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EXTENSIVE EXCHANGE OF RAT LIVER MICROSOMAL PHOSPHOLIPIDS

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Summary

Liver microsomal fractions were prepared from rats injected with a single dose of choline [^{14}C]methylchloride or with single or multiple doses of $^{32}P_i$. Exchangeability of microsomal phospholipids was determined by incubation with an excess of mitochondria and phospholipid exchange proteins derived from beef heart, beef liver or rat liver. Labeled phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were found to act as a single pool and were 85—95% exchangeable in 1—2 h. High latencies of mannose-6-phosphate phosphohydrolase activities and impermeability of microsomes to EDTA proved that phospholipid exchange proteins did not have access to the intracisternal space. If microsomal membranes are largely composed of phospholipid bilayers, the experiments suggest that one or more of the phospholipid classes in microsomal membranes undergo rapid translocation between the inner and outer portions of the bilayer.

Introduction

During the past several years phospholipid exchange proteins have been isolated from liver and from other tissues. These proteins appear to equilibrate the various phospholipids between organelles [1,2] and may also accelerate net transfer of phospholipids [3]. It is possible that also in vivo one or more of these exchange proteins function to distribute phospholipids within the cell.

It is not known whether the enzymes involved in phospholipid synthesis are localized on both the cisternal and the cytoplasmic sides of the endoplasmic reticulum. If the enzymes are located asymmetrically, some mechanism must exist for translocation of the phospholipid from one side of the bilayer to the other. This type of translocation has been shown to be exceedingly slow in sonicated phosphatidylcholine vesicles [4,5] but is faster in resealed red blood cell ghosts [6]. The present study addresses the question whether or not the

major phospholipids of liver microsomes are subject to transmembrane movement.

Materials and Methods

Chemicals. Choline [^{14}C]methylchloride, $^{32}P_i$ and L-[4,5-$^3H_2$ (N)]leucine were purchased from New England Nuclear (Boston, Mass.). Choline chloride was dissolved in 0.15 M NaCl before injection. [G-^{3}H] Adenosine 5′-monophosphate, diammonium salt was obtained from New England Nuclear. Adenosine 5′-monophosphoric acid, type IV (isolated from yeast) was obtained from Sigma (St. Louis, Mo.). Bovine serum albumin (Fraction V powder, free fatty acid poor) was purchased from Miles Laboratory Inc. (Kankakee, Ill.). Mannose 6-phosphate, prepared according to the method of Slein [7], and glucose 6-phosphate (Sigma, St. Louis, Mo.) were kindly donated by Dr. W.J. Arion, Cornell University.

Isolation procedure of the microsomal fraction. Male Sprague-Dawley albino rats from Blue Spruce Farms (Altamont, N.Y.), weighing 50—80 g, were maintained ad libitum on a Charles River laboratory chow diet (Agway, Inc., Syracuse, N.Y.) until their weights increased to 150—250 g. Microsomal phospholipids were labeled by injecting the rat intraperitoneally with 1—2 μCi choline [^{14}C]methylchloride or 5—10 μCi $^{32}P_i$/g body weight 1 h and 16 h prior to decapitation, respectively. Microsomal proteins were labeled in vivo with 2 μCi L-[4,5-$^3H_2$ (N)]leucine/g body weight injected 1 h before decapitation. Unless specified otherwise, the data in this study have been obtained with liver microsomes prepared according to a modified procedure of Nordlie and Arion [8]. All manipulations were carried out at 4° C. Livers were removed from rats starved for 24 h prior to decapitation. They were homogenized in ice-cold 0.25 M sucrose, 5 mM Tris/acetate, and 0.5 mM EDTA buffer, pH 7.4 (10%, w/v) for 1.5 min with a Potter-Elvehjem homogenizer, operating at an average speed of 1000 rev./min.

After removal of the nuclei (600 × g, 10 min) and mitochondria (twice 15 000 × g, 5 min) the microsomal fraction was pelleted at 105 000 × g_{av} for 60 min. The microsomal fraction was washed twice by redispersion with a Potter-Elvehjem homogenizer for 10—15 s in the same buffer, dilution to 5 ml/g liver, and recentrifugation at 105 000 × g_{av} for 30 min. Most of the diluted homogenized microsomal fractions were kept at —70° C overnight. Freezing was essential to maintain high glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9) activity (Arion, W.J. and Ballas, L.M., unpublished observations). The thawed suspension was centrifuged the next morning at 105 000 × g_{av} for 30 min. The final pellets were homogenized in the same buffer at a concentration of 10—15 mg protein/ml buffer. The microsomal fraction was finally centrifuged for 2 min at 8000 × g to remove aggregated membranes. Rough microsomal vesicles were prepared according to Dallner et al. [9,10].

Liver microsomes were also prepared from a sham-operated rat and a partially hepatectomized rat according to Higgins and Anderson [11]. Both rats received 0.5 mCi of $^{32}P_i$ per 100 g body weight, subcutaneously, immediately after surgery, and 0.25 mCi of $^{32}P_i$ intraperitoneally on each of the

following 2 days. On the third day, 65 h after surgery, each animal received 0.5 mCi of [^{3}H]leucine 1 h before removal of the liver.

Contamination of microsomes with plasma membranes was measured by the presence of 5'-nucleotidase (EC 3.1.3.5) according to Avruch and Hoelzl Wallach [12].

Mitochondria. Beef heart mitochondria were prepared according to the procedure of Green et al. [13]. They were subsequently washed twice by centrifugation in 0.25 M sucrose, 50 mM Tris/HCl, 1 mM EDTA buffer, pH 7.4, and stored at −20° C. Upon thawing, they were heated for 30 min at 60° C and rewashed once in 0.25 M sucrose, 5 mM Tris/acetate, 0.5 mM EDTA buffer, pH 7.4, before use. The heating effectively eliminated the lipase activity without destroying the phospholipid exchangeability [3]. Rat liver mitochondria were prepared as before [14] and heated to destroy lipase activity. The mitochondria were finally washed four times in 0.25 M sucrose, 5 mM Tris/acetate, 0.5 mM EDTA.

Phospholipid exchange protein purification. A protein specifically stimulating the exchange of phosphatidylcholine between membranes was isolated from beef liver according to Kamp et al. [15]. The protein fraction was stored at −20° C in 50% glycerol. A partially purified protein fraction, stimulating the exchange of phosphatidylcholine and phosphatidylinositol between membranes, was isolated from beef heart according to the method of Johnson and Zilversmit [16]. The protein fraction was stored at 4° C in 0.042 M Tris/acetate, 5 mM β-mercaptoethanol, pH 6.0. A partially purified protein fraction stimulating the exchange of all the major phospholipid classes and cholesterol was isolated from rat liver according to the method of Bloj and Zilversmit [17]. The protein fraction was stored at 4° C in 0.042 M Tris/acetate, 5 mM β-mercaptoethanol, pH 7.4.

Before use, all protein fractions were dialyzed overnight against 0.25 M sucrose, 5 mM Tris/acetate, 0.5 mM EDTA, pH 7.4, buffer. Their exchange activities were tested after dialysis, 1 day before the microsomal phospholipid exchange studies. One unit of exchange activity is defined as the transfer of 1 nmol of phosphatidylcholine per min under standard conditions [3].

Extensive microsomal phospholipid exchange. Extensive exchange was determined by measuring the transfer of [^{32}P]phospholipid or phosphatidyl-[^{14}C]choline from microsomes to mitochondria. Incubations were carried out in 20-ml glass counting vials or in conical 1.5-ml polypropylene micro test tubes with fitted caps (Brinkmann No. 2236411-1). In the latter, mixing was achieved by continuous tumbling action. Microsomal proteins were labeled with [^{3}H]leucine. ^{32}P,^{3}H- or ^{14}C,^{3}H-labeled microsomes (0.25—0.3 mg protein or 5—6 μg phospholipid phosphorus) were incubated at 30° C with an excess of preheated mitochondria (6.25 mg protein or 90 μg phospholipid phosphorus) and an excess of phospholipid exchange protein (units specified in each experiment) in 1 ml 0.25 M sucrose, 5 mM Tris/acetate, 0.5 mM EDTA, pH 7.4, containing 0.02% NaN_3 to inhibit microbial growth. After 50—60% of the labeled microsomal phospholipid had been transferred to mitochondria the latter were sedimented at 8000 × *g* for 2 min (Brinkmann centrifuge, model 3200) and replaced with fresh mitochondria to minimize reverse flow of isotopically labeled phospholipids, i.e. from mitochondria to microsomes. The

incubations were then continued for up to 20 h at 30° C. At various time points exchange was stopped by chilling an aliquot of the incubation mixture and removing the mitochondria by sedimentation. [^{3}H]Leucine incorporated in the microsomal proteins served as a non-exchangeable marker and quantitatively measured the microsomal membrane recovery during the incubation period. Microsomal recovery was best (85—90%) when polypropylene micro test tubes were used. The extent of total phospholipid exchange was calculated by expressing the $^{32}P/^3H$ or $^{14}C/^3H$ ratio of the remaining microsomes as a percentage of that of the original microsomes. Extensive exchange of individual phospholipid classes was measured by using singly labeled microsomal phospholipid classes, e.g. phosphatidyl[^{14}C]choline, or extracting aliquots of the remaining microsomal [^{32}P]phospholipids and separating the classes by thin-layer chromatography as described under the section on lipid determinations. The microsomal ^{32}P in each phospholipid class, after varying periods of exchange, was expressed as a percentage of the ^{32}P in the corresponding microsomal phospholipid class at time zero.

Microsomal membrane integrity. Two tests of membrane integrity were performed. Initially we measured the latency of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) with mannose 6-phosphate, based on the observation of Arion et al. [18] that mannose 6-phosphate does not penetrate the membrane and that the hydrolase is located on the cisternal side. In later experiments we measured the fraction of membrane phospholipid or protein present as vesicles impermeable to EDTA.

Glucose-6-phosphatase activity was measured in liver microsomes before and after addition of 0.4% sodium taurocholate according to the method of Arion et al. [18]. Assay mixtures contained 10 mg of bovine plasma albumin, 1 μmol of mannose 6-phosphate and 25—50 μg of microsomal protein per ml of 0.05 M Tris/cacodylate buffer, pH 6.5. The reaction was allowed to proceed for 30 min at 30° C and was terminated by the addition of 0.1 ml 50% trichloroacetic acid. The tubes were centrifuged for 10 min at 3000 rev./min and released P_i measured in the supernatant [19]. Microsomes disrupted with 0.4% sodium taurocholate were kept on ice for at least 30 min before assays were performed. Latency by this test is defined as the P_i released in the absence of taurocholate as a fraction of that released in the presence of taurocholate.

EDTA permeability of microsomal membranes was tested according to a modified procedure of Leskes et al. [20]. Microsomal fractions (0.3 mg protein/ml) were incubated for 60 min at 25° C in 0.175 M sucrose, 0.05 M Tris/cacodylic acid (pH 6.5), containing 1 μmol of glucose 6-phosphate and 2 μmol of $Pb(NO_3)_2$ per ml. Control microsomes were incubated with $Pb(NO_3)_2$ without glucose 6-phosphate, with or without 1 μmol inorganic phosphate. The reaction was stopped by transferring the mixtures to an ice bath. Non-specific binding of Pb^{2+} or $Pb_3(PO_4)_2$ to the outside surface of the membrane was reversed by incubating at 4° C for 1.5 h with 2 mM EDTA. The 1-ml samples were layered over 3.2 ml 2 M sucrose, and overlaid by 0.125 M sucrose to fill the centrifuge tube. The material was centrifuged at 64 000 × g_{av} for 60 min. The contents of each centrifuge tube were collected in three fractions: (1) the fraction containing 0.125 M sucrose, (2) the fraction containing the material at the interface between 0.125 and 2 M sucrose (open vesicles) and (3) the pellet

fraction (closed vesicles). The latter was recovered by sonication in 0.2 ml 5% sodium deoxycholate. The amount of [^{3}H]leucine-labeled microsomal protein and microsomal [^{32}P]phospholipids collected in each fraction was expressed as a percentage of the total radioactivity recovered in the centrifuge tube. The complete recovery of control microsomes in fraction 2 verified that the EDTA sufficed to solubilize accessible lead salts.

Lipid and protein determinations. Microsomal phospholipid extracts in 20 volumes of chloroform/methanol (2 : 1, v/v) were washed by the procedure of Folch et al. [21]. Microsomal phospholipids were separated, after addition of 250 μg of individual carrier phospholipids. Thin-layer chromatography was carried out on 500 nm plates on silica gel H with a solvent system of chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, by vol.) according to the method of Skipski et al. [22]. The combined phosphatidylinositol and phosphatidylserine fraction was subsequently resolved with the same solvent system and silica gel H, containing 0.03 g $(NH_4)_2SO_4$ per g of silica [23]. Thin-layer chromatography scrapings and aqueous samples were counted in the medium of Gordon and Wolfe [24]. Dried lipid extracts, or individual phospholipids, were counted in a toluene-based scintillation medium, containing 0.4% PPO and 0.01% POPOP. Phospholipid phosphorus was determined by the method of Bartlett [25]. Protein was determined according to Lowry et al. [26] or by the biuret reaction [27].

Results

Characterization of microsomal fractions

The microsomal fraction of liver consists largely of closed membrane vesicles originating primarily from the endoplasmic reticulum. A common contaminant in this fraction is derived from plasma membrane, the proportion present depending on the conditions of homogenization [28]. In our preparations about one half of the 5'-nucleotidase activity was associated with the lowest speed pellet (nuclear fraction and cellular debris). One fourth of the enzyme activity appeared in the microsomal fraction. The surface of the endoplasmic reticulum in the hepatocyte is approx. 37.5 times that of the plasma membrane [29]. If we assume that the amount of phospholipid per unit surface of plasma membrane equals that for endoplasmic reticulum, then contamination from the phospholipid of plasma membrane in our microsomal fraction represented only 0.7% of the total microsomal phospholipid.

Microsomal phosphatidyl[^{14}C]choline exchange

When microsomes were incubated with mitochondria in the absence of exchange protein, only 15% of the phosphatidylcholine was transferred in 20 h. Incubation of a small amount of labeled microsomes with an excess of unlabeled mitochondria in the presence of beef heart exchange factor resulted in an 85% loss of the labeled phosphatidylcholine from the microsomal fraction in 4 h (Fig. 1). The integrity of the microsomal vesicles was monitored by the latency of the glucose-6-phosphatase activity with mannose 6-phosphate as a substrate. During the first 3 h of incubation latency was 90% or more. At 6 h, latency of the enzyme still was 81%. It appears, therefore, that microsomal

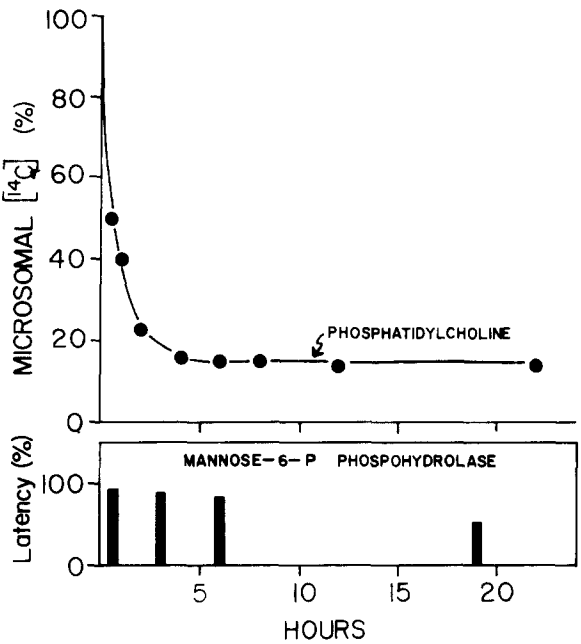

Fig. 1. Extensive exchange of microsomal phosphatidyl[^{14}C]choline with beef heart mitochondria and beef heart exchange protein fraction (5 units). The curve is a composite of three separate experiments. The latency of mannose-6-phosphate phosphohydrolase activity was determined at 0 and 45 min and at 3, 6 and 19 h on aliquots of microsomes subjected to exchange conditions.

phosphatidylcholine is nearly completely exchangeable during a time when membrane integrity is preserved.

In one experiment, in which rat liver microsomes had been labeled with [^{14}C]choline, the microsomal fraction was subfractionated by means of a sucrose gradient containing 15 mM CsCl [9,10]. The rough microsomal fraction was subjected to a 7.5 h exchange experiment in the presence of beef heart exchange protein. The results were essentially the same as those presented in Fig. 1 for the total microsomal fraction: in 90 min 82% of the labeled phosphatidylcholine had been removed by exchange, a fraction that remained constant up to 7.5 h.

In the next experiment [^{14}C]choline-labeled microsomes were exchanged against beef heart mitochondria in the presence of 2.5, 5 or 7.5 units of beef heart exchange protein. The curves in Fig. 2 (bottom) show the results of this experiment. Obviously the rate at which labeled phosphatidylcholine was exchanged from microsomes increased with larger amounts of exchange protein. In the insert in Fig. 2 the early parts of the exchange curves are replotted after subtracting 18% of the label originally present in the microsomal phosphatidylcholine fraction. The curves show a single exponential with slopes proportional to the concentration of exchange protein during the incubations. The pseudo first-order rate constants per unit of exchange protein for the three experiments were 0.11, 0.13 and 0.13 h^{-1}, respectively. These experiments are compatible with the conclusion that 82% of the phosphatidyl[^{14}C]choline behaves

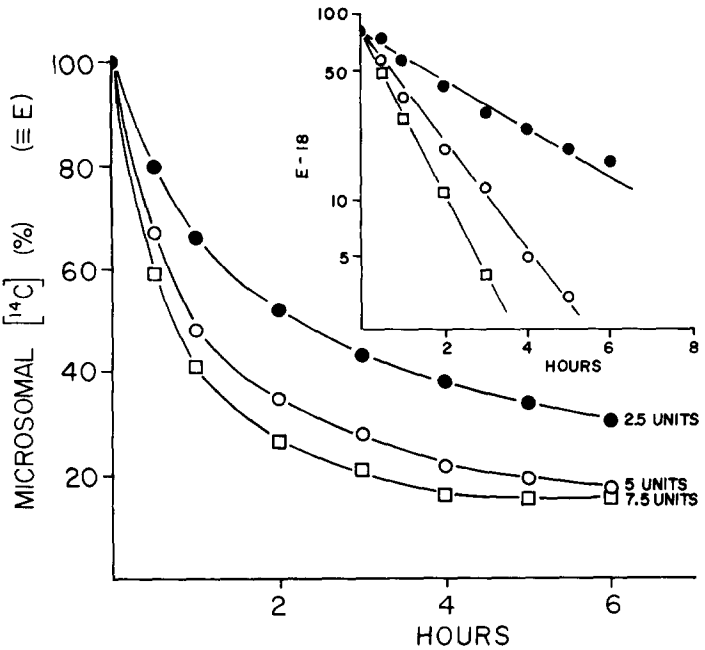

Fig. 2. Exchange kinetics of microsomal phosphatidyl[^{14}C]choline in the presence of increasing concentrations of beef heart exchange protein fraction (lower left). A semi-logarithmic graph of exchangeable phosphatidyl[^{14}C]choline (E-18) during the initial stages of exchange is shown in the upper right insert.

as a single phospholipid pool. The phosphatidylcholine exchange with the highest amount of exchange protein showed a half-time of about 45 min.

Microsomal phospholipid exchange after a single dose of $^{32}P_i$

In the next three experiments, summarized in Fig. 3 (left) exchange of phospholipids in microsomes from rats injected with $^{32}P_i$ was measured. In the presence of beef heart exchange protein 85—90% of the [^{32}P]phosphatidylcholine was exchangeable. Phosphatidylinositol also exchanged rapidly: in 2 h 70% of the [^{32}P]phosphatidylinositol was removed compared to 73—85% of the [^{32}P]phosphatidylcholine. At later times the phosphatidylcholine exchange remained at 85—90%. In the case of phosphatidylinositol, the ^{32}P remaining was quite variable, and fluctuated between 20 and 40%. Neither phosphatidylethanolamine, nor phosphatidylserine or sphingomyelin showed significant exchangeability in the presence of beef heart exchange protein. Integrity of microsomal membranes was measured by their ability to retain trapped $Pb_3(PO_4)_2$ in the presence of EDTA. Nearly 90% of microsomal ^{3}H-labeled protein (Fig. 3) and of microsomal [^{32}P]phospholipid was associated with vesicles impermeable to EDTA.

In most experiments beef heart mitochondria were used as acceptors for the labeled microsomal phospholipids. However, in one of the experiments shown in Fig. 3 (left) rat liver mitochondria were used as acceptor particles. The extent of phosphatidylcholine exchange was the same as when beef heart mitochondria were used.

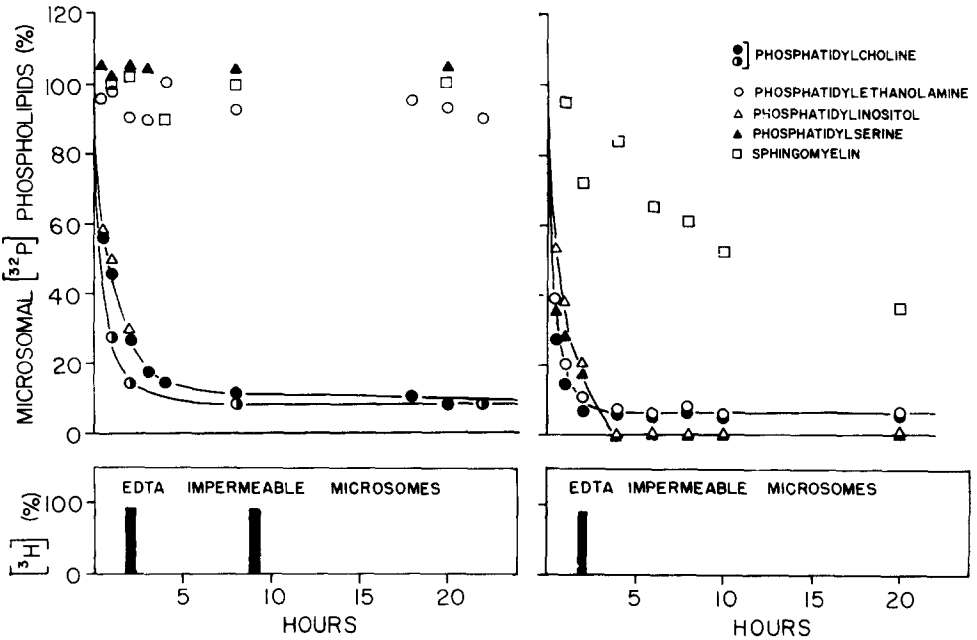

Fig. 3. (Left side) Extensive exchange of microsomal [^{32}P]phospholipids with beef heart mitochondria or rat liver mitochondria in the presence of beef heart exchange protein fraction (5 units). Integrity of the microsomal membranes is based on retention of $Pb_3(PO_4)_2$ in the presence of EDTA. ○, phosphatidylcholine exchange with beef heart mitochondria as acceptor (three experiments); other phospholipids are shown in different symbols. ●, phosphatidylcholine exchange with rat liver mitochondria as acceptor (one experiment). (Right side) Extensive exchange of microsomal [^{32}P]phospholipids with beef heart mitochondria in the presence of rat liver exchange protein fraction (9.5 units). Curves are a composite of two experiments: in one experiment exchange was measured in freshly prepared microsomes, in the other the microsomes were frozen at −70°C overnight. Integrity of microsomal membranes is based on retention of $Pb_3(PO_4)_2$ in the presence of EDTA.

In the next set of experiments either freshly prepared or frozen microsomes were incubated with exchange protein from rat liver. In the presence of this protein all major labeled phospholipids of the microsomes are transferrable to beef heart mitochondria (Fig. 3, right). Within 2 h 90% or more of [^{32}P]phosphatidylcholine and [^{32}P]phosphatidylethanolamine were lost from the microsomal fraction, whereas more than 80% of [^{32}P]phosphatidylinositol and [^{32}P]-phosphatidylserine were transferred. Only the transfer of sphingomyelin was relatively slow with a half-time of about 6 h. Apparently, even in microsomal preparations used within 6 h of killing the rats, the major phospholipids exchange rapidly and extensively. Membrane integrity at 2 h was still good; 90% of the microsomal [^{3}H]leucine was in vesicles impermeable to EDTA. During longer incubations 95—100% of most phospholipids were seen to be exchangeable with only sphingomyelin trailing at 65% exchange. Membrane integrity in the long term incubation experiments was not obtainable by the glucose-6-phosphatase latency or the EDTA permeability tests. Quantitative interpretation of both techniques rely on the activity of the microsomal glucose-6-phosphatase. Prolonged incubations caused a marked decrease in the activity of this enzyme in taurocholate-disrupted vesicles. As was shown earlier

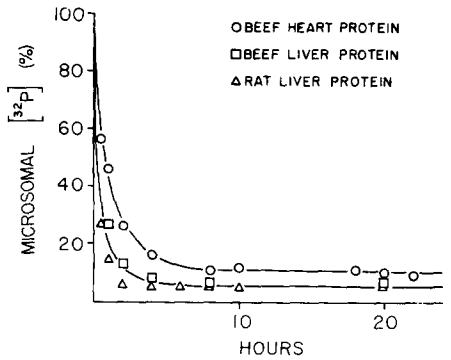

Fig. 4. Extensive exchange of microsomal [^{32}P]phosphatidylcholine with beef heart mitochondria in the presence of various exchange protein fractions. Beef heart and beef liver proteins (5 units), rat liver protein (9.5 units).

for the beef heart exchange protein [4], the rat liver exchange protein remained fully active during a 24 h incubation at 30° C.

It seemed possible that the extensive and rapid exchangeability of the major microsomal phospholipids could be induced by the exchange proteins. We therefore compared, in the same microsomal fraction, the exchange of microsomal phosphatidylcholine in the presence of three different exchange proteins. In 2—10 h 90—95% of the microsomal phosphatidylcholine was exchangeable with all three proteins (Fig. 4).

Microsomal phospholipid exchange after multiple doses of $^{32}P_i$

The following experiment was designed to assure that each microsomal phospholipid class is uniformly labeled throughout the membrane. From one rat 60% of the liver was removed. The animal was then given three doses of $^{32}P_i$ over a 3-day period. During this time the cell mass of the remaining liver approximately doubles. Thus at least 50% of the microsomes isolated from the liver were synthesized during the period of ^{32}P-labeling. A sham-operated rat was injected similarly, except that no liver tissue was removed. Microsomes from these animals, when incubated with mitochondria and rat liver exchange protein, showed phospholipid exchange curves indistinguishable from those in Fig. 3 (right side). In microsomes from the partially hepatectomized rat in 4—10 h about 85% of the [^{32}P]phosphatidylcholine, [^{32}P]phosphatidylethanolamine, [^{32}P]phosphatidylinositol and [^{32}P]phosphatidylserine had exchanged. In the sham-operated animal the corresponding exchangeable phospholipid fractions were 90—95%.

Discussion

From our results it is evident that microsomal phospholipids exchange rapidly and extensively and that more than 85% of each of the major phospholipids exchanges as if they were present in a single pool. According to Nilsson and Dallner [30,31] most of the phosphatidylethanolamine and phosphatidylserine are present on the cytoplasmic side of the microsomal membrane. In contrast, phosphatidylinositol is largely on the intracisternal surface, whereas

phosphatidylcholine appears to be nearly symmetrically distributed over both sides. These authors have also shown that 16 h after $^{32}P_i$ injection the labeled phospholipids on each side of the Golgi vesicle membranes have the same specific radioactivity. Our results with repeated $^{32}P_i$ injections, particularly in the partially hepatectomized rat, assure that phospholipid classes on both sides of the microsomal membranes are equally labeled. In view of these findings, and the extensive exchangeability of microsomal phospholipids, it seems likely that at least some of the phospholipids can cross the membrane much more rapidly than in sonicated phosphatidylcholine vesicles [4,5], viruses [32,33], red blood cell membranes [6,34—36] or inner mitochondrial membranes [37]. Only in spin-labeled membranes from electroplax [38] and *Acholeplasma laidlawii* [39] have very rapid transposition rates been reported.

Although phosphatidylcholine and phosphatidylethanolamine of microsomes are extensively exchangeable, the exchangeability of phosphatidylinositol was variable and appeared to depend somewhat on the exchange protein. Whereas the exchange protein from beef heart exchanged 60—80% of the phosphatidylinositol in different experiments, the rat liver protein exchanged 85—100% of this phospholipid. Phosphatidylserine exchange, which could only be studied with the rat liver protein, was extensive, whereas sphingomyelin exchange was too slow to reach a constant value in 22 h.

In our studies with microsomes the pseudo first-order rate constant for phosphatidylcholine exchange per unit of exchange protein was 0.12 h^{-1}. Thus, in the study with 7.5 units of exchange protein a single exponential decay curve was observed with a half-time of 45 min. It is possible that even shorter half-times might be observed in experiments with larger quantities of exchange protein, so that the upper limit for the half-time of phosphatidylcholine translocation is 45 min or less.

The interpretation that one or more of the microsomal phospholipids undergo rapid translocation is based on the supposition that the microsomal membrane is largely composed of a phospholipid-protein bilayer. In addition, several other conditions have to be met: (1) the microsomal vesicles must be impermeable to the exchange proteins, (2) the exchange proteins must not themselves facilitate the translocation, (3) the translocation must not result from compositional changes during the incubations, (4) the translocation should not be the result of the low ionic strength medium used for the preparation of microsomal fractions and (5) the labeled phospholipids must be present on both sides of the microsomal membrane.

The last condition has been ascertained by the results of Nilsson and Dallner [31] and by our experiments with repeated $^{32}P_i$ injections. Impermeability of microsomal vesicles to exchange proteins was demonstrated by the impermeability of microsomal vesicles to low molecular weight solutes (mannose 6-phosphate and EDTA) during a time when 80—90% of the phospholipids had exchanged. It seems unlikely that exchange proteins facilitated the translocation of phospholipids for the following reasons: (a) the proteins do not facilitate flip-flop of phospholipids in sonicated phosphatidylcholine vesicles [4,5] nor in viral membranes [32,33] and (b) when three different exchange proteins of widely different properties were used, the results for phosphatidylcholine exchange were the same.

Compositional changes of microsomes during incubations could not be determined directly, because of the large excess of mitochondria present in the incubation media. However, in at least some of the experiments such changes seem very unlikely. Beef liver exchange protein transfers only phosphatidylcholine and would therefore cause no significant changes in microsomal composition. Yet in experiments in which less specific exchange proteins were employed (Fig. 4) the extent of phosphatidylcholine exchange was identical. Furthermore, in one experiment with the beef heart exchange fraction the phospholipids of rat liver microsomes were exchanged with rat liver mitochondria. Presumably, these organelles were in equilibrium with one another before the exchange reaction, so that no driving force for net phospholipid transfer was present. Finally, we ascertained that no lysophosphatidylcholine was generated during the exchange incubations.

Microsomes are normally prepared in a medium containing sucrose with a low ionic strength buffer. It is possible that this medium might promote the extensive exchangeability of microsomal phospholipids. However, in one experiment we employed microsomes prepared in 0.15 M KCl, 0.05 M potassium phosphate buffer, pH 7.5, according to the procedure of Tangen et al. [40]. These microsomes showed the same rapid and extensive exchange of phosphatidylcholine as was observed in all other experiments with the beef heart exchange factor.

Although translocation of phospholipids in bilayered structures has usually been discussed as a flip-flop mechanism [41] in which the polar headgroups of the phospholipids have to pass through the hydrophobic core of the bilayer, the possibility of other mechanisms should not be overlooked. For example, a phospholipid molecule could pass from one side of the membrane to the other by facilitated transport [42] or by lateral diffusion through a membrane pore lined with a hydrophobic portion of a protein molecule. Lateral diffusion is very rapid [43] and could easily account for the translocation of labeled phospholipids such as that observed in our experiments. One might ask how membrane lipid asymmetry [42] can be maintained if lipid translocation is rapid. It seems possible that such asymmetry is dictated by the affinity of asymmetrically inserted proteins to the polar headgroups of specific phospholipid classes.

Acknowledgements

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