BBA 75594

EXCHANGE OF PHOSPHOLIPIDS BETWEEN MICROSOMES AND INNER AND OUTER MITOCHONDRIAL MEMBRANES OF RAT LIVER

M. C. BLOK, K. W. A. WIRTZ AND G. L. SCHERPHOF
Laboratory of Biochemistry, State University of Utrecht, Vondellaan 26, Utrecht (The Netherlands)
(Received October 30th, 1970)

SUMMARY

1. Sixty minutes after injection of ^{32}P-labelled phosphate into rats, the specific activities of phosphatidylcholine and phosphatidylethanolamine were determined in mitochondria, outer and inner mitochondrial membranes, and microsomes. The phospholipid specific activities of the outer membrane were lower than those of the microsomes and 3 to 4 times higher than those of the inner membrane. This is compatible with the idea that ^{32}P-labelled phospholipids, synthesized in the endoplasmic reticulum, exchange first with the outer mitochondrial membrane followed by an exchange between outer and inner mitochondrial membrane.

2. Mitochondria were incubated with microsomes in the presence of a 105000 $\times g$ supernatant fraction (the pH 5.1 supernatant). It was shown that both inner and outer mitochondrial membranes were involved in the exchange of phospholipids with microsomes. The phospholipids of the outer membrane exchanged to a larger extent than did those of the inner membrane.

3. Adenylate kinase activity was determined in mitochondria as an index for the integrity of the outer membrane. It was found that the integrity was not seriously affected by incubating mitochondria with or without microsomes and supernatant. Electron micrographs of the same mitochondria supported this conclusion. This endorses the view that *in vitro* phospholipids exchange between inner membrane and microsomes *via* the outer membrane.

4. Mitochondria isolated 60 min after injection of ^{32}P-labelled phosphate were incubated at 37° in 0.25 M sucrose–1 mM EDTA (pH 7.8). It was demonstrated that the 3- to 6-fold difference between the specific activities of outer and inner membrane phospholipids was partially eliminated. It is concluded that phospholipids exchange between inner and outer membrane inside the mitochondrion.

5. In the 105000 $\times g$ supernatant fraction, a protein is present which stimulates the exchange of phospholipids between microsomes and mitochondria. Such a stimulatory protein appears to be lacking in the mitochondrion. Differences in phospholipid exchange between microsomes and outer mitochondrial membrane as compared to phospholipid exchange between outer and inner mitochondrial membrane are discussed with respect to this observation.

INTRODUCTION

Syntheses of proteins by the mitochondria and the endoplasmic reticulum are the intertwined processes involved in the biogenesis of mitochondria in the liver[1,2]. Although *in vitro* transfer of proteins from microsomal membranes to mitochondria has been observed[3], the mechanism whereby proteins, synthesized in the endoplasmic reticulum, are incorporated into the mitochondrial structure remains poorly understood.

Similarly, it is not understood how phospholipids such as phosphatidylcholine and phosphatidylethanolamine, which are synthesized only in the endoplasmic reticulum[4,5], become part of the mitochondria. KADENBACH[6] suggested that *in vitro* phospholipids complexed to proteins are transferred from microsomes to mitochondria. However, it has been found in rat hepatoma cells[7] and in various microorganisms[8,9], that the incorporation of protein and phospholipid in membranes is not necessarily a synchronized process. This is in contrast with the conclusion of BEATTIE[10], based on the incorporation of isotopes, that *in vivo* protein and phosphatidylcholine are assembled simultaneously during mitochondrial biogenesis. Moreover, it was suggested by the latter author in view of the distribution of label over the inner and outer mitochondrial membrane, that the outer membrane was formed prior to the inner membrane.

It is doubtful, however, if *in vivo* the biogenesis of mitochondria with regard to the constituent phospholipids can be understood by determining specific activities. Specifically, *in vivo* the appearance of radioactivity in the mitochondrial phospholipids reflects an exchange of phospholipids between the mitochondria and the endoplasmic reticulum of the liver[11,12]. In view of this exchange process one would anticipate that phospholipids of the outer mitochondrial membrane become more rapidly labelled than phospholipids of the inner membrane after injection of [^{32}P]phosphate[4], [^{14}C]-choline[13], or [^{14}C]glycerol[10,14].

Since it has been shown that *in vitro* phospholipids exchange between mitochondria and microsomes[4,10,15–18], it was considered important to determine to what extent outer and inner membrane were involved in this exchange process.

MATERIALS AND METHODS

Preparation of subcellular fractions

Male rats (300–500 g) were injected with Triton WR 1339 (850 mg/kg body weight) as described by WATTIAUX *et al.*[19]. After 4 days, the animals, which had been fasted overnight, were sacrificed and the livers excised. After thorough rinsing of the minced livers with ice-cold 0.25 M sucrose containing 1 mM EDTA (pH 7.8) a 10 % homogenate was made in the same medium with two strokes of a Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 600 × *g* for 15 min in a swinging-bucket rotor of the IEC-PR-6 centrifuge to sediment nuclei, red blood cells, whole cells, and cell debris. The supernatant was centrifuged for 5 min at 15000 × *g* in the SS-34 rotor of a Sorvall centrifuge to sediment mitochondria. This subcellular fraction was washed twice with half the initial volume of sucrose–EDTA and was centrifuged each time as before.

The final pellet was resuspended in sucrose–EDTA (8 ml for the mitochondria

of one liver) and about 4 ml of this suspension were layered on top of a gradient with the following composition: 3 ml 20 %, 3 ml 25 %, 3 ml 30 %, 3 ml 35 %, 4 ml 45 %, 10 ml 55 %, and 8 ml 65 % sucrose. All sucrose solutions used were neutralized with solid Tris to pH 7 and contained 1 mM EDTA. The gradients were spun in the SW-27 rotor of a Beckman ultracentrifuge at 130000 × *g* for 30 min. The mitochondrial fraction was collected from the gradient, mixed with 5–6 times its volume of 0.25 M sucrose–1 mM EDTA, and centrifuged at 25000 × *g* for 15 min in the SS-34 rotor of a Sorvall centrifuge. The sedimented mitochondria were suspended in sucrose–EDTA in a concentration of 40 mg of protein/ml.

Inner and outer mitochondrial membrane fractions were prepared according to the method of SCHNAITMAN *et al.*[20]. The inner membrane fraction, isolated from 100 mg of mitochondrial protein, was washed twice with 30 ml of sucrose–EDTA, and was centrifuged each time at 9500 × *g* for 10 min in the SS-34 rotor.

The mitochondrial supernatant prepared from untreated livers as described above was centrifuged at 15000 × *g* for 20 min in the SS-34 rotor in order to remove lysosomes. The resulting supernatant fraction was centrifuged at 105000 × g_{max} for 90 min (angle rotor No. 30, Spinco) to isolate the microsomes. The microsomes were washed twice by resuspending the pellets first in 10 mM Tris–HCl (pH 8.6) and then in 1 mM Tris–HCl (pH 8.6), essentially following the procedure of WALLACH AND KAMAT[21]. The microsomal suspensions were centrifuged both times at 105000 × g_{max} for 60 min (angle rotor No. 30, Spinco).

The washed microsomes were resuspended in sucrose–EDTA (20 mg of protein per ml), subjected to ultrasonic irradiation for 1 min in the Branson Sonifier, and stored at −20°. The pH 5.1 supernatant was isolated from the 105000 × *g* supernatant by adjusting the pH to 5.1 with 3 M HCl, removing the precipitated material by centrifugation and readjusting the pH to 7.8 with solid Tris.

Radioactive subcellular fractions were obtained from rats injected intraperitoneally with 500 μC [^{32}P]phosphate (Philips Duphar) 20 h before excision of the livers, unless specified otherwise.

Protein was determined by the biuret method[22] or by the method of LOWRY *et al.*[23].

Enzyme assays

Succinate-cytochrome *c* reductase was assayed according to the method of TISDALE[24]. Monoamine oxidase was determined following the procedure of WEISBACH *et al.*[25]. Adenylate kinase was measured according to the method of SOTTOCASA *et al.*[26]. This enzyme was quantitatively released from mitochondria using the method of PFAFF AND SCHWALBACH[27].

Incubation

The incubations were performed with gentle agitation in a 37° water bath. The incubation mixture contained mitochondria and microsomes in 0.25 M sucrose–1 mM EDTA (pH 7.8). Further details on the incubation are given in the legends to tables and figures. After incubation, the samples were centrifuged promptly in the SS-34 rotor of the Sorvall centrifuge at 15000 × *g* for 10 min to separate the subcellular fractions. The mitochondria were washed once by resuspending in 0.25 M sucrose and subsequent centrifugation at 15000 × *g* for 5 min. The final pellets were resuspended

in a small volume of sucrose. Aliquots were taken for isolation of outer and inner mitochondrial membranes, for enzyme assays and for extraction of phospholipids. Microsomal phospholipids were extracted from the 15000 × *g* supernatant. When ^{32}P-labelled inner mitochondrial membranes were incubated with unlabelled outer membranes, separation of the two membrane fractions at the end of incubation was achieved by centrifugation for 5 min at 10000 × *g* in Sorvall tubes (No. 250) with adapters. The sedimented inner membranes were washed once and resuspended in a small volume of sucrose. Subsequently the outer membranes were sedimented at 150000 × *g* for 75 min (angle rotor No. 50, Spinco) and resuspended in sucrose. Aliquots of both suspensions were taken for enzyme assays and for extraction of phospholipids.

Lipid phosphorus and ^{32}P

Ice-cold 10 % trichloroacetic acid was added to aliquots of suspensions of the various subcellular fractions (1:1, v/v). After 15 min the supernatant was discarded. The precipitated material was suspended in 10 ml of chloroform–methanol (2:1, v/v) and extracted overnight. The extract was washed following the procedure of FOLCH *et al.*[28]. Individual phospholipids were separated by thin-layer chromatography. Thin-layer plates were prepared with Silicagel H (E. Merck, Darmstadt, Germany) according to the method of SKIPSKI *et al.*[29]. Chloroform–methanol–acetic acid–water (50:28:10:5, by vol.) was used as developing solvent. Lipids were detected by iodine vapor and eluted with 30 ml of chloroform–methanol (1:4, v/v) after the areas containing the lipid were scraped off into small columns. After elution, the samples were evaporated to dryness and dissolved in methanol. Lipid phosphorus was determined by the method of CHEN *et al.*[30] after destruction of the sample according to the procedure of AMES AND DUBIN[31]. Aliquots of the methanol solution (0.2 ml) were mixed with 16 ml of toluene containing 0.5 % 2,5-diphenyloxazole and 0.03 % of 1,4-bis-(5-phenyloxazolyl-2)-benzene. Radioactivity was measured with a Packard-Tricarb liquid scintillation spectrometer.

Electron microscopy

Mitochondria or microsomes were pelleted in a Beckman Microfuge or the Spinco L-65 ultracentrifuge, respectively. The pellets containing 0.5–1.0 mg of protein were fixed for 1 h at 4° in 1 % OsO_4, buffered with isotonic veronal–acetate, pH 7.2 (ref. 32). Dehydration was achieved with graded acetone–water mixtures at 4° and the samples were embedded in Araldite 502. Grey to white sections were cut on a Reichert OmU2 ultramicrotome and picked up on copper grids covered with a carbon-coated Parlodion film. The sections were stained on the grid with saturated uranyl acetate for 5 min and with lead citrate[33]. Electron micrographs were taken with a Siemens Elmiskop I microscope at an accelerating voltage of 60 kV and at nominal instrumental magnifications of 8000.

Chemicals

ADP and sodium azide were obtained from E. Merck (Darmstadt, Germany); cytochrome *c* from Fluka (Switzerland); NADP$^+$, glucose-6-phosphate dehydrogenase and hexokinase from Boehringer (Mannheim, Germany); kynuramine from Sigma (St. Louis, Mo., U.S.A.); and digitonin from Calbiochem (Los Angeles, Calif., U.S.A.).

RESULTS

Purity of subcellular fractions

Mitochondria were isolated from Triton-injected rats to assure that lysosomal contamination in this fraction would be minimal[19]. The extent of microsomal contamination in the mitochondrial fractions can be estimated from the data in Fig. 2. After a 20-min incubation of ^{32}P-labelled microsomes with unlabelled mitochondria the specific activity of phosphatidylethanolamine in the mitochondria was 3–4 % of the specific activity of this phospholipid in the microsomes. In this 20-min period, unlabelled microsomal particles present as contamination in the mitochondria will equilibrate with the ^{32}P-labelled microsomal particles[34]. Thus, the microsomal contamination must be less than 3–4 % since exchange of [^{32}P]phosphatidylethanolamine has contributed to this percentage. Electron microscopy substantiates this conclusion.

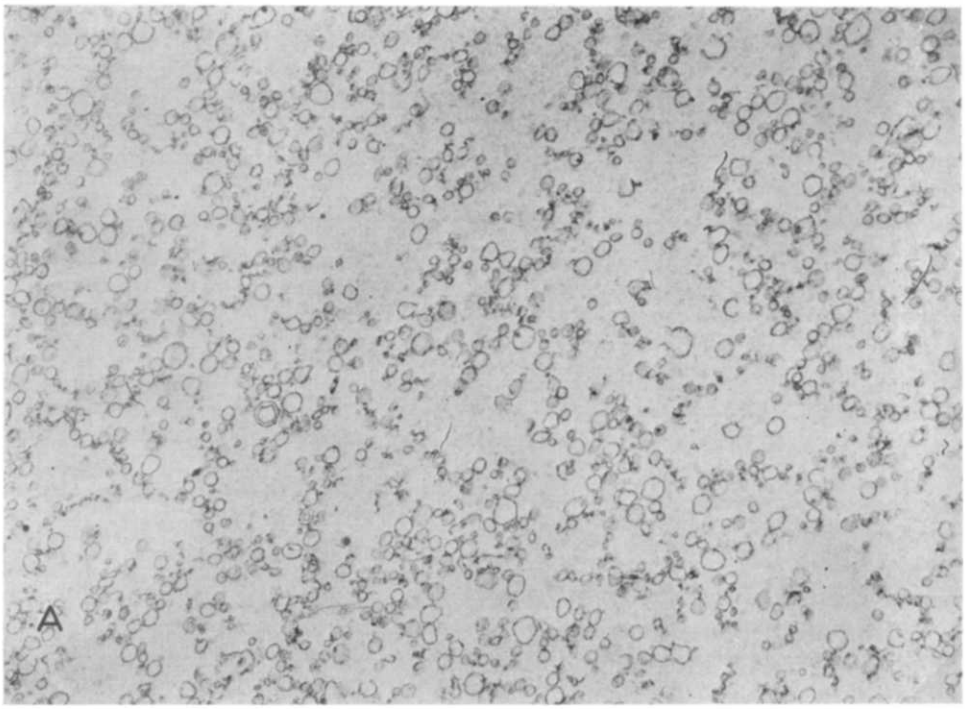
Fig. 1B shows mitochondria which have been incubated for 30 min at 37° in sucrose–Tris–EDTA. A picture of mitochondria immediately after isolation has the same overall appearance and is therefore omitted. Lysosomal structures were occasionally observed, but definitely accounted for less than 1 % of the total number of particles. Vesicular structures which could possibly be of microsomal origin were also encountered in extremely small proportions. Fig. 1C shows mitochondria isolated after a 30-min incubation in the presence of microsomes and supernatant fraction. It demonstrates that the procedure used to separate mitochondria from microsomes after incubation results in uncontaminated mitochondria. This is not surprising if one considers the small size of the microsomal particles shown in Fig. 1A.

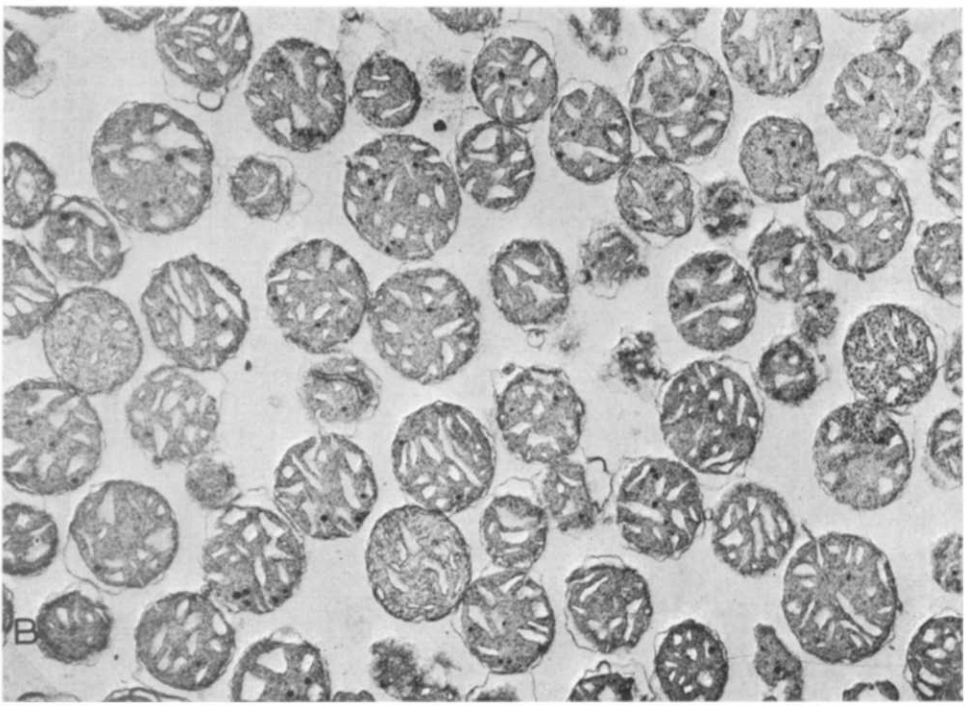
The specific activities of succinate-cytochrome *c* reductase and monoamine oxidase, marker enzymes for inner and outer mitochondrial membrane respectively, indicated that 7–8 % of the outer membrane fraction consisted of inner membrane material and that the inner membrane fraction contained 2 % of outer membrane material.

In vivo labelling of phospholipids of subcellular fractions

In a previous publication[11] we concluded that *in vivo* the rapid labelling of mitochondrial phospholipids after injection of [^{32}P]phosphate was due to an exchange of phospholipids between the endoplasmic reticulum and the mitochondria. Recently, JUNGALWALA AND DAWSON[12] provided direct evidence of the existence of this exchange process in isolated rat liver cells. In order to study this exchange process *in vivo* in greater detail, we determined the specific activities of phosphatidylcholine and phosphatidylethanolamine in intact mitochondria, outer and inner mitochondrial membranes, and microsomes 60 min after injection of [^{32}P]phosphate.

Table I shows that the highest specific activities of phospholipids were found in the microsomes, the lowest specific activities in the inner membrane fraction, whereas the outer membrane fraction had intermediate values. As for total mitochondria and microsomes, the specific activity ratio for phosphatidylethanolamine (0.18 $\pm$ 0.02) is lower than for phosphatidylcholine (0.46 $\pm$ 0.03). This suggests that phosphatidylethanolamine is involved to a lesser extent in the exchange between mitochondria and the endoplasmic reticulum than phosphatidylcholine. The same trend is apparent in the specific activity ratios of outer membrane phospholipid over microsomal phospholipid. A ratio of 0.81 $\pm$ 0.04 for phosphatidylcholine implies that close to isotopic equilibrium is reached within the given time interval.


A


B

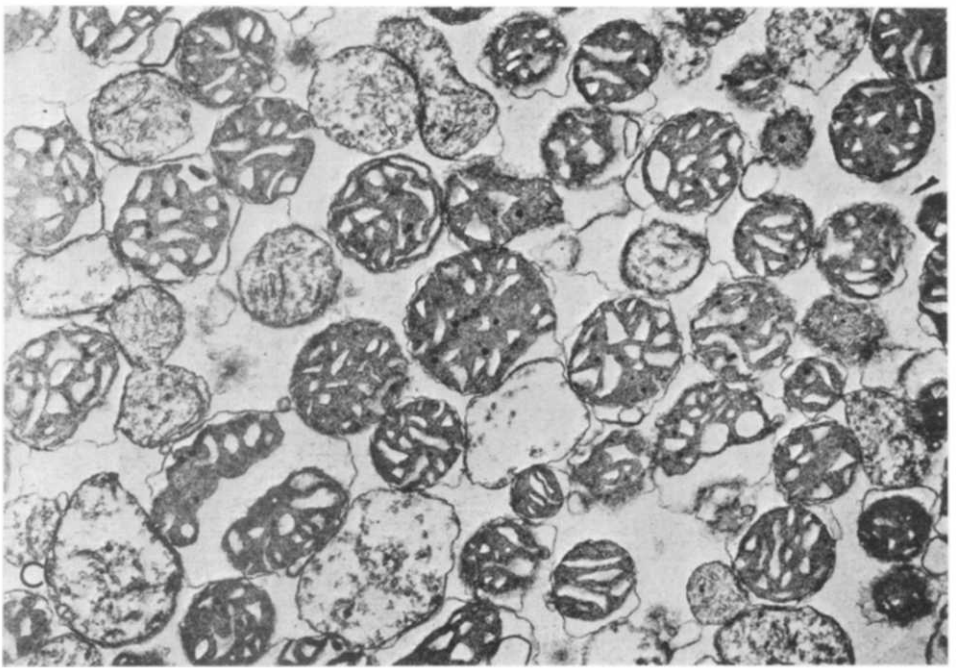


Fig. 1. A. Microsomal preparation after sonication; × 22400. B. Mitochondria incubated for 30 min at 37° in sucrose–Tris–EDTA; × 22400. C. Mitochondria isolated after incubation for 30 min at 37° in sucrose–Tris–EDTA in the presence of sonicated microsomes and supernatant proteins; × 22400.

In this respect, the approximately identical specific activity ratios of inner over outer mitochondrial membrane phosphatidylcholine and phosphatidylethanolamine (0.30 ± 0.09 and 0.26 ± 0.05, respectively) could mean that the rates of exchange of these two phospholipids inside the mitochondria are the same. This observation is at odds with what is observed for the exchange of these phospholipids between the endoplasmic reticulum and the outer mitochondrial membrane, where phosphatidylethanolamine is lagging behind phosphatidylcholine. Therefore, it is conceivable that *in vivo* another mechanism is operative in the transfer of phospholipids between the endoplasmic reticulum and the mitochondria than between outer and inner mitochondrial membranes.

In order to explore this possibility and to determine to what extent both the outer and the inner mitochondrial membrane are involved in the exchange of phospholipids with microsomes *in vitro*, various experiments were performed.

Exchange of phospholipids in vitro

^{32}P-labelled microsomes were incubated with unlabelled mitochondria in the presence of the pH 5.1 supernatant. After each incubation period, the specific activities of phosphatidylcholine and phosphatidylethanolamine, isolated from total mitochondria, inner and outer mitochondrial membranes, and microsomes were determined (see Fig. 2.). The decrease in specific activities of the microsomal phospholipids was compensated for by an increase in specific activities of the mitochondrial phospholipids. After 40 min of incubation, one could observe that 18% of the microsomal

TABLE I

INCORPORATION OF ^{32}P INTO MITOCHONDRIAL AND MICROSOMAL PHOSPHOLIPIDS

Microsomes, mitochondria, and mitochondrial subfractions were isolated 60 min after injection of [^{32}P]phosphate and specific activities of phospholipids were determined (see MATERIALS AND METHODS).

Expt. No.	*Phospholipid*	*Specific activity (counts/min per μg phospholipid-P)*				*Ratio of specific activities*		
		Microsomes	*Mitochondria*			*Mitochondria/ microsomes*	*Outer membrane/ microsomes*	*Inner membrane/ outer membrane*
			Intact	*Outer membrane*	*Inner membrane*			
1	Phosphatidylcholine	319	134	252	53	0.42	0.78	0.21
	Phosphatidylethanolamine	1753	279	670	140	0.16	0.38	0.21
2	Phosphatidylcholine	457	221	398	115	0.48	0.85	0.29
	Phosphatidylethanolamine	1270	248	518	158	0.19	0.41	0.31
3	Phosphatidylcholine	489	234	384	149	0.48	0.79	0.39
	Phosphatidylethanolamine	2723	535	1419	356	0.20	0.52	0.25
						mean of the ratios ± standard deviation		
	Phosphatidylcholine					0.46 ± 0.03	0.81 ± 0.04	0.30 ± 0.09
	Phosphatidylethanolamine					0.18 ± 0.02	0.44 ± 0.07	0.26 ± 0.05

phosphatidylcholine had exchanged, whereas only 3 % of the phosphatidylethanolamine had done so. The increase in specific activities was much higher for the outer mitochondrial membrane than for the inner mitochondrial membrane.

In the reverse experiment, ^{32}P-labelled mitochondria were incubated with unlabelled microsomes in the presence of pH 5.1 supernatant (Fig. 3). The drop in specific activity of phosphatidylcholine was more pronounced for the outer membrane than for the inner membrane. In fact, after about 10 min of incubation, the specific activity curve of outer membrane phosphatidylcholine intersected the corresponding curve for the inner membrane.

Because of the slow exchange of phosphatidylethanolamine between mitochondria and microsomes, differences in the specific activities of this phospholipid in outer and inner membrane did not become manifest in the course of the incubation.

The results in Figs. 2 and 3 show that the exchange of phospholipid between mitochondria and microsomes involves phospholipids of both inner and outer membrane. Phospholipids in the outer membrane appear to exchange more readily with microsomal phospholipid than phospholipids in the inner membranes. These observations provide indirect evidence that microsomal phospholipids exchange with those of the inner mitochondrial membrane by way of the outer mitochondrial membrane. Integrity of the outer mitochondrial membrane is required to prevent direct exchange of phospholipid between microsomes and the inner mitochondrial membranes. We checked this integrity both by enzyme assay and by electron microscopy.

Mitochondrial integrity

Various investigators[35,36] have shown that adenylate kinase is an enzyme which is localized in the intermembrane space of the mitochondrion. When the outer mitochondrial membrane is disrupted, adenylate kinase activity is released into the medium, thus providing a marker of outer membrane integrity.

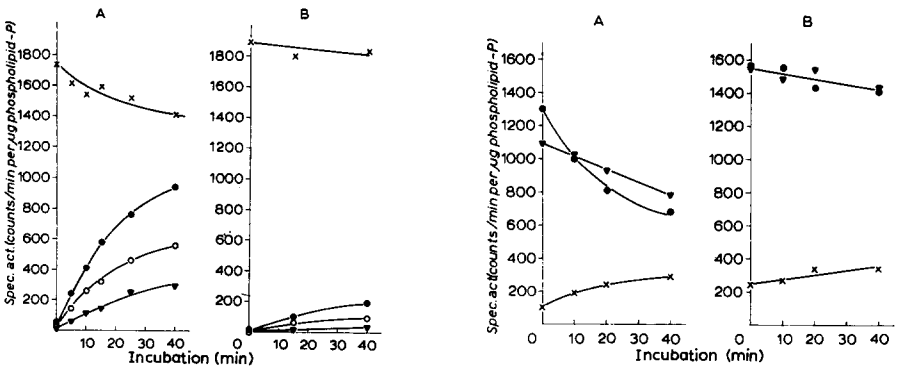


Fig. 2. Exchange of phosphatidylcholine (A) and phosphatidylethanolamine (B) between labelled microsomes and unlabelled mitochondria. Participation of outer and inner mitochondrial membrane in the exchange process. The incubation mixture contained 120 mg mitochondrial protein (240 µg phosphatidylcholine-*P*), 40 mg microsomal protein (400 µg phosphatidylcholine-*P*), and 85 mg pH 5.1 supernatant protein in a total volume of 30 ml 0.25 M sucrose–1 mM EDTA (pH 7.8). ×—×, microsomes; ○—○, intact mitochondria; ●—●, outer mitochondrial membrane; ▲—▲, inner mitochondrial membrane.

Fig. 3. See legend to Fig. 2. Unlabelled instead of labelled microsomes and labelled instead of unlabelled mitochondria were used in the incubations.

TABLE II

EFFECT OF INCUBATION ON THE RELEASE OF ADENYLATE KINASE ACTIVITY FROM MITOCHONDRIA

The incubation mixtures, containing in a total volume of 3.0 ml sucrose–EDTA 11.2 mg mitochondrial protein (column A), 4.5 mg microsomal protein and 7.4 mg pH 5.1 supernatant protein (column B), 11.2 mg mitochondrial protein, 4.5 mg microsomal protein and 7.4 mg pH 5.1 supernatant protein (column C) were incubated at 37° for different periods of time. Furthermore, 11.2 mg mitochondrial protein was incubated at 37° for 10 min in a total volume of 9 ml 20 mM triethanolamine buffer*. At the end of incubation, the subcellular fractions were sedimented at 150000 × *g* for 75 min (angle rotor No. 50, Spinco). The adenylate kinase activities were determined in the supernatant. Subtraction of the activities in column B from the activities in column C gives the corrected adenylate kinase activities (column D).

Time of incubation (min)	*nmoles $NADP^+$ reduced/3 ml supernatant per min*			
	A (mitochondria)	*B (microsomes plus supernatant)*	*C (mitochondria plus microsomes plus supernatant)*	*D (C − B)*
0	747	1086	1755	669
20	708	1101	1737	636
40	777	1095	1938	843

* 11.2 mg mitochondrial protein reduced 5967 nmoles $NADP^+$/min.

It is conceivable that during the incubation of mitochondria in the presence of microsomes and pH 5.1 supernatant, the outer mitochondrial membrane is gradually disrupted. Consequently, direct exchange of phospholipids between inner membranes and microsomes would become possible. The release of adenylate kinase was determined in the absence and presence of both the pH 5.1 supernatant and the microsomes (see legend to Table II).

Incubating mitochondria alone did not result in a release of adenylate kinase activity as indicated by the nearly identical activities in the mitochondrial supernatant at 0, 20, and 40 min of incubation (Table II, column A). The adenylate kinase activity already present at zero min of incubation resulted from resuspending the mitochondria before incubation. This activity accounted for about 10 % of the total activity present in the mitochondria.

Incubating mitochondria in the presence of pH 5.1 supernatant and microsomes also failed to affect significantly the mitochondrial integrity. This conclusion is based on the adenylate kinase activities present in the mitochondrial supernatant after incubation (Table II, column C). If this activity is corrected for the activity present initially in the pH 5.1 supernatant (Table II, column B), values resulted (Table II, column D) which are within 10 % of those obtained with mitochondria alone (Table II, column A).

Morphological examination revealed that mitochondria after a 30-min incubation in sucrose–Tris–EDTA retain the appearance they had immediately after isolation. Nearly all particles are surrounded by a double membrane structure while the matrix is generally in the condensed form, as described by HACKENBROCK[37] (Fig. 1B). Apparently, neither the isolation procedure nor the incubation at elevated temperature results in considerable detachment of the outer membrane structure. Incubation in the presence of microsomes and supernatant affected mitochondrial structure

slightly (Fig. 1C). Although most of the particles still had the condensed appearance, a significant proportion was swollen to various extents. At least 90% of the mitochondria had a clearly distinguishable outer membrane structure.

It follows from the enzyme assays and the electron micrographs that the integrity of the outer membrane is not seriously affected either by the isolation (see Fig. 1B) or by the incubation of the mitochondria (see Fig. 1C and Table II). This argues against a direct exchange of phospholipids between microsomes and inner mitochondrial membranes. Evidence of the ability of inner and outer mitochondrial membranes to exchange phospholipids *in vitro* is presented in the following section.

In vitro exchange of phospholipids inside the mitochondrion

1 h after the injection of [^{32}P]phosphate the specific activity of phosphatidylcholine and phosphatidylethanolamine was 3–5 times as high in the outer membrane as in the inner membrane of rat liver mitochondria (see Table I). If the phospholipids of inner and outer membrane are involved in an intramitochondrial exchange process, one would anticipate that incubation of mitochondria, isolated 1 h after the injection of [^{32}P]phosphate, would result in a decrease of specific activity of outer membrane phospholipids and an increase in the specific activity of inner membrane phospholipids.

Table III gives the specific activities for phosphatidylcholine and phosphatidylethanolamine. These phospholipids were isolated from total mitochondria, inner and outer mitochondrial membranes after incubation of ^{32}P-labelled mitochondria for different periods of time. In total mitochondria, the specific activities did not change during incubation. However, the specific activities of phosphatidylcholine and phosphatidylethanolamine increased in the inner mitochondrial membrane and decreased in the outer membrane. These data provide strong evidence that, inside the mito-

TABLE III

EXCHANGE OF PHOSPHOLIPIDS INSIDE THE MITOCHONDRION BETWEEN OUTER AND INNER MEMBRANE

Mitochondria were obtained from Triton-treated rats injected intraperitoneally with 500 μC [^{32}P]-phosphate 1 h before excision of the livers. See MATERIALS AND METHODS for the isolation of the mitochondria. Mitochondria (120 mg protein) were incubated in a total volume of 2.5 ml for the indicated periods of time. At the end of incubation, mitochondria were sedimented at 15000 × *g* for 5 min, the pellets were resuspended in a small volume of 0.25 M sucrose and outer and inner membrane were isolated according to the slightly modified method of SCHNAITMAN *et al.*[20].

Expt. No.	*Time of incubation (min)*	*Specific activity (counts/min per μg phospholipid-P)*					
		Mitochondria		*Outer membrane*		*Inner membrane*	
		Phosphatidylcholine	*Phosphatidylethanolamine*	*Phosphatidylcholine*	*Phosphatidylethanolamine*	*Phosphatidylcholine*	*Phosphatidylethanolamine*
1	0	221	248	398	518	115	158
	20	223	255	328	418	185	198
	40	212	—	287	391	195	211
	60	214	249	272	388	215	209
2	0	234	535	384	1419	149	356
	15	235	516	307	879	204	443
	30	214	495	273	819	195	454

TABLE IV

EXCHANGE OF PHOSPHOLIPIDS BETWEEN ISOLATED INNER AND OUTER MITOCHONDRIAL MEMBRANE FRACTIONS. EFFECT OF SOLUBLE MITOCHONDRIAL PROTEINS

Proteins of the intermembrane space (Fraction A) and of the matrix space (Fraction B) were isolated from mitochondria according to the method of KADENBACH[3]. These fractions were dialysed against sucrose–EDTA before incubation. ^{32}P-labelled inner mitochondrial membranes and unlabelled outer membranes were isolated as described in MATERIALS AND METHODS. 12 mg inner membrane protein (36 μg phospholipid-*P*) and 4 mg outer membrane protein (64 μg phospholipid-*P*) were incubated for 30 min at 37° in a total volume of 3 ml sucrose–EDTA with the various soluble protein fractions. At the end of incubation, inner and outer membranes were separated at 10000 × *g* for 10 min in the Sorvall centrifuge.

Protein added	*Specific activity (counts/min per μg phospholipid-P)*			
	Inner membrane		*Outer membrane*	
	Phosphatidyl-choline	*Phosphatidyl-ethanolamine*	*Phosphatidyl-choline*	*Phosphatidyl-ethanolamine*
	1391*	2073*		
None	1275	1928	65	163
4 mg Fraction A	1264	1903	75	186
4 mg Fraction B	1266	1922	75	148
4 mg pH 5.1 supernatant	938	1862	173	177

* Specific activities before incubation.

chondrion, phosphatidylcholine and phosphatidylethanolamine exchange readily between inner and outer membrane.

A search for a phospholipid-exchange protein in the mitochondrion comparable to the stimulatory factor in the 105000 × *g* supernatant[16,38] has been unsuccessful. Two fractions of soluble mitochondrial proteins have been isolated according to the method of KADENBACH[3]. At first, the mitochondria were suspended in a hypotonic solution of triethanolamine to release proteins of the intermembrane space (Fraction A). The triethanolamine-treated mitochondria were ultrasonically further disrupted in a phosphate buffer to release the proteins supposedly present in the matrix space of the mitochondria (Fraction B). Experiments were carried out to test the stimulatory activity of these protein fractions on the exchange of phospholipid between ^{32}P-labelled inner mitochondrial membranes and unlabelled outer membranes.

The results of Table IV indicate that whatever exchange occurs between inner and outer membrane phospholipids under those conditions, is not stimulated by mitochondrial protein fractions A or B. On the other hand, the pH 5.1 supernatant fraction shows a distinct effect on the exchange of phospholipids between these membrane fractions.

DISCUSSION

Various investigators[11,12,39] have provided evidence that in the rat liver cell, phospholipids exchange between the endoplasmic reticulum and the mitochondria. We have shown in this article that after injection of [^{32}P]phosphate, the specific activities of the outer membrane phospholipids were intermediate to the specific activities of microsomal and inner membrane phospholipids. This confirms the work of

McMURRAY AND DAWSON[4]. It also supports the view that *in vivo* phospholipids synthesized in the endoplasmic reticulum exchange with phospholipids of the inner mitochondrial membrane *via* the outer membrane.

In line with these *in vivo* results, we have shown that *in vitro* both inner and outer mitochondrial membranes are involved in the exchange of phospholipids with microsomes (Figs. 2 and 3). To what extent both inner and outer membrane phospholipids are replaced by microsomal phospholipids is evaluated as follows: Based on the data of SCHNAITMAN *et al.*[20] and STOFFEL AND SCHIEFER[40], we have calculated that 20 % of the mitochondrial phosphatidylcholine is present in the outer membrane fraction, 50 % in the inner membrane fraction, and 30 % in the fraction composed of inner and outer membrane material. It follows from these percentages and Fig. 2A that of the total [^{32}P]phosphatidylcholine transferred from microsomes to mitochondria after 40 min of incubation, 34 % is present in the outer membrane fraction and 28 % in the inner membrane fraction. The remaining 38 % was apparently present in the composite pellet, which we routinely discarded.

The more pronounced changes in the specific activities of the outer membrane phospholipids as compared to those of the inner membrane, reflect a close proximity of the outer membrane to the microsomal particles. In this respect the results *in vitro* show a close parallel with the observations *in vivo*. Moreover, both *in vivo* and *in vitro* the rate of exchange between mitochondria and microsomes appears to be lower for phosphatidylethanolamine than for phosphatidylcholine, the difference being more pronounced *in vitro* than *in vivo*.

The question remains whether or not *in vitro* certain areas of the inner mitochondrial membrane are in direct contact with the microsomes. If so, ^{32}P-labelled phospholipid could appear in or disappear from the inner membrane by-passing the outer membrane. Specifically, a part of the mitochondrial population as used in our incubations could have had a disrupted outer membrane because of the homogenization, although electron microscopy did not reveal such damage. It did reveal, however, that incubation of the mitochondria in the presence of microsomes and supernatant caused various degrees of swelling in some mitochondria. Nevertheless, more than 90 % of the mitochondria had retained their outer membrane. This supports the results of the adenylate kinase experiments and allows us to conclude that *in vitro* ^{32}P-labelled phospholipids arrive in or leave from the inner membrane primarily *via* the outer membrane.

This conclusion is substantiated by the observation that upon incubation of labelled mitochondria, the initial differences in the specific activities of the phospholipids between outer and inner membrane were partially eliminated (Table III). Phosphatidylcholine as well as phosphatidylethanolamine appeared to exchange rapidly between inner and outer membrane.

We failed to show the presence of a factor in the mitochondria comparable to the stimulatory factor in the 105000 $\times$ *g* supernatant, which could be involved in this intramitochondrial exchange process. At least *in vitro*, soluble protein fractions isolated from the mitochondria did not stimulate the exchange of phospholipids between outer and inner membrane (Table IV). A possible explanation for the exchange of phospholipids inside the mitochondria could be that inner and outer membrane are contiguous structures.

If one compares the exchange of phospholipids between microsomes and outer

mitochondrial membranes and between outer and inner mitochondrial membranes, an important difference becomes apparent. In the transfer of phospholipids from microsomes to outer membrane, phosphatidylethanolamine lags behind phosphatidylcholine *in vivo* as well as *in vitro*. This does not appear to be the case in the transfer of those phospholipids from outer to inner mitochondrial membrane. It is possible that this discrepancy is caused by the presence of the stimulating protein in the former instance and the absence of such a protein in the latter instance.

In general, it can be concluded that outer and inner mitochondrial membrane are in a state of communication by way of phospholipids. Further investigations will be required to ascertain the physiological significance of this phenomenon.

ACKNOWLEDGMENTS

The authors wish to thank Prof. Dr. L. L. M. van Deenen for his interest and advice, Dr. P. F. Elbers of the Biological Ultrastructure Research Unit of this university for placing one of his electron microscopes at our disposal, and Marijke Sanderse for expert technical assistance with the preparations of the samples for electron microscopy.

REFERENCES

1 D. S. BEATTIE, R. E. BASFORD AND S. B. KORITZ, *Biochemistry*, 5 (1966) 926.
2 D. S. BEATTIE, *J. Biol. Chem.*, 243 (1968) 4027.
3 B. KADENBACH, *Biochim. Biophys. Acta*, 134 (1967) 430.
4 W. C. MCMURRAY AND R. M. C. DAWSON, *Biochem. J.*, 112 (1969) 91.
5 F. B. JUNGALWALA AND R. M. C. DAWSON, *European J. Biochem.*, 12 (1970) 399.
6 B. KADENBACH, in L. ERNSTER AND Z. DRAHOTA, *Mitochondria, Structure and Function*, Vol. 17, Academic Press, London, 1969, p. 179.
7 P. G. W. PLAGEMANN, *Arch. Biochem. Biophys.*, 128 (1968) 70.
8 I. KAHANE AND S. RAZIN, *Biochim. Biophys. Acta*, 183 (1969) 79.
9 L. MINDICH, *J. Mol. Biol.*, 49 (1970) 433.
10 D. S. BEATTIE, *J. Membrane Biol.*, 1 (1969) 383.
11 K. W. A. WIRTZ AND D. B. ZILVERSMIT, *Biochim. Biophys. Acta*, 187 (1969) 468.
12 F. B. JUNGALWALA AND R. M. C. DAWSON, *Biochem. J.*, 117 (1970) 481.
13 F. L. BYGRAVE AND T. BÜCHER, *FEBS Letters*, 1 (1968) 42.
14 D. S. BEATTIE, *Biochem. Biophys. Res. Commun.*, 35 (1969) 67.
15 K. W. A. WIRTZ AND D. B. ZILVERSMIT, *J. Biol. Chem.*, 243 (1968) 3596.
16 K. W. A. WIRTZ AND D. B. ZILVERSMIT, *Biochim. Biophys. Acta*, 193 (1969) 105.
17 M. AKIYAMA AND T. SAKAGAMI, *Biochim. Biophys. Acta*, 187 (1969) 105.
18 A. B. ABDELKADER AND P. MAZLIAK, *European J. Biochem.*, 15 (1970) 250.
19 R. WATTIAUX, M. WIBO AND P. BAUDHUIN, in A. V. S. DE REUCK AND M. P. CAMERON, *Ciba Found. Symp. on Lysosomes, London, 1963*, Little and Brown, Boston, 1963, p. 176.
20 C. SCHNAITMAN, V. G. ERWIN AND J. W. GREENAWALT, *J. Cell Biol.*, 32 (1967) 719.
21 D. F. H. WALLACH AND V. B. KAMAT, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 8, Academic Press, New York, 1966, p. 164.
22 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
23 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
24 H. D. TISDALE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 213.
25 H. WEISBACH, T. E. SMITH, J. W. DALY, B. WITKOP AND S. UDENFRIEND, *J. Biol. Chem.*, 235 (1960) 1160.
26 G. L. SOTTOCASA, B. KUYLENSTIERNA, L. ERNSTER AND A. BERGSTRAND, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 448.
27 E. PFAFF AND K. SCHWALBACH, in E. QUAGLIARIELLO, S. PAPA, E. C. SLATER AND J. M. TAGER, *Mitochondrial Structure and Compartmentation*, Adriatica Editrice, Bari, 1967, p. 346.
28 J. FOLCH, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.

29 V. P. SKIPSKI, R. F. PETERSON AND M. BARCLAY, *Biochem. J.*, 90 (1964) 374.
30 A. S. CHEN, T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
31 R. N. AMES AND D. T. DUBIN, *J. Biol. Chem.*, 235 (1960) 769.
32 E. KELLENBERGER, A. RYTER AND J. SÉCHAUD, *J. Biophys. Biochem. Cytol.*, 4 (1958) 671.
33 J. H. VENABLE AND R. COGGESHALL, *J. Cell Biol.*, 25 (1965) 407.
34 T. W. O'BRIEN AND G. F. KALF, *J. Biol. Chem.*, 242 (1967) 2172.
35 M. KLINGENBERG AND E. PFAFF, in J.M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, Vol. 7, Elsevier, Amsterdam, 1966, p. 180.
36 D. BRDICZKA, D. PETTE, G. BRUNNER AND F. MILLER, *European J. Biochem.*, 5 (1968) 294.
37 C. R. HACKENBROCK, *J. Cell Biol.*, 37 (1968) 345.
38 K. W. A. WIRTZ AND D. B. ZILVERSMIT, *FEBS Letters*, 7 (1970) 44.
39 O. STEIN AND Y. STEIN, *J. Cell Biol.*, 40 (1969) 461.
40 W. STOFFEL AND H. G. SCHIEFER, *Hoppe-Seyler's Z. Physiol. Chem.*, 349 (1968) 1017.