esters or with values of equivalent chain lengths as given by HOFSTETTER *et al.*[42]. Phospholipid phosphorus was determined after wet ashing of the sample according to BARTLETT[43].

The ribosomal RNA of microsomal fractions was extracted by the method of SCHMIDT AND THANNHAUSER[44] and, after wet ashing, phosphate was estimated according to BARTLETT[43].

Determination of hexosamine and sialic acids

Sialic acids and hexosamines were extracted by the method of MOLNAR[45]. Samples containing 10–50 mg of proteins were precipitated by 5 % trichloroacetic acid at 2°. Sialic acids were extracted from the precipitate by hydrolysis in 5 % trichloroacetic acid for 1 h at 80°. After sedimentation of the residue, the supernatant fluid containing sialic acids was applied to a column of Dowex 1-X8 (200–400 mesh), Cl^- form and then the sialic acids were eluted by 40 ml of 0.2 M HCl. After concentration, sialic acids were determined by the WARREN method[46].

The hexosamines that were still bound to the residue were hydrolytically released by 4 M HCl for 4 h at 100° and after careful neutralization of the hydrolyzate the amino sugars were estimated by the ELSON–MORGAN reaction[47] slightly modified.

Electron microscopy

For sectioning, samples of each fraction were fixed for one hour in ice cold 2.5 % glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.2 and then for 1 h in ice-cold 2 % OsO_4 in the same buffer. Samples were dehydrated in a series of increasing concentrations of ethanol. Ethanol was removed with propylene oxide and the samples were embedded in Epon. After sectioning on a LKB ultronome III, samples were stained on the grid with 1 % uranylacetate in 70 % ethanol and with 0.2 % aqueous lead citrate. Electron micrographs were taken with a Philips EM 300 electron microscope at an accelerating voltage of 80 kV.

RESULTS

(*A*) *Isolation of the membranes*

Mitochondrial membrane

(*a*) *Purification of mitochondrial suspensions.* The final step in the isolation procedure of PARSONS *et al.*[15] for the outer mitochondrial membrane, involves a centrifugation on a 3-layer sucrose gradient. This procedure removes remaining fragments of inner membrane from the outer mitochondrial membrane. Nevertheless, this last step appeared quite inefficient for removing other endocellular membranes such as smooth endoplasmic reticulum and lysosomal membranes, which like outer mitochondrial membrane, have a buoyant density around 1.12–1.14[15,18]. Table I shows that repeated centrifugation passages through the 3-layer sucrose density gradient did not allow further separation of the outer membrane from the contaminant smooth endoplasmic reticulum and lysosomal membranes.

Since isolated membranes appeared to aggregate very easily, the present approach involved initial purification of the mitochondrial particles followed by fractionation into membranes of the purified organelles. Two methods were devised to purify whole mitochondrial preparations: (1) a flotation method and (2) a chemical method involving a gentle treatment with small amounts of digitonin.

Removal of lysosomes and plasma membranes by flotation. This first procedure is based on the observation[14] that lysosomes which have absorbed the non-ionic detergent Triton WR-1339 become lighter and may be separated from mitochondria by centrifugation on a sucrose gradient (Fig. 1). Table II shows that, when loaded with Triton WR-1339, lysosomes are found in the H-fraction (tritosomes) which remains on top of the sucrose gradient, together with contaminating plasma and reticulum membranes. However, it was noticed incidentally that a small percentage of mitochondria underwent alteration of their outer membrane during the purification process.

Removal of lysosomes and plasma membranes by treatment with small amounts of digitonin. Digitonin has been used by the Louvain group[48,49] to discriminate

TABLE I

EFFECT OF REPEATED PASSAGES THROUGH A 3-LAYER SUCROSE DENSITY GRADIENT ON THE ACTIVITIES OF MARKER ENZYMES IN AN OUTER MITOCHONDRIAL MEMBRANE FRACTION

Activities of marker enzymes in an outer mitochondrial membrane fraction, O_1, O_2, O_3 membrane fraction after the first, second and third centrifugations through a 3-layer sucrose gradient (51.3, 37.7 and 23.2%, w/v, sucrose, in 20 mM phosphate, pH 7.4[15]. *N.B.* NADPH cytochrome *c* reductase was measured here at pH 7.5, and not at pH 7.8 as everywhere else. Specific activities in nmoles·min^{-1}·mg^{-1} protein.

Fraction	*Monoamine oxidase*	*Cytochrome oxidase*	*Acid phosphatase*	*NADPH cytochrome c reductase*
Mitochondria	6.8	1100	73.1	5.6
Crude outer membrane	34.8	170	254.6	37.2
Purified outer membrane				
O_1	77.4	70	439.2	46.2
O_2	104	80	450.1	64.5
O_3	96.8	80	562.5	63.0
Microsomes	0.7	60	24.9	51.7

cholesterol-rich membrane such as plasma membrane from other endocellular membranes. Digitonin was therefore used under different conditions of incubation (concentration, temperature, duration) and its effect assessed quantitatively by

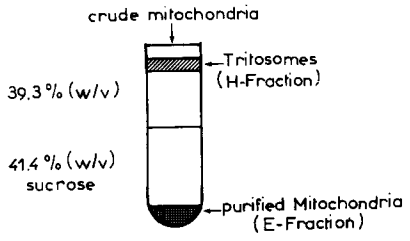

Fig. 1. Purification of mitochondria prepared from Triton WR-1339 injected rats. The two-layer sucrose gradient allows the separation of the tritosomes (H-fraction) from the purified mitochondria (E-fraction).

TABLE II

ELIMINATION BY FLOTATION OF TRITOSOMES, PLASMA, AND ENDOPLASMIC RETICULUM MEMBRANES FROM MITOCHONDRIAL PREPARATIONS

Mean of the specific activity of marker enzymes in liver mitochondria prepared from rats that had been injected with Triton WR-1339 4 days before. The E-fraction is the mitochondrial pellet collected after centrifugation of the non-purified mitochondria through a 2-layer sucrose gradient and the H-fraction is the light fraction (tritosomes) which remains on top of the gradient. (*cf.* METHODS and ref. 14). Specific activities in nmoles·min^{-1}·mg^{-1} protein ± S.D.; in parentheses: number of separate experiments.

Fraction	*Cytochrome oxidase*	*Monoamine oxidase*	*Acid phosphatase*	*Glucose-6-phosphatase*	*5'-Nucleotidase*
Non-purified mitochondria	1,590 ± 650 (7)	5 ± 2 (7)	64 ± 24 (7)	26 ± 13 (6)	48 ± 22 (3)
E-fraction	1,810 ± 540 (10)	6 ± 2 (10)	22 ± 9 (10)	15 ± 11 (8)	20 ± 4 (3)
H-fraction	161 ± 220 (7)	0.6 ± 1.1 (7)	950 ± 362 (7)	108 ± 66 (6)	447 ± 177 (3)

determining the specific activity of marker enzymes before and after treatment with digitonin. The conditions selected finally are those described in the legend of Table III. Electron microscopy served to verify that this slight digitonin treatment did not alter the outer mitochondrial membrane. Fig. 2 shows that, as indicated by the activity of marker enzymes (Table III), membranes such as plasma and reticulum membranes are found in the supernatant fluid.

This second method for removing the contaminant cytoplasmic membranes from a suspension of mitochondria proved to be comparably efficient to the preceding one. It has the advantage of being easier and quicker and of being applicable directly to an ordinary mitochondrial preparation without prior injection of Triton WR-1339 to the animals. The conditions of treatment must be strictly controlled in order to avoid the deleterious effect of digitonin on the outer mitochondrial membrane. Moreover, the isolation of the outer membrane by hypotonic treatment[15] leads to

lower yields with "digitonized" mitochondria than with untreated mitochondria, although the outer membrane of "digitonized" mitochondria appears well preserved under the electron microscope. The same observation holds for mitochondria purified by removal of tritosomes. The above mentioned limitations may be considered

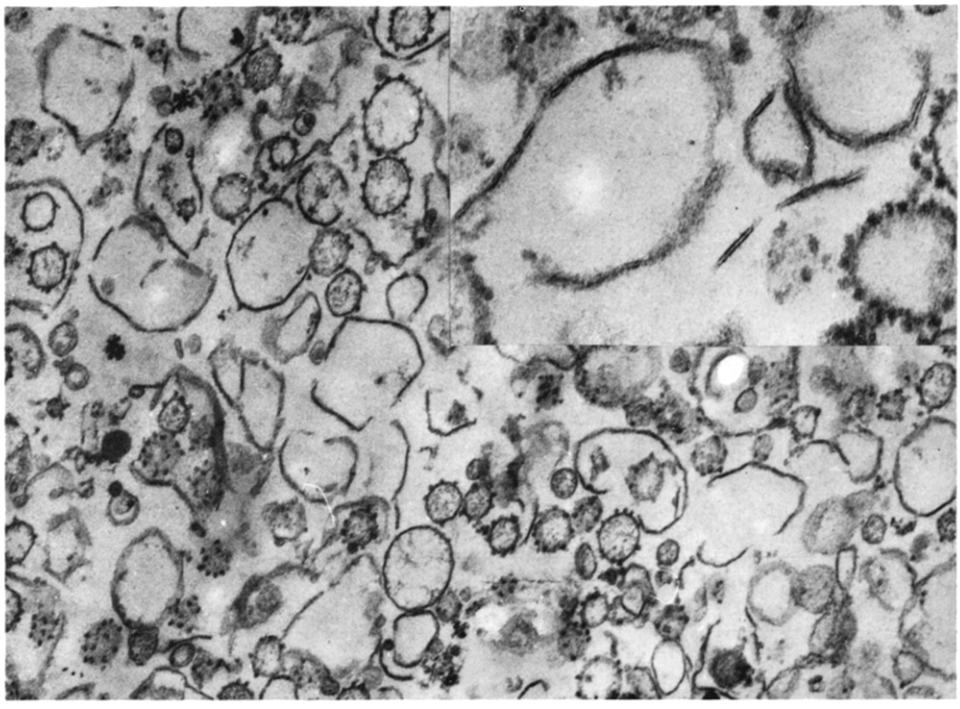

Fig. 2. Electron micrograph of the post-mitochondrial supernatant fluid after slight digitonin treatment (25 μg digitonin per mg of protein, 10 min at 0°). The material contained in the post mitochondrial supernatant was concentrated by high speed centrifugation before embedding. Sections stained with uranyl acetate and lead citrate × 28500. In insert details of rough endoplasmic reticulum and of plasma membrane characterized by stiff, three-layer segments × 78000.

TABLE III

PURIFICATION OF MITOCHONDRIAL PREPARATIONS BY SLIGHT DIGITONIN TREATMENT

Digitonin, in 0.27 M sucrose, is added to a cold suspension of mitochondria in 0.27 M sucrose (25 μg of digitonin per mg of protein, final digitonin concentration 0.25%, w/v), and kept for 10 min, in contact at 0° without stirring. The action of digitonin is stopped by dilution with 0.27 M sucrose and rapid centrifugation of the mitochondria. Specific activities in nmoles·min^{-1}·mg^{-1} protein; mean of 4 separate experiments ± S.D.

	Cytochrome oxidase	*Monoamine oxidase*	*Acid phosphatase*	*NADPH cytochrome c reductase*	*5'-Nucleotidase*
Mitochondria	2053 ± 650	5.1 ± 0.4	69 ± 21	5.6 ± 1.7	34 ± 20
Digitonin-treated mitochondria	2302 ± 440	5.9 ± 1.7	17 ± 6	4.3 ± 1.4	7 ± 5
Supernatant	74 ± 30	2.1 ± 1.8	379 ± 54	15.0 ± 7.6	175 ± 67

as a handicap for the use of "digitonized" mitochondria in large scale preparations of mitochondrial membranes. Furthermore, the two methods used to isolate mitochondrial membranes[15], [16], yielded the best resolution when applied to "untreated" mitochondria. Therefore, when solely the distribution between the inner and outer

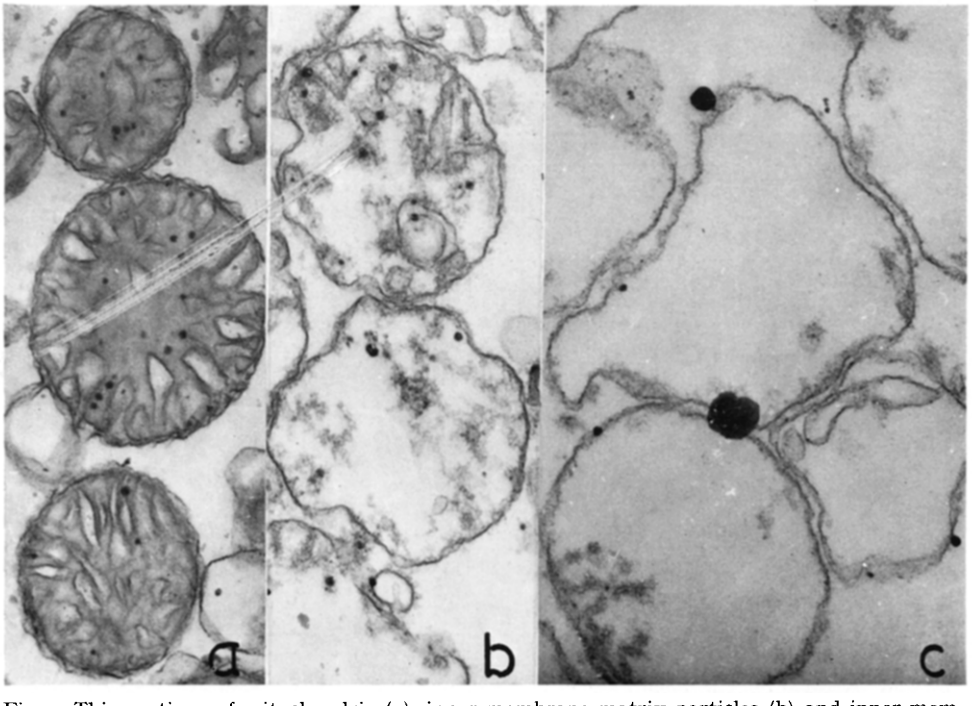

Fig. 3. Thin sections of mitochondria (a), inner membrane matrix particles (b) and inner membrane (c) obtained according to PARSONS *et al.*[15] (*cf.* Table IV-A). Note the enlargement of particles from left to right. Sections stained with lead citrate × 30000.

membrane of a truly mitochondrial component is to be studied, it is more advisable to use mitochondrial preparations purified only by careful washings and centrifugations.

(*b*) *Isolation of mitochondrial membranes.* By the method of PARSONS *et al.*[15] the outer membrane is detached from the inner mitochondrial membrane by swelling through a large amplitude in hypotonic phosphate buffer. This swelling causes soluble proteins from the matrix to leak out, but only partially. About 60 % of the original malate dehydrogenase content and 80 % of the original glutamate dehydrogenase content were found in the low speed pellet collected after swelling of mitochondria in 20 mM phosphate[15] (*cf.* METHODS). Observation under the electron microscope of thin sections of the low speed pellet[13] corroborated the results obtained with marker enzymes (Fig. 3), indicating that matrix proteins still remain in the low-speed pellet particles, even after their centrifugation through a sucrose density gradient. Therefore the particles so obtained, which had been called "inner membrane" by the authors of the method, are in this paper referred to "inner membrane *plus* matrix particles".

Extraction of the matrix material from the inner membrane space can be

achieved without breakage and fragmentation of the membrane by homogenization of the inner membrane *plus* matrix particles in a large volume of hypotonic phosphate buffer: 100 mg of proteins are rehomogenized in 100–200 ml of 20 mM phosphate buffer, pH 7.4, with a Potter–Elvehjem homogenizer, kept at 0° for 15 min, then collected by centrifugation at 20 000 × *g* for 10 min, resuspended in a small volume of 20 mM phosphate buffer and then recentrifuged through the discontinuous sucrose gradient (25.2 %, 37.7 %, 51.3 %, w/v, 30 min at 39 000 × *g* in a SW-39 Spinco rotor) described by PARSONS *et al.*[15]. The inner membrane gathers at the interface between the 37.7 and 51.3 %, w/v, sucrose layers, whereas the insufficiently extracted inner membrane *plus* matrix particles sediment to the bottom of the tube. The yield of inner membrane varies from one experiment to another. It may be necessary to repeat one further time the last extraction and the centrifugation on the three-layer sucrose gradient to obtain the final separation described above. From the activity of cytochrome oxidase, it can be shown that practically all the inner membrane may be collected at the second interface of the gradient. Although they appear under the electron microscope (Fig. 3c) to be largely devoid of matrix material they still contain some malate dehydrogenase activity (0.5 μmole NADH formed per min per mg protein in inner membrane particles *versus* 2.1 in matrix extract and 1.7 in inner membrane *plus* matrix particles) and glutamate dehydrogenase activity (0.04 μmole NADH per min per mg protein *versus* 0.1 in matrix extract). The different extractibility of these two enzymes points to different forces of association with the fibrous network of the matrix[50].

The outer membrane fractions obtained by the Parsons method had a low cytochrome oxidase activity but, on the other hand, exhibited an increased activity of other marker enzymes such as acid phosphatase, NADPH cytochrome *c* reductase, 5′-nucleotidase, which proved to be very difficult to eliminate. As already shown in Table I, endocellular membranes, once isolated, tend to aggregate and cannot be separated by centrifugation on sucrose density gradient even if their densities are different. Since the actual purification of the outer membrane by density gradient centrifugation is doubtful and the recovery very poor, the lipid analyses were performed on the high speed pellet (*cf. Methods* and [15])obtained from purified mitochondrial preparations (see above).

The method of SCHNAITMAN AND GREENAWALT[16] based on the removal of the outer membrane by treatment with digitonin (150 μg digitonin per mg of protein), was also used to separate inner and outer mitochondrial membranes. In Table IV are compared the mitochondrial fractions obtained by the Parsons method (upper part of Table IV) and those obtained according to SCHNAITMAN AND GREENAWALT (lower part of Table IV). In the latter case no monoamine oxidase activity could be detected in the inner membrane matrix particles, where the other measured marker enzymes have also very low activities. The inner membrane *plus* matrix particles obtained by the digitonin method[16] appear also to give purer inner membrane fractions than PARSONS' method. Nevertheless digitonin seems to solubilize some of the cytochrome oxidase of the inner membrane or inactivates it, since lower values for the specific activity of that enzyme were found consistently in that type of preparation. A possible deleterious effect of digitonin on the structural organization of the inner membrane is illustrated by the observation that inner membrane obtained by the digitonin method[16] contains fewer high-affinity binding sites for atractyloside

TABLE IV

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF MITOCHONDRIAL MEMBRANE PREPARATIONS

Specific activities expressed in nmoles·min^{-1}·mg^{-1} protein. A: fractionation by swelling in 20 mM phosphate[15]. B: fractionation by digitonin treatment[16].

Fraction	*Cytochrome oxidase**	*Monoamine oxidase*	*Acid phosphatase*	*5'-Nucleotidase*	*NADPH cytochrome c reductase*	*Glucose-6-phosphatase*	*Phospholipid-P/protein (μmoles P/mg)*	*Cholesterol/phospholipid-P (molar ratio)*
A. Mitochondria	1340 ± 460 (31)	5 ± 1 (31)	60 ± 23 (15)	33 ± 15 (20)	6 ± 4 (7)	25 ± 12 (17)	0.16 ± 0.04 (10)	0.10 ± 0.04 (4)
Inner membrane *plus* matrix	2920 ± 1160 (23)	3 ± 1 (24)	9 ± 4 (9)	10 ± 6 (11)	5 ± 3 (4)	9 ± 2 (4)	0.20 ± 0.04 (8)	0.053 ± 0.026 (5)
Inner membrane	5510 ± 2550 (49)	3 ± 1 (45)	12 ± 9 (12)	6 ± 6 (8)	3 ± 1 (6)	—	0.34 ± 0.06 (9)	0.060 ± 0.025 (5)
HSP (outer membrane)	370 ± 260 (25)	61 ± 25 (27)	222 ± 79 (17)	131 ± 67 (12)	30 ± 11 (9)	96 ± 22 (5)	0.46 ± 0.09 (8)	0.12 ± 0.04 (5)
B. Inner membrane *plus* matrix	2300 ± 830 (5)	<0.1 (3)	0.4 ± 0.6 (5)	1 ± 1 (5)	2 ± 1 (4)	3 ± 2 (5)	0.20 ± 0.04 (4)	<0.006 (2)
Inner membrane	2950 ± 1220 (7)	<0.1 (4)	1 ± 1 (5)	2 ± 4 (5)	<0.1	1 ± 1 (4)	0.29 ± 0.04 (3)	<0.005 (2)
HSP (outer membrane)	1208 ± 550 (4)	31 ± 13 (4)	159 ± 94 (3)	93 ± 44 (3)	12 ± 6 (6)	72 ± 27 (3)	0.37 ± 0.10 (3)	0.132 (2)

* The presence of inner membrane may also be assessed by the cytochrome *a* content. For instance the content in cytochrome *a* measured by the absorbance between 605–630 nm with a $\varepsilon_{mm} = 16$[51] was found to be: 0.153, 0.188, 0.611, 0.043 nmole/mg protein in mitochondria, inner membrane matrix particles, inner membrane and crude outer membrane[15] respectively. Nevertheless while 10 μg of mitochondrial proteins are sufficient to carry out a cytochrome oxidase test, 8 mg of proteins are necessary to measure the cytochrome content in a Cary M.15 spectrophotometer.

than the inner membrane obtained after hypotonic swelling as described above (P. V. VIGNAIS, unpublished results).

The method of SCHNAITMAN AND GREENAWALT[16] permits a reasonable recovery of protein in the high speed pellet (crude outer mitochondria membrane), but that fraction still contains a high cytochrome oxidase activity, while exhibiting a rather poor monoamine oxidase activity. The above results clearly illustrate the possibilities and limitations of two of the methods currently used to prepare inner and outer mitochondrial membrane. The hypotonic method is preferred for the preparation of outer membrane. On the other hand, the digitonin method allows isolation of purer inner membrane, as assessed by the activities of marker enzymes. Nevertheless recent results point to possible deleterious effects of digitonin on the organization of the inner membrane, for instance on ADP translocase.

Smooth and rough endoplasmic reticulum membranes

The study of the isolation and analysis of endoplasmic reticulum membranes was motivated by the finding[18] that our inner mitochondrial membrane preparations could be contaminated by rough endoplasmic membrane and that outer mitochondrial membrane was contaminated by smooth endoplasmic reticulum. In Table V are reported enzymic analyses of microsomal, smooth endoplasmic reticulum and rough endoplasmic reticulum preparations. This latter resembles that of microsomes, on the contrary, the smooth endoplasmic reticulum fraction seems to be more heterogeneous and corresponds to an enrichment not only of endoplasmic reticulum membrane but also of plasma and lysosomal membranes.

The sedimentation behaviour of the membranes collected in the smooth endoplasmic reticulum fraction depends on the concentration of $MgCl_2$; whereas in the presence of 1 mM $MgCl_2$ the original microsomal proteins divide in about equal proportion into the two endoplasmic reticulum membrane fractions, in the presence of 5 mM $MgCl_2$ about 70 % of the protein is recovered in the rough endoplasmic reticulum fraction. This may explain the apparent different distribution of NADPH cytochrome *c* reductase according to the isolation conditions (Table V). In any case, and in agreement with others[52,53], D-glucose-6-phosphatase was found more active in the rough endoplasmic reticulum membrane.

The smooth endoplasmic reticulum fraction contains, besides some lysosomal and plasma membranes, different types of endoplasmic reticulum membranes. By further centrifugation through a density-gradient it is possible to obtain a subfraction which, when examined after negative staining by electron microscopy, contains particles resembling small bags having extended filaments, as found in Golgi apparatus[54]. On the other hand, after treatment of the rough endoplasmic reticulum fraction by EDTA, as the rough endoplasmic reticulum membrane loses its attached polysomes* its sedimentation behaviour changes and polysome-free rough endoplasmic reticulum membrane moves into the smooth endoplasmic reticulum fraction.

On the specificity of microsomal marker enzymes

BRUNNER AND BYGRAVE[55] have questioned the choice of NADPH cytochrome *c* reductase and D-glucose-6-phosphatase as marker enzymes for microsomes. They

* Assessed by the ratio RNA-P/phospholipid-P which dropped from 1.08 for rough endoplasmic reticulum to 0.49 after EDTA treatment; a value of 0.81 being found for the starting microsome fraction and 0.31 for smooth endoplasmic reticulum.

TABLE V

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF MICROSOMAL AND PLASMA MEMBRANE PREPARATIONS

Specific activities in nmoles·min^{-1}·mg^{-1} of protein (mean and S.D.; in parentheses: number of experiments).

Fraction	*Acid phosphatase*	*5'-Nucleotidase*	*NADPH cytochrome c reductase*	*Glucose 6-phosphatase*	*Phospholipid-P/protein (μmoles P/mg)*	*Cholesterol/phospholipid-P (molar ratio)*
Microsomes$_1$*	34 ± 13 (33)	61 ± 28 (12)	94 ± 10 (5)	192 ± 54 (30)	0.318 ± 0.030 (12)	0.120 ± 0.022 (11)
Smooth endoplasmic reticulum$_1$	75 ± 29 (14)	174 ± 66 (9)	187 ± 68 (3)	220 ± 79 (16)	0.468 ± 0.103 (11)	0.237 ± 0.051 (12)
Rough endoplasmic reticulum$_1$	30 ± 21 (12)	7 ± 3 (4)	62 ± 39 (4)	286 ± 105 (13)	0.330 ± 0.044 (11)	0.065 ± 0.014 (11)
Microsomes$_5$**	31 ± 3 (5)	56 ± 22 (5)	99 ± 18 (5)	108 ± 29 (5)	0.322 ± 0.029 (5)	0.100 ± 0.003 (3)
Smooth endoplasmic reticulum$_5$	117 ± 54 (5)	246 ± 100 (4)	65 ± 37 (5)	111 ± 61 (5)	0.412 ± 0.105 (6)	0.287 (2)
Rough endoplasmic reticulum$_5$	22 ± 8 (5)	37 ± 8 (5)	135 ± 36 (5)	144 ± 52 (5)	0.310 ± 0.033 (6)	0.053 ± 0.027 (3)
Plasma membrane before gradient	49 ± 15 (5)	608 ± 191 (7)	8 ± 2 (4)	21 ± 2 (3)	0.260	0.46
after gradient	80 ± 49 (4)	961 ± 166 (5)	10 ± 4 (4)	9 (2)	0.368	0.761

* Fractions isolated in the presence of 1 mM $MgCl_2$.
** Fractions isolated in the presence of 5 mM $MgCl_2$.

consistently found high specific activities of those two enzymes in outer membrane preparations and therefore concluded that these two enzymes were intimately associated with the outer mitochondrial membrane as well as the microsomal membrane of rat liver mitochondria (the two membranes are similar in other respects, for instance in lipid composition). Such an assertion calls for the following remarks.

(1) D-Glucose-6-phosphatase[23] and NADPH cytochrome *c* reductase[24] are localized in microsomes. Both D-glucose-6-phosphatase and NADPH cytochrome *c* reductase exhibit increases in their specific activities as non-membrane proteins are removed from the rough endoplasmic reticulum fraction.

(2) It is true that outer mitochondrial membrane preparations may have high D-glucose-6-phosphatase activity, as was the case for the experiment reported in Table VI. In that experiment there was an apparent discrepancy between the results given by the two microsomal marker enzymes: whereas the activity of NADPH cytochrome *c* reductase was 4 times higher in microsomes than in the outer mitochondrial membrane fraction, the activity of D-glucose-6-phosphatase was only twice as high. It could, therefore, have been concluded that 60 % of the D-glucose-6-phosphatase found in the outer membrane does really belong to that fraction. To check this point, the true D-glucose-6-phosphatase was inactivated by preincubation at pH 5 for 1 min at 37°[56], and the phosphatase activity measured again at pH 6.5 with D-glucose-6-phosphate as substrate. The activities given on line 3, Table VI, are those calculated by subtracting the amount of phosphate released by unspecific phosphatases which are unaltered by heating at pH 5 and which represent 60 % of the activity measured in the outer mitochondrial membrane fraction. This

TABLE VI

COMPARISON OF THE SPECIFIC ACTIVITY OF NADPH CYTOCHROME *c* REDUCTASE, D-GLUCOSE-6-PHOSPHATASE AND MONOAMINE OXIDASE IN MITOCHONDRIA, OUTER MITOCHONDRIAL MEMBRANE AND MICROSOMES

Specific activities given in nmoles·min^{-1}·mg^{-1} protein.

Experiment	*Mitochondria*	*Crude* outer membrane*	*Microsomes*	*Ratio outer membrane / microsomes*	*Ratio outer membrane / mitochondria*
NADPH cytochrome *c* reductase		19.9	88.4	0.22	
Glucose 6-phosphatase (total)**		95	190	0.50	
True glucose 6-phosphatase activity***		34	152	0.22	
NADPH cytochrome *c* reductase+	6	30	94	0.32	5.0
Glucose 6-phosphatase	25	96	192	0.50	3.8
Monoamine oxidase	5	61	<0.1	>600	12.2

* High speed pellet in the method of PARSONS *et al.* (*cf.* METHODS).

** Total activity (measured at pH 6.5).

*** Difference between total activity and that found after inactivation of the true glucose 6-phosphatase by incubation of the fraction 5 min at pH 5 and at 37°.

+ Mean values of the specific activities reported in Tables IV and V.

unspecific phosphatase activity equals 1/2 and 1/3 of the acid phosphatase activity, measured with *p*-nitrophenyl phosphate as substrate, in microsomes and outer membrane, respectively. After correction was made for the non-specific phosphatases, the ratio of the specific activities found in the two fractions for the two marker enzymes was the same.

(3) When the mean of the specific activities of marker enzymes reported in Tables IV and V is used to compare outer mitochondrial membrane with microsomes, it is seen (lower part of Table VI) that the presence of NADPH cytochrome *c* reductase parallels that of D-glucose-6-phosphatase, but that both are distributed quite differently from monoamine oxidase.

(4) When cytochrome P-450 was used to estimate a microsomal contamination the data obtained correlated with those given by the measure of NADPH cytochrome *c* reductase activity[18]. We therefore conclude with VAN TOL[57] that NADPH cytochrome *c* reductase and glucose-6-phosphatase are true enzyme markers for microsomes.

(B) Lipid composition of membranes

The lipid analyses were performed on fractions obtained by the different methods noted in Section A. The results of analyses of two types of submitochondrial membrane fractions (P = fractions obtained by the method of PARSONS *et al.*[15], D = fractions obtained by digitonin treatment according to SCHNAITMAN AND GREENAWALT[16]), are reported in Tables IV and XI, and analyses of two types of submicrosomal fractions (isolated either in the presence of 1 mM or 5 mM $MgCl_2$), in Tables V and XII.

Phospholipid content. As shown in Table IV, the phospholipid content expressed as μmoles of lipid-P per mg of protein increases progressively from a mean average value of 0.16 for mitochondria to 0.34 for inner membrane particles and to 0.46 for crude outer membrane (values higher than 0.5 have been found for purified outer membrane). As mentioned in Section A, the so-called inner membrane particles still contain some matrix material and therefore, the amount of 0.34 μmole of phospholipid-P per mg of protein probably represents an underestimated value of the true phospholipid content of the inner membrane.

The phospholipid content of microsomal and plasma membranes was found to be roughly the same as that of mitochondrial membranes (0.32–0.47 μmole of lipid-P per mg of protein). In order to avoid fluctuations due to the variable amount of non-membrane proteins in particles, the cholesterol content of each fraction has been expressed as a function of the lipid phosphorus content.

Cholesterol content of mitochondrial membranes. Attention has recently been drawn to the finding that mitochondria are practically devoid of cholesterol[49]. In our experiments, the mean value of the molar ratio cholesterol: phospholipid-P, which was 0.10 in whole mitochondria and 0.13 in outer mitochondrial membrane, was much lower in the case of inner membrane preparations. It decreased from a value of 0.06 in the case of inner membrane prepared by hypotonic treatment of mitochondria to a value of 0.01 and lower for inner membrane obtained by the digitonin method; the latter is relatively free of contaminant cholesterol-rich membranes such as plasma membranes (Table IV).

In order to check whether digitonin may extract some cholesterol originally

present in the inner membrane, subcellular fractions such as microsomes, smooth endoplasmic reticulum and rough endoplasmic reticulum membranes were analyzed for their cholesterol content before and after a digitonin treatment analogous to the one used by SCHNAITMAN AND GREENAWALT[16] to fractionate mitochondria (1.50 mg of digitonin per 10 mg of mitochondrial protein for 10 min at 0°). The results reported in Table VII show that, in the membranes collected after treatment with digitonin, the cholesterol content was quite comparable to that determined before digitonin treatment. Therefore it seems reasonable to conclude that the inner mitochondrial membrane contains either only minute amounts or probably no cholesterol at all.

TABLE VII

CHOLESTEROL CONTENT OF MICROSOME FRACTIONS BEFORE AND AFTER DIGITONIN TREATMENT

Smooth and rough endoplasmic reticulum were obtained according to MOLNAR[17] in the presence of 1 mM $MgCl_2$ as described in METHODS. Digitonin treatment was carried out according to SCHNAITMAN AND GREENAWALT[16]: an amount of 0.15 mg of digitonin per mg of protein was added to a suspension of endoplasmic reticulum membranes (final digitonin concentration: 1 % (w/v) and kept in contact for 10 min at 0°. The digitonin action was stopped by diluting twice with 0.27 M sucrose; the membranes were collected by centrifugation and the lipids extracted as described in METHODS.

Conditions	*Fraction*	*Acid phosphatase**	*5'-Nucleotidase**	*Cholesterol/phospholipid-P***
Control	Microsome	30	67	0.122
	Smooth endoplasmic reticulum	72	184	0.287
	Rough endoplasmic reticulum	31	39	0.107
After digitonin treatment	$Microsome_D$	39	43	0.111
	Smooth endoplasmic $reticulum_D$	43	195	0.271
	Rough endoplasmic $reticulum_D$	12	43	0.103

* Specific activities in nmoles·min^{-1}·mg^{-1} protein.
** Molar ratio.

The outer mitochondrial membrane preparations contain enough lysosomal and plasma membranes to account for the cholesterol found in the outer membrane fraction. However, when whole mitochondria are analyzed for their cholesterol content after purification by tritosome flotation and by "slight" digitonin treatment (25 μg digitonin per mg of protein), 10 min at 0°, which does not result in a morphological change of the outer membrane, the drop in cholesterol is comparatively smaller than the decrease of the specific activities of 5'-nucleotidase and acid phosphatase (Table VIII). These results strongly suggest that mitochondria may contain some cholesterol which is located only in the outer mitochondrial membrane.

Comments on the hexosamine and sialic acid content of mitochondria. Along the same lines it might be thought that plasma membrane, which is richer in glycoproteins than endomembranes[58,7], could account for the presence of hexosamines and sialic acids in the mitochondrial preparations. However, as shown in Table VIII, after purification of the mitochondrial suspension the decrease in carbohydrates is less than that of the activity of marker enzymes for contaminant membranes.

TABLE VIII

CHOLESTEROL, HEXOSAMINE, AND SIALIC ACID CONTENT OF WHOLE MITOCHONDRIAL PREPARATIONS BEFORE AND AFTER PURIFICATION

E is a purified fraction of mitochondria obtained from rats which have been injected with Triton WR-1339 (*cf.* ref. 14). E "digitonin" is an E-fraction treated with a small amount of digitonin (25 μg/mg protein). Enzymatic activities in nmoles·min^{-1}·mg^{-1} protein. Sialic acid and hexosamines content in nmoles/mg protein.

	Cytochrome oxidase	*Malate dehydrogenase*	*Monoamine oxidase*	*Acid phosphatase*	*Arylsulfatase*	*5'-Nucleotidase*	*NADPH cytochrome c reductase*	*Cholesterol/phospholipid-P**	*Sialic acid*	*Hexosamine*	*Hexosamine/sialic acid**
Mitochondria	2056	720	4.6	56	3.05	24.8	5.3	0.092	3.74	14.9	4.0
E-fraction	3826	1050	7.4	49	3.97	16.8	6.1	0.031	1.21	12.7	10.5
E "digitonin"	4053	1100	5.4	17	1.54	5.6	5.0	0.026	1.06	9.05	8.5

* Molar ratio.

TABLE IX

TOTAL GLYCERIDES AND TRIGLYCERIDES CONTENT IN INTRACELLULAR MEMBRANES

Mitochondrial membranes were obtained by the method of PARSONS[15]. Tritosomes were Triton-filled lysosomes. Rough endoplasmic reticulum and smooth endoplasmic reticulum were prepared from 1 mM Mg^{2+} microsomes.

Fraction	*Glycerides**	
	Total	*Triglyceride*
Mitochondria		
Inner membrane *plus* matrix	13.2	13.3
Inner membrane	8.8	8.1
Outer membrane	17.3	—
Tritosomes		
H-fraction	17.3	13.2
Microsomes		
Total	36.8	22.4
Smooth endoplasmic reticulum	47.6	10.7
Rough endoplasmic reticulum	46.6	18.5

* nmoles/mg protein.

In accordance with findings by other authors[59], we found hexosamines and sialic acids in rat liver mitochondria distributed as follows respectively in the inner and outer membranes[15]: 1.0 and 5.5 nmoles of *N*-acetylneuraminic acid per mg of protein and 10.4 and 20.6 nmoles of hexosamines per mg of protein.

Glyceride content. Although glycerides are not believed to be structural components of biological membranes[5], we measured the content of total glycerides and triglycerides in mitochondria, microsome and tritosome preparations (Table IX). Triglycerides but no diglycerides were found in submitochondrial membrane preparations. In microsomes that contained three to four times more glycerides than mitochondria, diglycerides and monoglycerides were found as well as triglycerides.

Cholesterol content of microsomal and plasma membranes. Table V records the specific activities of enzyme markers and the lipid content of microsomes, smooth endoplasmic reticulum, rough endoplasmic reticulum and plasma membrane fractions.

The nature of the membranes collected as rough endoplasmic reticulum and smooth endoplasmic reticulum on the basis of a difference of density varies according to the $MgCl_2$ concentration: in the presence of 1 mM $MgCl_2$, about 50 % of the microsomal protein is recovered in each fraction whereas in the presence of 5 mM $MgCl_2$ 30 % is found in the smooth endoplasmic reticulum fraction and 70 % in the rough endoplasmic reticulum fraction. This difference in the partition of smooth endoplasmic reticulum and rough endoplasmic reticulum membranes is correlated by a difference in the distribution of the NADPH cytochrome *c* reductase activity, which suggests a heterogeneous composition of the microsomal membranes as already reported by DALLMAN *et al.*[60]. It is to be noted that, in the presence of 5 mM $MgCl_2$. the smooth endoplasmic reticulum and rough endoplasmic reticulum membrane fractions are more contaminated by other membranes such as lysosomal and plasma membranes as shown by the increased acid phosphatase and 5'-nucleotidase activities.

The cholesterol values reported in Table V for rat liver microsomal fractions are in good agreement with those obtained by PASCAUD *et al.*[61] who, for the cholesterol: phospholipid-P ratio, found values of 0.130, 0.260 and 0.068 for microsomes, smooth endoplasmic reticulum and rough endoplasmic reticulum, respectively. Besides free cholesterol, esters of cholesterol were found in microsomes: molar ratios of cholesterol esters/phospholipid-P equal to 0.041 and 0.009 were found in microsome and rough endoplasmic reticulum fractions respectively.

In view of the lower amount of contaminant membrane present in the rough endoplasmic reticulum fraction, as assessed by enzymic evaluation, it may be concluded that the rough endoplasmic reticulum may be more representative of the native endoplasmic reticulum than the smooth endoplasmic reticulum. This statement is corroborated by the presence in the preparation of smooth endoplasmic reticulum of contaminant plasma-membrane especially rich in cholesterol (*cf.* Table V: cholesterol: phospholipid-P = 0.76 in the plasma membrane).

Phospholipid composition of mitochondria and mitochondrial membranes. The phospholipid distribution in mitochondria and mitochondrial membranes is given in Table X. As already shown in Table IV, the inner membrane matrix particles and the inner membrane fraction are contaminated only very slightly by other cytoplasmic membranes; their phospholipid composition should therefore be really representative. In the inner membrane, the two main phospholipids: phosphatidyl-

TABLE X

PHOSPHOLIPID COMPOSITION OF RAT LIVER MITOCHONDRIAL MEMBRANES

The inner and outer mitochondrial membranes were prepared either by the method of PARSONS *et al.*[15] (P), or by digitonin action (D). The phospholipid content is expressed as total phosphorus percentage. Data represent mean ± S.D.

	Mitochondria (5)	*Inner membrane plus matrix* *P* (7)	*Inner membrane* *P* (5)	*Inner membrane plus matrix* *D*	*Inner membrane* *D* (6)	*Outer membrane* *P* (5)	*Outer membrane* *D* (4)
Lysophosphatidyl choline	1.4 ± 0.3	0.7 ± 0.2	0.6 ± 0.2	—	0.6 ± 0.6	—	1.8 ± 1.0
Sphingomyelin	2.4 ± 0.9	1.0 ± 0.2	2.0 ± 0.7	1.1	0.8 ± 1.0	2.2 ± 1.3	4.4 ± 0.9
Phosphatidyl choline	40.5 ± 2.9	39.2 ± 3.8	40.5 ± 2.5	38.5	37.9 ± 1.0	49.4 ± 5.5	45.4 ± 2.5
Phosphatidyl inositol	6.6 ± 1.5	3.6 ± 1.1	1.7 ± 1.2	1.8	2.0 ± 1.5	9.2 ± 3.6	8.4 ± 4.2
Phosphatidyl ethanolamine*	34.7 ± 1.3	39.9 ± 3.1	38.8 ± 2.6	38.6	38.3 ± 1.5	34.9 ± 5.2	30.4 ± 4.7
Cardiolipin (*plus* phosphatidic acid)	14.8 ± 1.2	15.4 ± 2.1	17.0 ± 2.0	20.0	20.4 ± 1.9	4.2 ± 1.5	9.5 ± 3.4

* By a two-dimensional thin-layer chromatography[38] of the phospholipids from whole mitochondria, a phosphatidyl serine spot could be isolated which represented 0.9% of the total lipid phosphorus.

TABLE XI

FATTY ACID DISTRIBUTION IN THE MAIN PHOSPHOLIPIDS OF MITOCHONDRIAL MEMBRANES

P = mitochondrial membranes were obtained by the method of PARSONS *et al.*[15]. D = mitochondrial membranes were obtained by digitonin treatment[16]. Each figure is the mean of two separate determinations and is expressed as area percent. Mitos., mitochondria; I.M., inner membrane; O.M., outer membrane.

Fatty acid	*Phosphatidyl choline*					*Phosphatidyl inositol*			*Phosphatidyl ethanolamine*					*Cardiolipin*		
	Mitos.	*I.M. P*	*O.M. P*	*I.M. D*	*O.M. D*	*Mitos.*	*O.M. P*	*O.M. D*	*Mitos.*	*I.M. P*	*O.M. P*	*I.M. D*	*O.M. D*	*Mitos.*	*I.M. P*	*I.M. D*
14:0	0.4	0.5	0.5	0.3	0.4	—	—	2.0	0.3	0.5	0.2	1.2	0.6	0.2	0.5	1.0
16:0	27.0	27.8	31.5	22.1	27.0	26.3	30.2	27.3	26.6	24.8	30.6	19.6	25.3	7.0	7.7	6.4
16:1	3.9	3.9	4.6	3.6	4.1	5.8	6.0	3.8	3.2	1.8	2.2	1.0	2.5	7.6	6.2	5.9
18:0	21.6	20.5	26.1	18.0	21.0	38.4	35.3	30.6	27.3	27.7	26.1	24.8	25.5	3.6	4.8	3.9
18:1	13.0	13.6	13.3	16.2	13.5	14.0	14.4	18.2	12.0	8.8	8.2	13.0	10.0	19.9	19.2	20.3
18:2	12.4	14.0	11.0	15.8	13.5	4.2	8.2	5.1	5.4	4.7	6.3	8.5	4.7	58.8	57.8	59.4
20:3	1.3	1.4	1.0	1.0	1.1	—	—	—	—	0.4	0.3	1.9	0.1	1.2	2.4	2.5
20:4	17.7	15.8	10.7	18.5	15.7	7.6	5.9	8.6	22.0	23.8	21.4	23.5	22.8	1.8	1.4	0.6
22:6	2.9	2.5	1.5	3.8	3.5	3.2	—	4.5	3.2	7.5	4.8	5.6	8.0	—	—	—
Percent saturated	49.0	48.8	58.1	40.4	48.4	64.7	65.5	59.9	54.2	53.0	56.9	45.6	51.4	10.8	13.0	11.3
Ratio satd./unsatd.	0.95	0.95	1.38	0.69	0.94	1.86	1.89	1.47	1.15	1.13	1.32	0.85	1.07	0.12	0.15	0.12
ΣC_{18}	47.0	49.6	50.4	50.0	48.0	56.6	57.9	53.9	44.7	41.2	40.6	46.3	40.2	82.3	81 8	83.6

choline and phosphatidylethanolamine, are present in about equal amounts (a little less than 40 % each), cardiolipin accounts for practically 20 % of the total lipid phosphorus. These values are in good agreement with those already found in other laboratories[15,62-66]. The outer mitochondrial membrane contains more phosphatidylcholine and less phosphatidylethanolamine than the inner membrane, a distribution intermediary between that of inner membrane (Table X) and of microsomes (Table XII). The presence of cardiolipin in the outer membrane, which parallels that of the cytochrome oxidase activity, reflects the contamination of the outer membrane preparation by inner membrane. As already mentioned by others[15,63,66], the outer mitochondrial membrane is markedly richer in phosphatidyl inositol than the inner membrane.

Fatty acid distribution in mitochondrial phospholipid. The fatty acid distribution in phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and cardiolipin has been determined for each type of mitochondrial membrane preparation (Table XI). Nevertheless, for the sake of clarity, the fatty acid pattern of the phosphatidyl inositol found in the inner mitochondrial membrane and of the cardiolipin found in the outer mitochondrial membrane preparations was not reported in Table XI. The following conclusions may be drawn:

(a) the fatty acid composition of each of the main four phospholipid classes was essentially the same for each phospholipid species in all submitochondrial membrane fractions. This confirms other reports[4,63,64].

(b) cardiolipin is the most highly unsaturated phospholipid (only 10 % of the fatty acids are saturated) and contains more than 80 % of C_{18} fatty acids: linoleic acid (18:2) representing more than 60 % of the total, a percentage lower,* however, than that reported for guinea pig mitochondria[67] or beef heart mitochondria[13] or even rat liver mitochondria[62,64,68]. It was in cardiolipin that the highest content of palmitoleic (16:1) was found.

A paradoxically higher degree of saturation was found in the so-called cardiolipin found in lipid extracts from outer membrane fractions. This discrepancy is probably accounted for by the presence in outer membrane of phosphatidic acid which requires further chromatography for complete differentiation from cardiolipin[69]. Actually phosphatidic acid represents less than 1 % of the total mitochondrial phospholipids; it is not detected in extracts from inner membrane matrix particles even after labelling with radioactive fatty acids, while it could be detected in outer membrane thanks to its rapid labelling[70]. If the total mitochondrial phosphatidic acid is located in the outer membrane, its contribution to the cardiolipin spot is not negligible and may modify quite substantially its fatty acid composition (the proportion of phosphatidic acid present has always been too low to allow a specific analysis).

(c) Phosphatidylinositol is distinguishable from the other phospholipids by its high content of saturated fatty acids (60–65 % saturated), among which stearic acid predominates, and the low percentage of polyunsaturated fatty acid, oleic acid (18:1) being the principal unsaturated fatty acid.

(d) No more than half of the fatty acids found in phosphatidylcholine and phosphatidylethanolamine are saturated. Phosphatidylethanolamine contains more

* This might be due to the diet being marginally deficient in essential fatty acids as reflected by a somewhat high content of eicosatrienoic acid (20:3).

arachidonic acid (20:4) and long chain polyunsaturated fatty acids than phosphatidyl choline.

Phospholipid composition of microsomes and microsomal membranes. In contrast with the inner mitochondrial membrane, but similarly to the outer mitochondrial membrane, microsomes contain more phosphatidylcholine (60 % of the total microsomal phospholipid phosphorus) less phosphatidylethanolamine (only 25 %) and about 10 % of phosphatidylinositol. The enzymatic activities reported in Table V and the lipid analyses in Table XII permit comparison of the 5'-nucleotidase activity and the sphingomyelin content of each submicrosomal fraction: the rough endoplasmic reticulum fraction isolated in the presence of 1 mM $MgCl_2$ which seems to be the most representative of the endoplasmic reticulum membrane, probably does not contain any sphingomyelin as lipid constituent.

Fatty acid distribution in microsomal phospholipids. With regard to the phospholipid composition, the fatty acid pattern of the major phospholipids (phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine) are very similar in microsome and rough endoplasmic reticulum fractions; they differ slightly in smooth endoplasmic reticulum fraction (Table XIII).

The fatty acid distributions in phosphatidylcholine and phosphatidylethanolamine are quite similar. In both there is about the same proportion (23 %) of palmitic acid (16:0) and stearic acid (18:0). However, phosphatidylethanolamine is more highly unsaturated, being richest in arachidonic acid (20:4) (23 % versus 17 % in phosphatidylcholine) and docosahexenoic (22:6) (7–10 % *versus* in phosphatidylcholine). There is no significant difference between the fatty acid composition of the lecithins of microsomes, endoplasmic reticulum membrane, mitochondria and submitochondrial fractions. The same remark may be extended to the fatty acid composition of the phosphatidylethanolamines of those fractions.

The fatty acid composition of phosphatidyl inositol differs markedly from that of phosphatidylethanolamine and phosphatidylcholine in that stearic acid (18:0) represents 45 % of the total fatty acids, and palmitic acid (16:0) nearly 20 % (Table XIII). As in mitochondria, phosphatidylinositol is the most saturated of the microsomal phospholipids (60 % saturated) but, unlike mitochondrial phosphatidyl inositol, where the main unsaturated fatty acid was oleic acid (18:1), microsomal phosphatidyl inositol was found to contain more than 20 % of arachidonic acid (20:4).

Phospholipid and fatty acid distribution in plasma membrane. The phospholipid composition of a plasma-membrane fraction characterized by a nucleotidase activity of 1086 nmoles of P_i released per min per mg of protein (Table XIV) is given in Table XV. In agreement with other authors[41,69,71,72], plasma membrane was found to have a high content (23 %) of sphingomyelin, the major phospholipid being phosphatidyl choline (43 %), phosphatidyl ethanolamine representing a little less than 20 % of the total.

The fatty acid pattern of the major phospholipids is given in Table XVI. Phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine are all characterized by a high degree of saturation, palmitic acid (16·0) and stearic acid (18:0), present in about equal proportions, amounting up to 65 % of the total fatty acids. Unsaturated fatty acids are essentially represented by linoleic acid (18:2) and arachidonic acid (20:4). Since sphingomyelin also contains a high percentage

TABLE XII

PHOSPHOLIPID COMPOSITION OF RAT LIVER MICROSOMES AND MICROSOMAL MEMBRANES

Results expressed as total phosphorus percentage ± S.D. In parentheses: number of separate experiments.

	Microsomes (1 mM Mg^{2+}) (6)	*Smooth reticulum* (1 mM Mg^{2+}) (4)	*Rough reticulum* (1 mM Mg^{2+}) (4)	*Microsomes* (5 mM Mg^{2+}) (8)	*Smooth reticulum* (5 mM Mg^{2+}) (4)	*Rough reticulum* (5 mM Mg^{2+}) (6)
Lysophosphatidyl choline	2.0 ± 1.0	2.9 ± 0.7	2.9 ± 0.8	2.0 ± 0.4	1.9 ± 0.9	1.5 ± 0.8
Sphingomyelin	4.0 ± 0.5	6.3 ± 0.4	2.4 ± 0.6	4.2 ± 0.7	12.3 ± 0.4	3.9 ± 1.3
Phosphatidyl choline	58.7 ± 4.0	54.4 ± 4.6	59.6 ± 1.0	59.1 ± 1.5	55.4 ± 0.2	60.2 ± 3.1
Phosphatidyl inositol	8.2 ± 1.5	8.0 ± 2.9	10.1 ± 1.0	9.2 ± 1.2	6.7 ± 0.4	9.0 ± 1.8
Phosphatidyl ethanolamine (*plus* phosphatidyl serine)*	25.5 ± 2.8	26.1 ± 2.9	23.5 ± 3.4	24.1 ± 1.8	21.5 ± 1.9	23.3 ± 2.6
Cardiolipin (*plus* phosphatidic acid)	1.6 ± 1.3	2.4 ± 0.5	1.2 ± 2.0	1.0 ± 0.5	1.9 ± 1.7	1.2 ± 0.6

* By two-dimensional thin-layer chromatography[38] of a microsome extract it could be estimated that phosphatidyl serine represents 3.9% of the total lipid-P.

TABLE XIII

FATTY ACID DISTRIBUTION IN THE MAIN PHOSPHOLIPIDS OF MICROSOMAL MEMBRANES

Values (area %) are expressed as mean ± S.D. (4 experiments).

Fatty acid	*Phosphatidyl choline*			*Phosphatidyl inositol*			*Phosphatidyl ethanolamine*		
	Microsomes	*Smooth*	*Rough*	*Microsomes*	*Smooth*	*Rough*	*Microsomes*	*Smooth*	*Rough*
14:0	0.8 ± 0.5	0.4 ± 0.4	0.5 ± 0.6	2.5	—	—	tr.	0.2 ± 0.2	0.3 ± 0.4
16:0	24.5 ± 1.2	28.6 ± 1.2	22.7 ± 0.8	19.3	16.4	16.5	22.6 ± 1.6	26.1 ± 2.1	21.5 ± 1.2
16:1	3.3 ± 0.7	3.1 ± 0.6	3.6 ± 1.3	1.8	6.0	5.5	2.3 ± 0.2	2.7 ± 1.2	1.5 ± 0.2
18:0	21.0 ± 0.6	26.5 ± 3.2	22.0 ± 1.6	45.0	45.4	43.2	23.4 ± 1.1	25.5 ± 2.0	23.5 ± 1.8
18:1	12.3 ± 0.6	10.6 ± 1.3	11.1 ± 0.2	7.2	7.4	7.5	9.8 ± 2.0	9.9 ± 2.0	8.3 ± 0.3
18:2	17.7 ± 1.0	14.9 ± 0.8	16.1 ± 1.6	3.2	2.3	3.4	10.3 ± 2.0	10.9 ± 3.8	10.2 ± 1.7
20:3	1.2 ± 1.0	1.4 ± 0.6	1.8 ± 0.5	—	2.0	1.2	tr.	0.7 ± 0.5	—
20:4	15.8 ± 1.2	14.0 ± 1.8	19.7 ± 1.9	21.4	18.8	22.8	23.1 ± 2.8	15.8 ± 2.6	22.9 ± 4.8
22:6	2.9 ± 1.2	0.7 ± 1.1	2.9 ± 1.7	—	—	—	7.2 ± 0.7	7.2 ± 3.3	11.3 ± 2.6
Percent saturated	46.3	55.5	45.2	66.8	61.8	59.7	46.0	51.8	45.3
Σ satd./Σ unsatd.	0.87	1.24	0.82	1.99	1.69	1.48	0.87	1.19	0.8_4
Σ C_{18}	51.0	52.0	49.2	55.4	54.1	54.1	43.5	46.3	42.0

TABLE XIV

SPECIFIC ACTIVITIES OF MARKER ENZYMES OF THE PLASMA MEMBRANE FRACTION AND THE TRITOSOME FRACTION USED FOR LIPID ANALYSIS

Enzymatic specific activities in nmoles·min^{-1}·mg^{-1} protein. N.D., non detected.

	5'-Nucleotidase	*Acid phosphatase*	*NADPH cytochrome c reductase*	*Cytochrome oxidase*	*Monoamine oxidase*	*Phospholipid-P/protein**	*Cholesterol/phospholipid-P***
Plasma membrane	1086	148	15.2	143	N.D.	0.368	0.761
H-Fraction (Tritosomes)	337	908	7.7	—	N.D.	0.21	0.490

* μmoles phospholipid per mg protein.
** Molar ratio.

TABLE XV

PHOSPHOLIPID ANALYSIS OF A TRITOSOME AND A PLASMA MEMBRANE FRACTION

Analysis of the plasma membrane and the tritosome fractions described in Table XIV

	Phospholipid-P (%)	
	Plasma membrane	*Tritosomes (H-fraction)*
Lysophosphatidyl choline	1.8	2.9
Sphingomyelin	23.1	16.0
Phosphatidyl choline	43.1	41.9
Phosphatidyl inositol	6.5	5.9
Phosphatidyl serine	3.7	N.D.*
Phosphatidyl ethanolamine	19.8	20.5
Not identified		12.9
Phosphatidic acid	2.0	

* N.D., non detected.

TABLE XVI

FATTY ACID DISTRIBUTION IN THE MAIN PHOSPHOLIPIDS OF TRITOSOMES AND PLASMA MEMBRANE

Area (%) mean of two experiments.

Fatty acid	*Plasma membrane*				*Tritosomes*		
	Sphingomyelin	*Phosphatidyl choline*	*Phosphatidyl inositol*	*Phosphatidyl ethanolamine*	*Phosphatidyl choline*	*Phosphatidyl inositol*	*Phosphatidyl ethanolamine*
16:0	36.1	32.8	30.7	30.6	29.9	36.3	28.5
16:1	4.0	2.9	8.4	1.2	2.9	7.5	4.6
18:0	25.6	34.9	36.6	31.3	24.4	27.1	27.2
18:1	2.1	10.2	13.2	10.1	10.5	13.5	12.3
18:2	1.0	8.1	2.9	6.5	8.7	2.4	5.4
20:0	0.7	—	—	—	—	—	—
20:3	—	1.1	—	0.9	2.5	2.1	1.3
20:4	17.7	8.4	8.0	16.5	18.4	11.1	13.4
22:0	2.8	—	—	—	—	—	—
22:6	—	1.6	—	2.9	2.8	—	7.0
24:0	9.8	—	—	—	—	—	—
Percent saturated	75.0	67.7	67.3	61.9	54.3	63.4	55.7
Ratio satd./unsatd.	3.03	2.10	2.06	1.62	1.17	1.73	1.26
ΣC_{18}	28.7	53.2	52.7	47.9	43.6	43.0	44.9

of a saturated fatty acid, it is clear that all phospholipids contribute to the high saturation of the plasma membrane, as already reported by COLEMAN[73].

Comments on the phospholipids of lysosomes. Whereas the estimated contamination of plasma membrane by lysosomal membrane (5–10 %) allows its phospholipid composition to be estimated, it is much more difficult to make a good estimation of the true phospholipid composition of lysosomes. The tritosome preparations had a high 5′-nucleotidase activity (Table II) and the lysosome fraction obtained between mitochondria and microsomes by differential centrifugation is a very mixed fraction having a low acid phosphatase activity (compared to a tritosome fraction). However it appears that the sphingomyelin content of the tritosome fraction, 16 % in Table XV and up to 21 % in other experiments, is sufficiently high for sphingomyelin to be considered as a genuine component of the lysosomal membrane[74].

DISCUSSION

The cholesterol values found in this study are in good agreement with those already published in the literature.

In rat liver microsomes, molar ratios of cholesterol: phospholipid equal to 0.111[75], 0.110[76], 0.130[61] and 0.100-0.120 (this study) have been found; some others were much higher: 0.240[77], 0.310[73]. Comparable values were found in guinea pig microsomes: 0.11-0.17[78] and in pig liver microsomes: 0.092[76].

In mitochondria, the cholesterol: phospholipid molar ratio has been found to be 0.180[73], 0.111[75], 0.063[79], 0.100[71], 0.062[80] and 0.10 (this study). For mitochondrial membranes isolated after swelling, cholesterol: phospholipid molar ratios of 0.030 and 0.062 were found for inner and outer membrane of guinea pig mitochondria[78] and 0.039 and 0.13-0.25 for inner and outer membrane of rat liver mitochondria[77]. Up to very recently cholesterol was considered as a normal constituent of all cellular membranes, but in most of the former studies the amount of contaminant cholesterol-rich membrane such as plasma membrane was not assessed. Recently, the studies of the group of LOUVAIN[48,49] cast some doubt on the belief in an even distribution of cholesterol in rat liver cellular membranes, since it was shown that digitonin alters the density[48] and the morphological aspect[49] of the plasma membrane in particular. It was, therefore, inferred that the cholesterol found in mitochondria and microsome preparations could come from contaminant plasma membranes. In agreement with this, it is shown in this study that the amount of cholesterol found in mitochondrial membrane fractions dropped drastically with the elimination of plasma membrane as assessed by the 5′-nucleotidase activity of the fractions (Table V), which corroborates the concept that plasma membrane is a typically cholesterol-rich cytoplasmic membrane. In lipid extracts of digitonin inner membrane fractions only traces of cholesterol could be detected. Since the digitonin treatment when applied to microsomes and microsomal membranes did not appear to modify their cholesterol content (Table VII), it is concluded that the inner mitochondrial membrane does not contain cholesterol. It must be pointed out, however, that digitonin-cholesterol complexes have been observed at the level of mitochondria and even inside mitochondria in mouse liver cells[81].

Plasma membrane is characterized by a high molecular ratio of cholesterol to phospholipid, values found are 0.76 (this study), 0.74[72], 0.7[69,82], 0.6[25,71].

Our results on the phospholipid composition of mitochondria and mitochondrial membranes are in agreement with those of other laboratories[15,62,-66]. We found most of the mitochondrial phosphatidyl inositol in the outer membrane. The amount of phosphatidyl inositol found in the inner membrane fraction was often barely detectable and could have arisen from contaminant membranes.

The phospholipid compositions of microsomal membranes reported here agree with the values of KLEINIG[76] and KEENAN AND MORRÉ[41] but differ from those of MANGANIELLO AND PHILLIPS[83] who, using a different fractionation procedure, found only 2% of phosphatidylinositol*, more sphingomyelin, and less phosphatidyl choline. The results of DALLNER *et al.*[85] (45% of phosphatidylcholine and 10% of sphingomyelin) would indicate a higher percentage of plasma membrane in their microsome fractions, since it appears that the sphingomyelin content practically parallels the 5'-nucleotidase activity. The lipid composition of microsomal membranes characterized by a high content in phosphatidylcholine (60%) and the presence of phosphatidylinositol (10%), is comparable with the lipid composition of nuclear membrane[76,86], and of outer mitochondrial membrane (this study and ref. 15). On the other hand, some phospholipid species seem to be specific for certain membrane: for instance, sphingomyelin for plasma membrane and cardiolipin for inner mitochondrial membrane.

In agreement with MACFARLANE *et al.*[87] PASCAUD[88] and GETZ *et al.*[62] for whole mitochondria and microsomes, with STOFFEL AND SCHIEFER[63] and PARKES AND THOMPSON[67] for microsomes and submitochondrial membranes, and with KEENAN AND MORRÉ[41] for submicrosomal membranes, it was found that the fatty acid composition of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol was fairly similar in mitochondrial and microsomal membranes: the fatty acid pattern being more characteristic of the phospholipid species than of the membrane. However, the fatty acid pattern of the phosphatidylcholine and phosphatidyl ethanolamine contained in plasma membrane differs by its high degree of saturation (67% saturated, palmitic (16:0) and stearic (18:0) acid representing more than 30% each). Plasma membrane would differ from endomembranes by control mechanisms for positioning the fatty acids residues in phospholipids, these mechanisms acting either by selection of the phosphatidic acid molecules used as precursors of phosphatidylethanolamine or phosphatidylcholine or by an intermediate deacylation–reacylation cycle that might be catalyzed by enzymes shown to be present in plasma membrane such as phospholipase A[89–91] and lysophospholipid reacylase[92].

Due to the difficulty in purifying extensively outer mitochondrial membrane and smooth endoplasmic reticulum membrane fractions it is hazardous to regard as significant the differences found in the fatty acid composition of their phospholipids. In particular the higher saturation found in the phospholipids contained in the outer membrane fraction (Table XI and ref.[93]) could be due to contaminant plasma-membrane phospholipids (*cf.* Table XVI). KEENAN AND MORRÉ[41] have found the phospholipids of the Golgi apparatus to be slightly more saturated. The Golgi together with the plasma membranes present in the smooth endoplasmic

* The mode of extraction of the phospholipids may also be involved: we found for instance that the method of BLIGH AND DYER[84] extracted much less phosphatidyl inositol than the method of DAWSON *et al.*[32].

reticulum fraction may account, at least in part, for the slightly higher saturation of phosphatidylcholine and phosphatidylethanolamine found in that fraction.

Based on the cholesterol content and the degree of saturation of their phospholipids, it appears that, among the cytoplasmic membranes, the inner mitochondrial membrane and the plasma membrane are the most typically different. It is probably significant that a very low amount (bordering on absence) of cholesterol in the inner mitochondrial membrane goes with a high degree of unsaturation of phospholipids, while a high amount of cholesterol, as found in plasma membrane, goes with a large percentage of saturated acids. Since cholesterol is known to have a "condensing" effect on phospholipid monolayers[94,95] an effect which increases with the saturation of fatty acids, these two factors, the high cholesterol content and the high degree of saturation should contribute to the rigidity of plasma membrane. On the other hand, the absence of cholesterol and the degree of unsaturation of phosphatidylethanolamine and cardiolipin in particular, may confer flexibility to the inner mitochondrial membrane.

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The data presented here on lipid composition of lysosomes and plasma membrane are in good agreement with those obtained by HENNING *et al.*[96] in a similar study.

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