Protein-Mediated Phospholipid Translocation in the Endoplasmic Reticulum with a Low Lipid Specificity†

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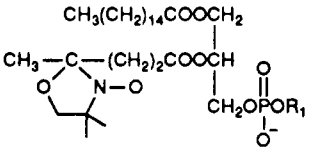
ABSTRACT: The outside–inside translocation rate of various amphiphilic spin-labeled phospholipids has been measured in rat liver endoplasmic reticulum vesicles. The eight spin-labels tested experienced a fast flip-flop rate with the same half-time of approximately 20 min at 37 °C. The stationary distribution of these phospholipid analogues was ca. 45% on the inner vesicular leaflet and 55% on the external one, showing that there is no net enrichment of some lipid in one layer under the experimental conditions used. The initial rate of translocation was reduced 4-fold if membranes were preincubated with *N*-ethylmaleimide (2 mM) and was about an order of magnitude lower in liposomes made from the extracted lipids. An apparent saturability of the transbilayer diffusion can be deduced from the variation of the initial velocity of the relocation kinetics vs the amount of analogue incorporated in the membrane. Moreover, translocation rates of two different spin-labeled phospholipids introduced simultaneously in the membrane were almost equally reduced by the presence of the other lipid. On the other hand, no competition between the water-soluble dibutyroylphosphatidylcholine and the amphiphilic spin-labeled phospholipids could be detected. Overall, these results suggest that phospholipid translocation in the endoplasmic reticulum is a protein-mediated process with a low specificity, which tends, in the absence of any other metabolic event, to equilibrate the phospholipid composition of the two membrane halves.

In the endoplasmic reticulum (ER), phospholipid biosynthesis is an asymmetric process since the active sites of the enzymes are located on the cytoplasmic face of the membrane (Coleman & Bell, 1978; Hutson & Higgins, 1982; Bell et al., 1981). Yet, the newly synthesized lipids are rapidly transferred across the membrane to the cisternal leaflet (Hutson & Higgins, 1982, 1985). Measurements of phospholipid transbilayer movement in ER vesicles clearly indicate that the lipid diffusion in this system is a fast process, with a characteristic time of a few minutes for phosphatidylethanolamine and its methylated derivatives (Hutson & Higgins, 1982, 1985); similar values were obtained for phosphatidylcholine (Bishop & Bell, 1985; Van den Besselaar et al., 1978; Van Duijn et al., 1986), lysophosphatidylcholine, and glycerophosphocholine (Kawashima & Bell, 1987). Using exchange proteins, Zilversmit and Hughes reported half-times below 45 min for phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, but sphingomyelin did not reach equilibrium in 22 h (Zilversmit & Hughes, 1977). This rapid transbilayer movement of lipids does not take place in multilamellar liposomes made from ER lipids (Van den Besselaar et al., 1978).

In 1985, Bishop and Bell used a water-soluble phosphatidylcholine (dibutyroyl-PC) and showed that the rapid passage through the ER membrane is sensitive to protein modifications either by proteases or by chemical reagents. This led them to postulate the existence of a phosphatidylcholine transporter in the endoplasmic reticulum. Their hypothesis was sustained by a later report of Backer and Dawidowicz (1987), who succeeded in reconstituting this "flippase" activity into liposomes, from solubilized ER membranes. However, apart from a comparison between phosphatidylcholine and the lyso derivative (Kawashima & Bell, 1987), no data are available concerning the phospholipid specificity of this transporter from ER. By contrast, the *aminophospholipid translocase* from red cells has a high specificity for the aminophospholipids (PS and PE) and does not efficiently transport the lyso derivatives (Morrot et al., 1989). Another difference is the requirement for cytosolic ATP in the case of the aminophospholipid translocase, while the activity of the microsomal transporter does not seem to require an energy source.

In the present work, we have measured the transmembrane diffusion of several spin-labeled phospholipids in rat liver ER using the spin-label assay previously designed for the determination at 37 °C of the translocation rates of phospholipids in human erythrocytes (Calvez et al., 1988; Morrot et al., 1989). The technique takes advantage of the partial water solubility of spin-labeled phospholipids having one relatively short chain. This solubility has several advantages: first, it facilitates membrane labeling; second, it allows one to monitor lipid translocation by bovine serum albumin extraction of the spin-labeled lipids remaining in the outer monolayer. Three classes of spin-labeled phospholipids were utilized. The first class of spin-labels possessed a short β-chain bearing a nitroxide:



Four different head groups were used: choline (PC*),[1] serine

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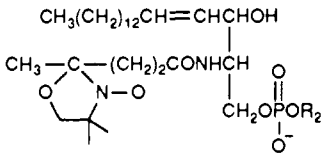
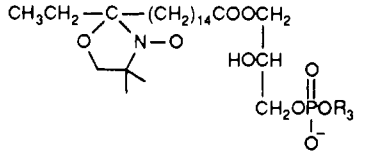
(PS*), ethanolamine (PE*), and monomethylated ethanolamine (PME*). The second class of spin-labels had a ceramide backbone:



Two different head groups were used for this class of lipids: choline (SpC*) and serine (SpS*). The third class of spin-labels had a nitroxide on the α-chain:



The head groups used were again choline (LPC*) and serine (LPS*).

All these molecules were transported at approximately the same rate to an equilibrium distribution where they were equally distributed between the two layers. The velocity of the diffusion was reduced by the action of *N*-ethylmaleimide upon the microsomal proteins. This protein-mediated (facilitated) diffusion was saturable with respect to the substrate available and exhibited a low (phospholipid) substrate specificity as inferred from competition experiments.

Materials and Methods

Microsome Preparation. Endoplasmic reticulum membranes were isolated from rat liver by differential centrifugations after homogenization, according to Coleman and Bell (1978). After isolation, membranes were kept at −20 °C in aliquots and thawed immediately before use. After thawing, membranes were diluted in 1 mM $MgSO_4$ and 50 mM Tris-HCl buffer, pH 7.4 (buffer A), to a final phospholipid concentration of 4 mM. They were then allowed to stand 30 min at 37 °C and subjected to an extra 5-min incubation in the presence of 5 mM (final concentration) diisopropyl fluorophosphate, in order to minimize any phospholipase A_2 activity (Bishop & Bell, 1985). At that stage, in some instances, membranes were treated by *N*-ethylmaleimide (2 mM final concentration) for 15 min.

Multilamellar Vesicle Preparation. Microsomal lipids were solubilized from membranes in chloroform/methanol according to Folch et al. (1957). To form the multilamellar vesicles, lipids were dried from the organic solution and then dispersed ($\geq$10 μmol·mL^{-1}) in buffer A, supplemented with 10% (w/v) Dextran T70 (Pharmacia), by heating at 60 °C for 5 min and vortexing. The vesicle suspension was then adjusted to a phospholipid concentration of 4 mM in buffer A. The presence of Dextran inside the vesicles allowed them to pellet by centrifugation.

Spin-Labeling. The synthesis of the various spin-labeled phospholipids has been described in Morrot et al. (1989). 16-Doxylstearic acid was synthesized according to Hubbell and McConnell (1971). An aliquot of the desired spin-label in chloroform was deposited in a glass tube and dried under nitrogen. The dried film was resuspended in buffer A by vigourous vortexing and heated at 37 °C. Phospholipid translocation assay was initiated by addition of 1 volume of spin-label suspension to 1 volume of microsome suspension. The final concentration of spin-labels was calculated to correspond to $\sim$1% of the membrane phospholipids. In competition experiments, PC* and LPS* were introduced simultaneously in the incubation. ESR spectroscopy showed that total incorporation of any probe in the membranes was achieved in less than 90 s as the spectra were characteristic of diluted spin-labeled lipids embedded in a bilayer and not of probes tumbling rapidly in water or forming micelles with strong spin–spin interactions (Seigneuret et al., 1984; Morrot et al., 1989). All the incubations were carried out at 37 °C.

ESR Spectroscopy and Kinetics Assay. ESR spectra were recorded with a Varian E109 spectrometer equipped with a temperature-control device and connected to a Tektronix 4051 computer. Spin-label translocation kinetics were determined by the intensity of the signal obtained by extraction of the probes from the outer monolayer by bovine serum albumin (BSA) (Morrot et al., 1989). For this purpose, at various times, 80-μL aliquots were taken from the incubation medium and mixed with 80 μL of a 2% fatty acid free bovine serum albumin (Sigma) solution (w/v). After 1 min on ice, the mixture was centrifuged for 2.5 min at 14800*g* in an Eppendorf tube and the supernatant kept. Comparison of the double integral of the ESR spectra coming from the supernatant and the pellet at time 0 showed that albumin extracts more than 95% of the labels incorporated in the outer layer as it does in erythrocytes (Calvez et al., 1988; Morrot et al., 1989). The difference between the amount of extractable probe at time *t*, evaluated by the ESR spectrum intensity, and that at time 0 gave the amount of probe located on the cisternal leaflet of the microsomes or on one of the internal leaflets of the liposomes at time *t*. No spin reduction took place at 37 °C during the 3-h incubation, and no hydrolysis of the β-chain could be detected when the membranes had been incubated with DFP. In a few experiments, two different spin-labeled lipids were added simultaneously and each BSA aliquot contained the signal of the two probes. Since the probes at the 4th position of the β-chain give a different line shape than the probes at the 16th position of the α-chain, when bound to BSA, a spectral deconvolution was possible. It required the storage of the line shapes obtained with each spin-label separately. Spectral subtraction was carried out from the composite spectra to obtain the percentage of each probe in the mixture.

Miscellaneous. Dibutyroylphosphatidylcholine was synthesized from glycerophosphocholine cadmium salt and butyric acid, following Samuel's procedure (Samuel et al., 1985).

Results

Panels A–D of Figure 1 show the outside–inside transmembrane diffusion of the four major phospholipid analogues of the microsomes. The kinetics obtained with PC*, PS*, PE*, and SpC* are very similar and establish a transmembrane equilibrium where $\simeq$45% of the probe is resistant to BSA extraction; the half-time of the diffusion process is approximately 20 min. The same half-time and initial rate were obtained with the lyso derivatives (LPC* and LPS*) and with monomethylphosphatidylethanolamine (PME*). Finally, SpS*, which corresponds to a sphingomyelin where the choline

[1] Abbreviations: PC*, 1-palmitoyl-2-[4-(2,4,4-trimethyl-3-oxy-2-oxazolidinyl)propanoyl]phosphatidylcholine; PE*, 1-palmitoyl-2-[4-(2,4,4-trimethyl-3-oxy-2-oxazolidinyl)propanoyl]phosphatidylethanolamine; PME*, 1-palmitoyl-2-[4-(2,4,4-trimethyl-3-oxy-2-oxazolidinyl)-propanoyl]phosphatidyl-*N*-monomethylethanolamine; PS*, 1-palmitoyl-2-[4-(2,4,4-trimethyl-3-oxy-2-oxazolidinyl)propanoyl]phosphatidylserine; SpC*, [*N*-[4-(2,4,4-trimethyl-3-oxy-2-oxazolidinyl)propanoyl]-*trans*-4-sphingenyl]-1-phosphocholine; SpS*, [*N*-[4-(2,4,4-trimethyl-3-oxy-2-oxazolidinyl)propanoyl]-*trans*-4-sphingenyl]-1-phosphoserine; LPC*, 1-[15-(2-ethyl-4,4-dimethyl-3-oxy-2-oxazolidinyl)pentanoyl]lysophosphatidylcholine; LPS*, 1-[15-(2-ethyl-4,4-dimethyl-3-oxy-2-oxazolidinyl)pentanoyl]lysophosphatidylserine; DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; ESR, electron spin resonance.

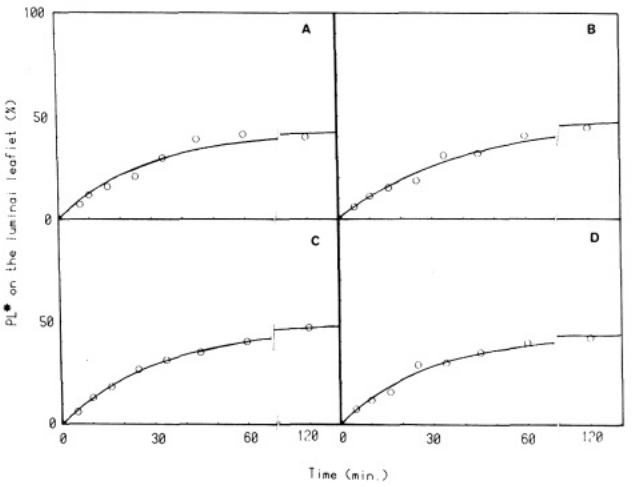


FIGURE 1: Kinetics of reorientation of PC* (A), SpC* (B), PS* (C), and PE* (D) in rat liver microsomes at 37 °C. Each phospholipid analogue was introduced as 1% of the quantity of endogenous lipids. Experimental values were obtained by the technique of "back-exchange" with BSA, as described under Materials and Methods. Curves are fitted according to the equation of an equilibrated diffusion: $X(t) = X_{eq}[1 - \exp(-kt)]$, where $X(t)$ is the amount of nonextractable label at time t and X_{eq} is the amount of nonextractable label at equilibrium.

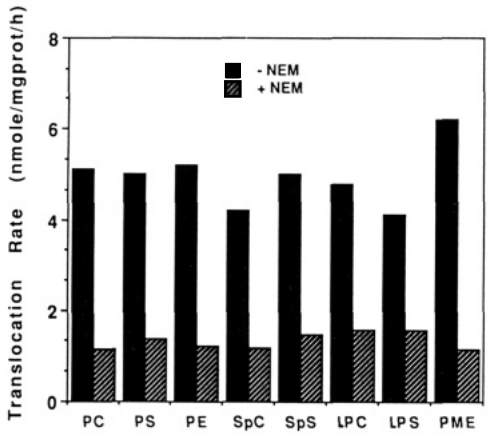


FIGURE 2: Initial diffusion rates of the various analogues in intact microsomes (solid columns) or in microsomes treated with NEM (hatched columns; see Materials and Methods). The analogues were introduced on the microsomal outer leaflet as 1% of the endogenous lipids and their reorientation kinetics followed as in Figure 1. The initial rate corresponds to kX_{eq}.

head group is replaced with a serine, gives similar kinetics. Figure 2 shows a comparison of the initial rates of translocation obtained with eight spin-labeled phospholipids. Similar experiments were attempted with a spin-labeled fatty acid (16-doxylstearic acid). With such a spin-label, the intensity of the signal after BSA extraction did not change during incubation (data not shown). Presumably, this reflects a very fast flip-flop (half-time of equilibration $\ll$ 1 min).

Consequences of Protein Modification. When the microsomes were incubated with the SH group reagent *N*-ethylmaleimide (2 mM), the translocation of the phospholipid analogues was noticeably reduced (Figure 2), confirming the involvement of a protein in the phenomenon. No striking differences could be noticed between the various phospholipids, which moved from the outer to the inner layer at an approximately 4-fold lower rate than in intact membranes.

Saturability and Specificity. We have followed the reorientation kinetics of PC* according to its membrane concentration. It can be seen (Figure 3) that the diffusion rate increased with increasing amounts of PC* (from 0.9 to 4.6%

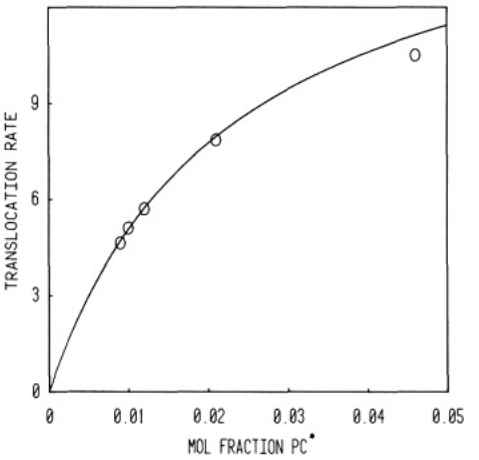


FIGURE 3: Variation of the initial translocation rate [in nmol/(h·mg of protein)] as a function of the mole fraction of PC* incorporated in the microsomal membrane. The analogue was introduced at time 0 in the microsomal outer leaflet, and the diffusion kinetics were followed as in Figure 1.

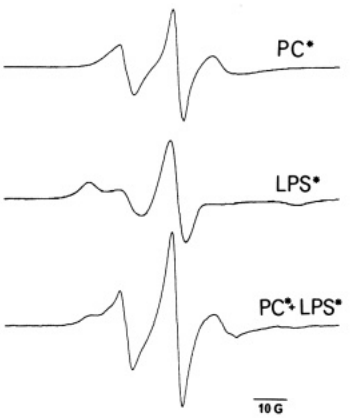


FIGURE 4: ESR spectra obtained with spin-labeled phospholipids adsorbed on BSA: PC* (top trace), LPS* (middle trace), and an equimolar mixture of the two previous molecules (bottom trace). Spectra were recorded on a Varian E-9 spectrometer operating at 9.15 GHz, field scan 100 G, modulation amplitude 2 G. The bar represents 10 G. By computer subtraction, it is possible to quantify the contribution of each spin-label in a composite spectrum.

of the membrane lipids), but not proportionally to the amount of label present, arguing for a saturable phenomenon. The experimental maximal rate could not be obtained as it would require such a high amount of amphiphilic spin-labeled probe that membrane damages would occur. However, data linearization led to a maximal rate of approximately 15 nmol/(h·mg of microsomal protein) with an apparent K_m of ca. 0.02 (in mole fraction). The same was true for LPS* (not shown). These observations allowed us to study the transporter specificity by introducing simultaneously in the microsomal membrane equal amounts of PC* and LPS*, and by following their individual reorientation. This was rendered possible by the different position of the paramagnetic doxyl group along the acyl chain in each molecule. On PC*, the doxyl residue is located at the top of the *sn*-2 chain, and when adsorbed on BSA, it generates an ESR spectrum whose line widths are intermediate (Figure 4). As to LPS*, the presence of the nitroxide at the bottom of the *sn*-1 chain gives rise to a broadline spectrum. It is thus possible, by computer subtraction, to quantify the contribution of each component in a composite spectrum. Using this technique, it can be seen that the presence of the two probes in the membrane reduced by the same factor the individual diffusion rates of each analogue (Figure 5).

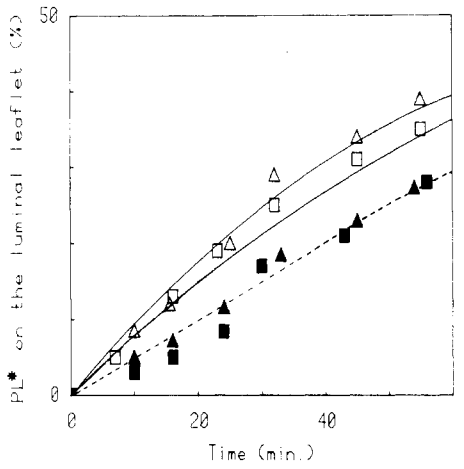


FIGURE 5: Transmembrane reorientation kinetics of PC* (△, ▲) and LPS* (□, ■) introduced separately (open symbols) or simultaneously (closed symbols), each at 1% of the endogenous lipids, in the microsomal membrane. In the competition experiment, the kinetics of each analogue were inferred by spectral subtraction (see Figure 4 legend).

By contrast, the transmembrane diffusion of PC* (40 μM) was not influenced by the presence of up to 20 mM unlabeled dibutyroyl-PC (not shown). Thus, water-soluble molecules do not compete efficiently with amphiphilic lipids.

Diffusion in Liposomes. Multilamellar liposomes were prepared from a microsomal lipid extract. The presence of multiple bilayers limits the study to the rate of transmembrane diffusion of the probes and does not allow one to determine the stationary distribution of a lipid between the two halves of a bilayer. In these membranes, the three glycerophospholipids PC*, PE*, and PS* diffused at an equivalent rate. The initial rate of incorporation is approximately one-third the rate detected in NEM-treated microsomes (data not shown). As to SpC*, it diffused at an even slower rate than the three other probes, a point that was already described for liposomes made from human erythrocyte lipids (Zachowski et al., 1985).

DISCUSSION

In the erythrocyte membrane, the phospholipid analogues used throughout this study proved to be very accurate reporters of the behavior of physiological phospholipids, i.e., molecules with two long natural fatty acids esterified on the glycerol moiety: the same type of experiment as the one reported here led to an equilibrium distribution of the probes (Calvez et al., 1988; Morrot et al., 1989) identical with that of endogenous phospholipids (Verkleij et al., 1973), and the kinetics of transmembrane redistribution of the spin-labeled analogues were superimposable on those obtained with long-chain, radioactive phospholipids (Tilley et al., 1986). It is thus most probable that the results obtained with the spin-labels in the microsomes could be extended to endogenous phospholipids under the same conditions, i.e., in the absence of lipid metabolism.

The diffusion of phospholipid analogues between the two halves of the microsomal membrane is rather independent of the nature of the lipid. Both the diffusion constant and the equilibrium plateau experienced by the phospholipids studied here were quite similar regardless of the polar head group (choline, serine, or ethanolamine or its methylated derivative) or the backbone (glyceride or ceramide) or the presence of absence of a fatty acid chain. All the phospholipids tested in the present work have a relatively short β-chain; therefore, we cannot exclude a small influence of long acyl chains. Nevertheless, our data with spin-labels appear to be rather consistent with previous work on lipid transverse diffusion in microsomes. Zilversmit and Hughes, in particular, found essentially the same fast diffusion and the same absence of specificity, although in their work, based on the use of exchange proteins, sphingomyelin appeared to diffuse more slowly than the spin-labeled sphingomyelin (Zilversmit & Hughes, 1977). A diffusion rate with a half-time of 20 min is not a general feature of all spin-labeled lipids, as indicated by the results obtained with the 16-doxylstearic acid. The constant intensity of the ESR signal originating from fatty acids extracted with BSA signifies that during the 1-min incubation all free fatty acids exchange between the inner and outer monolayers. Alternatively, one might interprete the latter experiment as being indicative of a very slow diffusion. However, it was already reported that transmembrane diffusion of a fluorescent fatty acid occurs with characteristic time well below 1 s (Doody et al., 1980).

The plateaus of stationary transmembrane distribution of the spin-labeled phospholipids, as deduced from Figure 1, were comprised in the range 40–45% of the analogue located on the inner vesicular leaflet. This can be considered as an equilibration between the two membrane halves, assuming that microsomal vesicles of 200-nm average diameter (Dallner & Nilsson, 1966; Stier et al., 1978) and 4-nm bilayer thickness exhibit an inner leaflet area of ca. 92% that of the outer leaflet. This raises the question of asymmetric distribution of endogenous phospholipids between the two membrane halves. Results drawn from attack of the microsomal membrane by phospholipases (A_2 or C) often led to contradictory results: for instance, PE was found equally distributed (Sundler et al., 1977) or preferentially outside (Nilsson & Dallner, 1977) or inside (Higgins & Dawson, 1977). Since it is known that studies with phospholipases can be misleading when fast transbilayer movements of lipids are possible, as in the case of microsomes, it is reasonable to be confident with the results obtained with the spin-labels and to conclude that there is no marked asymmetry in these membranes.

If there is no accumulation of a phospholipid on either membrane layer in the absence of de novo synthesis, the transmembrane movement of these molecules is a diffusion. When the membranes were treated with sulfhydryl reagents, the diffusive process was slowed (4-fold), and the rate of diffusion was the same whatever the phospholipid tested. This effect of NEM on lipid transmembrane diffusion is in accordance with the results of Bishop and Bell (1985) on the diffusion of dibutyroyl-PC in microsomal vesicles. However, this slower diffusion did not prevent the phospholipids from equilibrating in the membrane, which explains why no effect of sulfhydryl reagents could be detected in long-lasting incubations (Backer & Dawidowicz, 1987). Note that, even when the facilitated diffusion is inhibited, the transbilayer motion of the probes is still 3 times faster than in pure lipidic vesicles. This could be due to unspecific enhancement of lipid flip-flop caused by the presence of intrinsic proteins in a lipid bilayer (De Kruijff et al., 1978; Zachowski et al., 1985).

The involvement of a limited number of specific microsomal proteins is sustained by the saturability of the diffusion which is revealed by increasing the amount of phospholipid analogue in the membrane. Even though all the spin-labeled phospholipids used in this study exhibited similar kinetics parameters, one could wonder whether they are all transported by a unique protein or each possesses its own carrier. Competition experiments between a diacyl-PC molecule and a monoacyl-lyso-PS suggested that at least these two lipids, and probably

the other tested, utilized the same carrier to be translocated between the two membrane halves. The absence of competition with dibutyrol-PC may be taken solely as reflecting the low membrane partitioning of the latter lipid. In fact, dibutyroyl-PC and long-chain PC transports could be controlled by two different aspects of the same system, as the maximal rates of uptake of these molecules differed by 1 order of magnitude, the highest being the one of the short-chain lipid (Bishop & Bell, 1985). This would mean that the limiting step of phospholipid diffusion is the transbilayer reorientation of the hydrophobic fatty acid chains. In conclusion, at this stage, we have not proven the involvement of a unique protein in microsomal phospholipid translocation; however, the wide variety of phospholipid translocated (including nonphysiological lipids) and the occurrence of competition between some of the lipids tested suggest the existence of a single phospholipid flippase in microsomes.

The high rate of lipid movement has been correlated with the presence in microsomal membranes of nonbilayer structures (Van Duijn et al., 1986) as detected by the presence of an isotropic component in ^{31}P nuclear magnetic resonance spectra. These structures would be responsible for a fast exchange of molecules between the two layers. The same phenomenon does not exist in liposomes made with lipids extracted from microsomes. Moreover, after addition of *N*-ethylmaleimide to microsomes, the fast transverse diffusion of lipids is slowed down, which is also indicative of a protein involvement. Surprisingly, the fast translocation was also obtained with a water-soluble phospholipid, dibutyroyl-PC (Bishop & Bell, 1985). The nonbilayer structures may, in this case, play the role of an aqueous pore. One may imagine that these bilayer defects occur at the periphery of a well-defined protein where a polar cleft accommodates the polar head group of a phospholipid or the hydrosoluble lipid.

It is interesting to know if the translocation rates determined here may have some physiological relevance. Considering that there is 15 mg of microsomal protein/g wet weight of liver (Bishop & Bell, 1985), the translocation system can transport 3.75 nmol of PC/(min·g wet weight). As it was reported (Pritchard & Vance, 1981) that PC synthesis in cultured hepatocytes reached 3.7 nmol/(min·g wet weight), the phospholipid translocation system seems sufficient to assume the transmembrane equilibration of the newly synthesized phospholipids.

Acknowledgments

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