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EXCHANGE OF PHOSPHOLIPIDS BETWEEN MICROSOMES AND MITOCHONDRIAL OUTER AND INNER MEMBRANES

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SUMMARY

1. ^{32}P-labelled phospholipids are transferred from rat liver microsomes to rat liver mitochondria when the particles are incubated in the presence of the soluble cytoplasmic fraction. Labelled phospholipids are incorporated mainly into the outer mitochondrial membrane, and only after disruption or detachment of the outer membrane could a substantial uptake of the label by the inner membrane be observed. The exchange of phospholipids between microsomes and the outer mitochondrial membrane was essentially complete within 15–20 min under experimental conditions, whereas the exchange between microsomes and exposed inner mitochondrial membranes proceeded for at least 60 min.

2. Free fatty acids added to the mitochondrial suspension in tracer amounts could easily penetrate across the outer membrane and be bound by the inner membrane.

3. Liver slices incubated in the presence of [^{14}C]choline incorporated the label into both microsomes and mitochondria. A chase applied after 60 min labelling produced a decrease of the specific activity of microsomal lecithin, but the specific activity of mitochondrial lecithin continued to increase. [^{14}C]Choline was mainly incorporated into lecithin of the outer mitochondrial membrane, about as little as 20% being found in the inner membrane.

4. The results are interpreted in the sense that the exchange of phospholipids between the inner and outer membranes of intact mitochondria either does not occur or is qualitatively different and quantitatively much slower than a direct exchange between microsomes and the outer mitochondrial membrane.

INTRODUCTION

Mitochondria are deficient in enzyme systems for a complete synthesis of complex phospholipids[1]. Consequently, the synthetic ability of mitochondria is most likely limited mainly to phosphatidic acid[2–7], the other phospholipids, especially those containing a nitrogen base, being produced in other parts of the cell, mainly

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in the endoplasmic reticulum. An exchange of phospholipids between microsomes and mitochondria was discovered by WIRTZ AND ZILVERSMIT[8] and has been recently studied in several laboratories[5,9–14]. Although this exchange has been demonstrated directly with isolated particles only[5,8,9,11], its occurrence in intact cells is also postulated on the basis of indirect evidence[12,13,15]. This may account for the fact that labelled precursors either injected into experimental animals or added to cell suspensions become rapidly incorporated into mitochondrial phospholipids[5,13,16–19]. However, one may not exclude the possibility that mitochondria present in intact cells, in contrast to isolated mitochondria, do possess an intrinsic enzyme system for the complete synthesis of complex phospholipids. It also remains to be elucidated whether the exchange of phospholipids occurs only between microsomes and the outer mitochondrial membrane, or whether the inner membrane is also involved; in other words whether an intermembrane exchange of phospholipids within the mitochondrion is possible.

The present investigation provides further evidence for the exchange of phospholipids between the endoplasmic reticulum and mitochondria in intact liver cells, but shows that this exchange, both in isolated particles and in tissue slices, mainly concerns the outer mitochondrial membrane.

MATERIAL AND METHODS

Albino rats of the Wistar strain, about 6 months old, of both sexes were used. They had free access to food (standard laboratory diet) until they were killed. For the preparation of labelled sub-cellular particles, rats were injected with $^{32}P_i$, about 1 mC per each animal, 20 h prior to killing. Doubly labelled microsomes were obtained from rats injected first with $^{32}P_i$ and then, 1 h prior to killing, with either [1-^{14}C]-leucine or a mixture of ^{14}C-labelled amino acids, 50 μC per each animal. All labelled precursors were introduced intraperitoneally dissolved in sterile 0.9 % NaCl.

Liver mitochondria and microsomes were isolated by conventional procedures[20]. Outer mitochondrial membranes and mitochondria stripped of the outer membrane (the so-called inner membrane–matrix particles or mitoplasts) were obtained by the procedure of either SCHNAITMAN AND GREENAWALT[21] or SOTTOCASA *et al.*[22] as modified by WOJTCZAK AND SOTTOCASA[23]. In experiments with tissue slices mitochondrial membranes were obtained by subjecting the mitochondria to phosphate swelling according to the method of PARSONS *et al.*[24] followed by contraction with 0.9 M sucrose. Mitoplasts were collected by centrifugation at 13000 $\times$ g_{max} during 10 min and outer membranes by sucrose-gradient centrifugation as described by SOTTOCASA *et al.*[22] in the 3 $\times$ 23 ml swing-out rotor of MSE-65 ultracentrifuge operating at 30000 rev./min (130000 $\times$ g_{max}) during 3 h. Inner membranes were obtained by sonifying the mitoplasts and separating the membranes by centrifugation at 100000 $\times$ g.

Liver slices, about 1 mm thick, were hand-cut with a razor-blade.

Rotenone-insensitive NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were determined as markers for the outer[22] and inner membranes, respectively. The intactness of the outer membrane in whole mitochondria was determined by measuring the rate of oxidation of external reduced cytochrome *c*. As shown elsewhere[23,25] the outer mitochondrial membrane is impermeable to cytochrome *c*, so that the rate of the oxidation of external cytochrome *c* by mitochondria, as

compared to the rate of its oxidation by these particles solubilized with a non-ionic detergent, *e.g.* Lubrol, can indicate the degree of damage of the outer membrane[5,6].

Labelled microsomes were incubated at 20° with unlabelled mitochondria in the medium containing unlabelled post-microsomal supernatant, 250 mM sucrose, 1 mM EDTA and 1 mM Tris–HCl, pH 7.4. Incubation was terminated by a rapid centrifugation of this mixture at 13000 × *g*, and the resulting mitochondrial pellet was washed twice with 250 mM sucrose *plus* 1 mM EDTA to purify it from adhering microsomes.

In experiments with liver slices, 500-mg portions of the slices were incubated in 5-ml samples of the incubation medium at 37° under constant gentle shaking. The medium was Krebs–Ringer solution, phosphate buffered (pH 7.4) with $CaCl_2$ omitted[27], containing, in addition, 5 mM glucose and 0.6 mM [*Me*-^{14}C]choline (1.25 μC/μmole). After incubation, the medium was removed by rapid filtration through a Büchner funnel and the slices were washed 3 times with the ice-cold Krebs–Ringer solution not containing choline. The wet tissue was then homogenized in cold 250 mM sucrose and sub-cellular structures were isolated as described above. In pulse-chase experiments, after the incubation with [^{14}C]choline the slices were rapidly washed with Krebs–Ringer solution and transferred into the medium of identical composition as the original incubation medium but containing unlabelled choline.

Phospholipids were extracted from pellets of mitochondria, mitoplasts and mitochondrial membranes with a mixture of methanol and chloroform (1:2, v/v) according to FOLCH *et al.*[28] or as described previously[29]. Lipids were separated and identified by thin-layer chromatography on Silica gel G (Merck A. G., Darmstadt, Germany). Chromatograms were developed with a mixture of chloroform, methanol and water (60:25:4, v/v/v) according to WAGNER *et al.*[30]. Spots were visualized by iodine vapour, eluted with a mixture of chloroform and methanol (1:1, v/v) and assayed for phospholipid phosphorus[31] and radioactivity.

Radioactivity was measured with a thin-window Geiger counter or, in the case of doubly labelled material, with a scintillation spectrometer (Packard, La Grange, Ill., U.S.A.).

Protein was determined by the biuret method[32].

[^{32}P]Phosphate was obtained from the Institute of Nuclear Research, Świerk, Poland, and [*Me*-^{14}C]choline, [^{14}C]leucine, fatty acids and a mixture of amino acids from either Radiochemical Centre, Amersham, England, the Institute of Nuclear Research, Świerk, Poland, or the Institute for Research, Production and Application of Radioisotopes, Prague, Czechoslovakia.

RESULTS

Exchange of phospholipids in isolated particles

Preliminary results fully confirmed reports of other authors[5,8–14] that phospholipids exchange between microsomes and mitochondria and that this process is enhanced by the post-microsomal supernatant. When doubly labelled microsomes were used (^{32}P in phospholipids and ^{14}C in proteins) the results showed that the uptake of radioactive phospholipids by the mitochondrial fraction was substantially higher than the uptake of labelled proteins, indicating that the former was not due to microsomal contamination only. Contrary to earlier observations of KADENBACH[11],

in our hands the exchange was energy independent and was not inhibited by cyanide and 2,4-dinitrophenol.

The time-course of the exchange was different for whole mitochondria and mitochondria without the outer membrane (the so-called mitoplasts). With whole mitochondria the exchange was fast during the first 15 min, after which time the radioactivity of the mitochondrial phospholipids remained essentially constant or changed only slightly (Fig. 1A). Contrary to this, the exchange of labelled phospholipids between microsomes and mitoplasts proceeded at a high rate usually during at least 60 min and attained a higher level than with whole mitochondria (Fig. 1A).

Fig. 1B shows that the degree of damage of the outer mitochondrial membrane of whole mitochondria, as measured by the rate of oxidation of external ferrocytochrome *c*, amounted to 10% and increased slightly during the incubation. At the

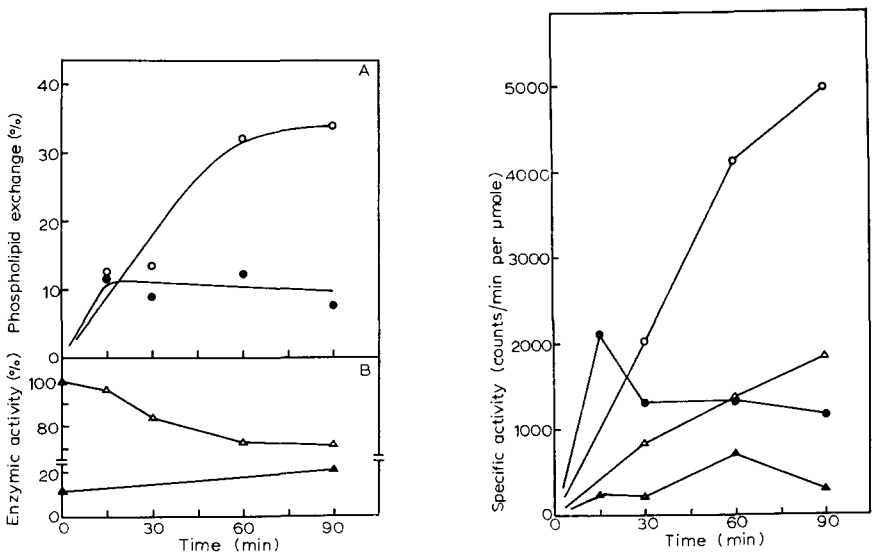

Fig. 1(A). Exchange of phospholipids between microsomes and mitochondria or mitoplasts. The incubation mixture contained microsomes (11.5 mg protein) labelled with ^{32}P in phospholipids, post-microsomal supernatant (40 mg protein) and mitochondria (28 mg protein) or an equivalent amount (16 mg protein) of mitoplasts obtained by the procedure of SOTTOCASA *et al.*[22,23]. Total volume was 8.0 ml, temperature 20°. The uptake of labelled phospholipids by mitochondria (●—●) and mitoplasts (○—○) is expressed as a percentage of total labelled phospholipids initially present in microsomes. (B). Assays for the damage of mitochondria during incubation with microsomes and the subsequent re-isolation. Same experiment as in 1 (A). △—△, activity of rotenone-insensitive NADH–cytochrome *c* reductase in re-isolated mitochondria (assay for the loss of the outer membrane); ▲—▲, accessibility of the inner membrane to external cytochrome *c* (assay for the rupture or detachment of the outer membrane[25,26]). This assay was performed on mitochondrial suspension prior to re-isolation.

Fig. 2. Exchange of lecithin and phosphatidylethanolamine between microsomes and mitochondria or mitoplasts. Conditions: microsomes (5.7 mg protein) containing lecithin of specific activity 14800 counts/min per μmole and phosphatidylethanolamine 11300 counts/min per μmole, post-microsomal supernatant (34 mg protein) and mitochondria (30 mg protein) or mitoplasts (13.5 mg protein). Other conditions as in Fig. 1A, except that the medium also contained serum albumin, 0.6 mg/ml, to protect mitochondria against swelling. The curves represent changes in specific activities of individual phospholipids in whole mitochondria and mitoplasts. ●—●, lecithin in mitochondria; ▲—▲, phosphatidylethanolamine in mitochondria; ○—○, lecithin in mitoplasts; △—△, phosphatidylethanolamine in mitoplasts.

same time there was also a partial loss of the outer membrane, as shown by the loss of NADH–cytochrome *c* reductase of re-isolated mitochondria. Occasionally, the initial degree of damage of the outer membrane was higher or its increase during the incubation was more pronounced. In those cases the incorporation of phospholipids into whole mitochondria increased steadily, similarly, though not as fast as in mitoplasts (not shown).

In Fig. 1A the exchange is expressed per the whole sample of mitochondria and their equivalent of mitoplasts. If calculated per mg protein or as the specific activity of phospholipids, the difference between mitochondria and mitoplasts appears even higher. This is shown in Fig. 2 where, in addition, the exchange of two main mitochondrial phospholipids, lecithin and phosphatidylethanolamine, is followed separately. It is evident that lecithin exchanges faster than phosphatidylethanolamine, but the difference in the rate of incorporation into mitochondria and mitoplasts is observed with both phospholipids.

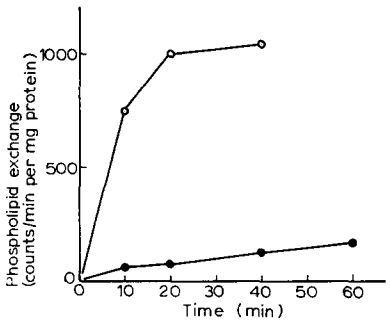

Fig. 3. Exchange of phospholipids between microsomes and inner and outer membranes of 'intact' mitochondria. Mitochondria (58 mg protein) were incubated with the post-microsomal supernatant (85 mg protein) and ^{32}P-labelled microsomes (25 mg protein, 4000 counts/min per mg protein) under conditions similar to those in Fig. 1A. After incubation mitochondria were separated by centrifugation and the membranes were isolated by the procedure of SCHNAITMAN AND GREENAWALT[21] followed by sonication. The curves represent incorporation of ^{32}P-labelled phospholipids into the outer (○—○) and inner (●—●) membranes.

Whole mitochondria incubated with labelled microsomes were subsequently separated into outer and inner membranes. The incorporation of labelled phospholipids into these membranes is shown in Fig. 3. It is evident that the uptake of labelled phospholipids by the outer membrane is rapid and reaches a plateau after 20 min (in other experiments even earlier), whereas the uptake by the inner membrane proceeds slowly during the whole 60 min of incubation. In Fig. 3 the exchange is expressed per mg membrane protein. It is well known that the content of phospholipids in the outer membrane is higher than in the inner one, *e.g.* according to data of LÉVY AND SAUNER[33] for rat liver mitochondria, the phospholipid to protein ratios amount to 0.60 and 0.34 in the outer and inner membranes, respectively. Nevertheless, one can easily conclude from data of Fig. 3 that, even calculated per mg membrane phospholipid instead of mg membrane protein, the exchange is more rapid and higher with the outer membrane than with the inner one.

The results described so far show that, in intact mitochondria, the exchange of phospholipids with microsomes concerns mainly, if not exclusively, the outer membrane. Only when this membrane is ruptured or removed can the inner membrane

participate in this exchange to an appreciable degree. A conclusion can thus be drawn that, in isolated intact mitochondria, the intermembrane exchange of phospholipids is either very slow or non-existent. To determine whether this is due to intrinsic properties of this membrane pair or to a specific situation in the intact mitochondrion the following experiment was performed. Outer mitochondrial membranes obtained from rats injected with $^{32}P_i$ and therefore containing ^{32}P-labelled phospholipids were incubated with unlabelled mitoplasts. After separation of the membranes it was found (Table I) that the exchange proceeded similarly as with microsomes. To ascertain a better comparison, the amount of labelled microsomes used in this experiment was approximately the same as that of labelled outer membranes. These results show that an exchange of phospholipids can in fact occur between the isolated mitochondrial membranes. Therefore, the lack of the intermembrane exchange, or its slow rate,

TABLE I

EXCHANGE OF PHOSPHOLIPIDS BETWEEN ISOLATED MEMBRANES

Mitoplasts (24.4 mg protein) were incubated with post-microsomal supernatant (53 mg protein) and either microsomes (0.93 mg protein) or outer membrane fragments (0.90 mg protein) containing ^{32}P-labelled phospholipids. Incubation conditions as in Fig. 1. The exchange is expressed as percentage of total labelled phospholipids initially present in microsomes or outer membrane fragments.

Incubation time (min)	*Phospholipid exchange*	
	Microsomes–mitoplasts (%)	*Outer membranes–mitoplasts* (%)
30	40	40
60	53	56

TABLE II

BINDING OF OLEATE BY MITOCHONDRIAL MEMBRANES *

Samples A and B: Mitochondria (130 mg protein) were incubated at 0° with 290 nmoles of sodium [1-^{14}C]oleate (2.6 μC) in 20 ml of 250 mM sucrose *plus* 1 mM Tris–HCl (pH 7.4) *plus* 0.1 mM 2,4-dinitrophenol *plus* 1 μM rotenone. After 5 min the mixture was centrifuged at 13000 × *g* for 10 min and mitochondrial pellet thus obtained was treated according to SOTTOCASA *et al.*[22,23] and further on as described in the MATERIALS AND METHODS to separate the membranes. Lipids were extracted therefrom[29] and free fatty acids were separated by extraction with an alkaline water–ethanol mixture[34]. Sample C: Same as Samples A and B except that [1-^{24}C]oleate was added after the sonication step of the membrane separation procedure[22,23].

Sample	*Specific binding (counts/min per mg protein)*		*Specific binding ratio outer membranes/inner membranes*
	Outer membranes	*Inner membranes*	
A	2090	1020	2.1
B	2650	940	2.8
C	3460	2080	1.7

* Experiment carried out in collaboration with Dr. Karel Vizek.

in intact mitochondria is due to a situation in the particles rather than to properties of the membranes themselves.

Transport of free fatty acids between mitochondrial membranes

In view of the suggestion that phospholipids are not easily transferable between the two membranes of intact mitochondria it became interesting to examine the transfer of free fatty acids. Small amounts of ^{14}C-labelled palmitate or oleate were added to suspensions of mitochondria under conditions where both oxidation and esterification were minimized by addition of an uncoupler and rotenone. Under these conditions and within the quantity range used most of the added fatty acid remained unesterified and all of it became bound to mitochondria. It was found (Table II) that the binding to the outer membrane was, per mg protein, somewhat higher than to the inner membrane. However, an essentially similar distribution of the label was observed when the fatty acid was added to already disrupted mitochondria (Table II, Sample C), indicating that the uneven distribution was due to the difference in binding ability between the two membranes towards fatty acids rather than to a permeability barrier. It can thus be concluded that external free fatty acids can easily penetrate the outer mitochondrial membrane and be bound to both the outer and the inner membranes. It should be pointed out here that the amounts of the fatty acid added (*e.g.* 2 nmoles oleate/mg mitochondrial protein) were not enough to produce mitochondrial swelling or any other gross alterations of mitochondrial functions (*cf.* ref. 35). Therefore, binding by the inner membrane must not be due to the disruption of the outer membrane.

Phospholipid synthesis and transport in liver slices

In order to ascertain whether phospholipid exchange, or its absence, observed in isolated intracellular structures also occurs in intact cells, a series of experiments with liver slices was performed. The slices were incubated in a medium containing [*Me*-^{14}C]choline and its incorporation into lecithin was followed. It is now generally agreed that the transfer of phosphocholine moiety from CDP-choline to diglyceride proceeds mainly or exclusively in the endoplasmic reticulum[1-3,36]. Therefore, the presence of labelled lecithin outside this structure in our experiments should suggest a transport of this phospholipid. As shown in Fig. 4, phospholipids of both microsomes and mitochondria were labelled. In most of the experiments, the incorporation of the label into mitochondrial lecithin was preceded by a lag of about 30 min, whereas no such lag was observed with microsomes. The incorporation into both kinds of structures reached a plateau after about 90 min, the specific radioactivity of lecithin in microsomes being then twice as high as that in mitochondria.

When slices pre-incubated for 60 min with [^{14}C]choline were transferred into the medium containing unlabelled choline, the specific activities of lecithin in mitochondria and microsomes underwent opposite changes. In mitochondria, the activity increased almost linearly during the subsequent 120 min, while the activity of the microsomes decreased (Fig. 5A). When a similar chase experiment was performed with slices pre-incubated for 120 min with labelled choline, *i.e.* until the plateau of specific activity was reached, the activity of both fractions decreased, but the decrease was faster in microsomes than in mitochondria (Fig. 5B).

The observation that the specific activity of lecithin in mitochondria never

attained the level obtained with microsomes may suggest that only a part of the mitochondrial lecithin is exchangeable with microsomal lecithin. To investigate this point, mitochondria isolated from slices were separated into outer and inner membranes and the level of [^{14}C]choline incorporated into lecithin of both membranes was measured separately. Due to structural alterations and increased fragility of mitochondria isolated from slices pre-incubated in Krebs–Ringer solution[37] neither the digitonin procedure[21] nor the method of SOTTOCASA *et al.*[22,23] appeared effective in the clean separation of the two membranes. Therefore, the methods of simple swelling in phosphate buffer[24] followed by contraction in hypertonic sucrose[23] were used to detach the outer membrane. In this way a relatively pure outer membrane fraction was obtained. However, the yield was very low and as little as 35–50 % of outer membranes were recovered in the outer membrane fraction, the remaining 50–65 % being still attached to inner membrane–matrix particles.

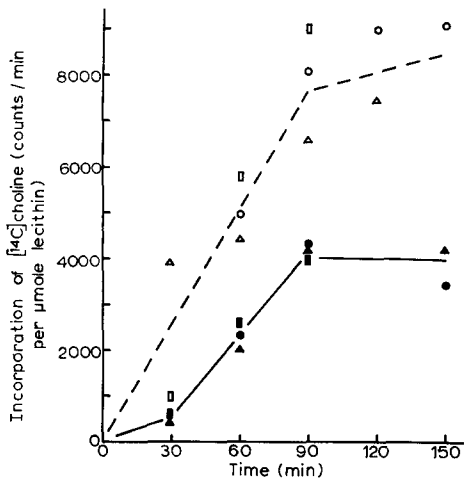

Fig. 4. Incorporation of [*Me*-^{14}C]choline into phospholipids of mitochondria and microsomes in liver slices (three experiments). Full symbols and solid line, mitochondria; open symbols and dashed line, microsomes.

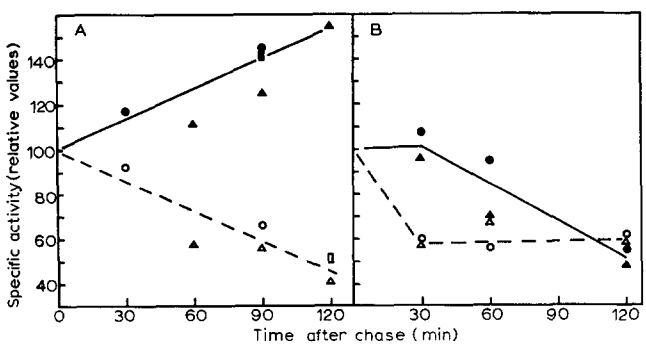

Fig. 5. Incorporation of [*Me*-^{14}C]choline into mitochondria and microsomes of liver slices during pulse-chase labelling. (A) Chase applied after 60 min. (B) Chase after 120 min. For changes in labelling with time, see Fig. 4. Specific radioactivity of each fraction at the onset of chase was taken as 100. Full symbols and solid lines, mitochondria; open symbols and dashed lines, microsomes.

These experiments showed that the specific labelling of lecithin in the outer membrane fraction increased faster than in whole mitochondria and in the inner membrane fraction. Due to the high degree of contamination of the latter fraction by the outer membrane, a direct comparison of the data is misleading. Therefore, the values were recalculated to obtain the true labelling of both membranes as a function of the incubation time. This is shown in Fig. 6 from which it is evident that the labelling of lecithin in the outer mitochondrial membrane increases much faster and attains a much higher level than in the inner membrane.

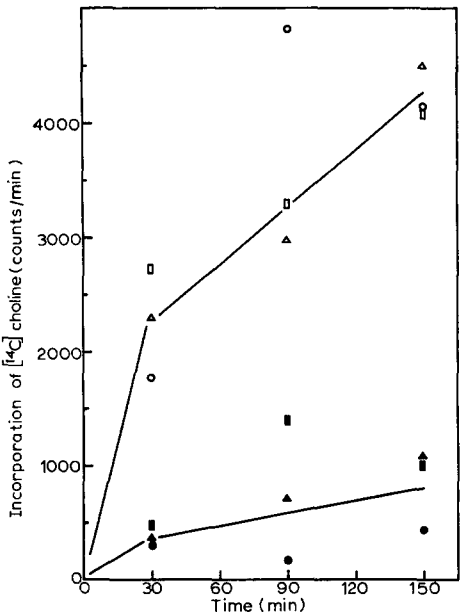

Fig. 6. Net labelling of lecithin in outer and inner mitochondrial membranes in liver slices. The data represent total labelling (per sample) of individual membranes and are calculated from the amount of [*Me*-^{14}C]choline incorporated into each membrane fraction, taking into account the yield of the membranes recovered and the cross contamination. Full symbols, inner membrane; open symbols, outer membrane (three experiments).

DISCUSSION

The present investigation provides a further support to the view that the exchange of phospholipids between sub-cellular structures, observed on isolated particles[5,8,9,11], occurs in intact cells as well[5,12,13,17,19,38]. This is indicated in experiments with tissue slices by the lag in labelling of mitochondrial lecithin upon incubation with [^{14}C]choline, as well as by changes in labelling of mitochondria and microsomes after the chase. The experiments with liver slices also show that the specific radioactivity of mitochondrial phospholipids never attains the level observed in the microsomal fraction. This may be the result of two factors. Firstly, there may occur alterations in the structure or composition of the cells during the incubation of slices, stopping or slowing down a further exchange of phospholipids. Secondly, only a portion of mitochondrial lecithin may be freely exchangable, the other portion being rather inert. The first possibility is supported by our observations[37] that some

soluble cytoplasmic enzymes leak out of the slices and that the ultrastructure of the slices undergoes distinct destructive changes during incubation in Krebs–Ringer solution. This may cause a deficiency of the exchange factor[5,8–10,39] after prolonged incubation. However, the other possibility, *i.e.* that only a part of mitochondrial phospholipids is rapidly exchangable, can not be excluded. In fact, the labelling of the outer mitochondrial membrane increased faster that of the inner membrane. This may point to phospholipids of the outer membrane as being rapidly exchangable and to those of the inner membrane as being relatively inert in this respect. As a matter of fact, it has been found[17] that phospholipids of the outer mitochondrial membrane have a higher turnover than those in the inner membrane.

A more direct support of the view that phospholipids of the outer mitochondrial membrane, but not those of the inner membrane, are readily exchangable is given by experiments on isolated particles. However, it is worthwhile to note that this is true for intact mitochondria only. When the inner mitochondrial membrane is exposed to a direct contact with microsomes by removal of the outer membrane, it can also exchange phospholipids with microsomes. This may lead to controversial interpretation of experimental results. KADENBACH[11] and recently SAUNER AND LÉVY[40] and BLOK *et al.*[41] reported on the incorporation of labelled microsomal phospholipids into both outer and inner mitochondrial membranes. A slow incorporation into the inner membrane of whole mitochondria was also observed in the present investigation. It should be, however, taken into account that isolated liver mitochondria may contain a certain proportion of particles whose outer membrane has been disrupted or even completely detached[26]. This portion was determined as 10 % in the present investigation and a similar figure was obtained by BLOK *et al.*[41] using a different procedure. Unfortunately, no information on the degree of intactness of the outer membrane in their mitochondrial preparations is given by KADENBACH[11] and SAUNER AND LÉVY[40]. It seems therefore likely that the slow incorporation of labelled phospholipids into the inner membrane of whole mitochondria is made possible by a direct access of labelled microsomes and the phospholipid-exchange protein[39] to the inner membrane of damaged particles.

It is difficult to estimate whether this direct exchange accounts for all the incorporation of labelled phospholipids into the inner membrane, or whether there is still room for a true exchange between the outer and the inner membranes of isolated intact mitochondria, as recently postulated by SAUER AND LÉVY[40] and BLOK *et al.*[41]. A similar difficulty appears when the results of experiments with tissue slices are interpreted. Although rupture and detachment of the outer membrane of mitochondria *in situ* are rather unlikely, it seems possible that such changes may occur during isolation of the particles. As will be described elsewhere[37], mitochondria undergo structural alterations during incubation of liver slices and become more fragile during homogenization of the tissue. Therefore, the exposure of the inner membrane to a direct contact with microsomes and outer membrane fragments may take place during homogenization. This could be responsible, at least partly, for the incorporation of labelled phospholipids into the inner membrane. Thus, it can be concluded that in intact mitochondria, both isolated and *in situ*, the outer membrane is mainly, or even exclusively, engaged in the exchange of phospholipids with external membraneous structures endoplasmic reticulum or microsomes. The present investigation does not exclude a direct exchange between the two membranes in the intact mitochondrion,

but makes it rather unlikely. If, however, such exchange does occur, it is much slower, and possibly qualitatively different, than a direct exchange between microsomes and outer or inner mitochondrial membranes.

Two explanations can be proposed for the slowness or a complete lack of the intermembrane phospholipid exchange in mitochondria. Firstly, it is known[5,8–10] that the exchange is mediated by a macromolecular factor, most likely of protein nature[39], present in the cytoplasm. As shown by BLOK *et al.*[41], no such factor can be found in the intermembrane space of liver mitochondria. Secondly, nothing is known about the mobility of phospholipids within mitochondrial membranes. If phospholipids bound to the outer surface of the outer membrane cannot penetrate to the inner surface of this membrane*, they cannot eventually pass to the inner membrane. Thus, the limiting factor for the phospholipid exchange inside the mitochondrion would be represented by the intermembrane space according to the first explanation, and by the outer membrane itself according to the second explanation.

In contrast to phospholipids, free fatty acids were found freely to penetrate through the outer mitochondrial membrane and to be bound by the inner membrane. This was to be expected since externally added fatty acids are known to affect oxidative phosphorylation, electron transport and translocation of adenine nucleotides (*cf.* ref. 35), all processes occurring in the inner membrane.

In view of the present investigation the origin and turnover of phospholipids of the inner mitochondrial membrane remains unclear. It has been shown[17–19] that phospholipid precursors introduced into living animals become incorporated into phospholipids of the inner mitochondrial membrane. This may occur by three mechanisms: (1) phospholipid biosynthesis in the inner membrane or matrix; (2) exchange of phospholipids between the inner membrane and endoplasmic reticulum, *via* the outer membrane; and (3) genesis of new mitochondria. In view of the present knowledge[2–7] the first mechanism is unlikely. On the basis of the present investigation the second mechanism also remains doubtful. Thus, an assumption may be proposed that incorporation of phospholipids into the inner mitochondrial membrane occurs at the time of formation of the mitochondrion, whatever the nature of this process is. This is compatible with earlier studies on half-life time of mitochondrial proteins and phospholipids[42,43] and with more recent investigations[44] on incorporation of protein and phospholipid precursors into mitochondrial membranes.

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