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RAPID TRANSMEMBRANE MOVEMENT OF PHOSPHATIDYLCHOLINE IN SMALL UNILAMELLAR LIPID VESICLES FORMED BY DETERGENT REMOVAL

RUTH M. KRAMER *, HANS-JOACHIM HASSELBACH ** and GIORGIO SEMENZA

Eidgenössische Technische Hochschule, Laboratorium für Biochemie, ETH-Zentrum, CH-8092 Zürich (Switzerland)

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Summary

Small unilamellar phosphatidylcholine vesicles, formed by solubilizing phosphatidylcholine with sodium cholate and removing the detergent by gel filtration, have been studied in their interaction with phospholipid exchange protein. The exchange of phosphatidylcholine between the vesicles and erythrocyte ghosts was greatly stimulated by the phosphatidylcholine-specific exchange protein from bovine liver. It was found that 95% of the phosphatidylcholine was readily available for exchange within 3 h at 37°C. In similar vesicles prepared by sonication only 70% of the phosphatidylcholine was rapidly exchangeable. Our results indicate that the transmembrane movement of phosphatidylcholine across the bilayer of vesicles prepared by the cholate technique is a relatively fast process. The results are discussed with respect to the presence of trace amounts of lipid-associated cholate which may facilitate the transbilayer exchange of phosphatidylcholine.

Introduction

When sodium cholate is removed from mixed phosphatidylcholine/cholate micelles by gel filtration, single-walled phosphatidylcholine vesicles of uniform

* Present address: Boston V.A. Medical Center, 150 South Huntington Avenue, Boston, MA 02130, U.S.A.

** Present address: Max-Planck-Institut für medizinische Forschung, Organische Chemie, Jahnstrasse 29, D-6900 Heidelberg, F.R.G.

Abbreviation: buffer A, 100 mM NaCl, 10 mM sodium phosphate, pH 7.6.

size (300 Å in diameter) are formed [1]. These vesicles have been extensively characterized and were found to have similar structural and physicochemical properties as compared to sized sonicated vesicles [1]. The cholate-removing procedure has also been successfully used to incorporate purified integral membrane proteins into phospholipid bilayers [2—4]. Such artificial membrane systems may serve as suitable models to study structural and dynamic properties of protein-lipid interactions in membranes. It was therefore of obvious interest to first characterize the dynamic organization of lipids in pure lipid vesicles formed by the cholate method.

The transmembrane distribution and movement of lipids have been extensively studied in artificial and biological membranes [5,6]. Using techniques involving phospholipid exchange proteins [7—9], chemical labeling [10] and ^{13}C-NMR [11] it has been shown that the inside-outside translocation of phosphatidylcholine in unilamellar vesicles is an extremely slow process with a half-time of several days. We have investigated the transbilayer movement of phosphatidylcholine in small unilamellar vesicles formed by the cholate procedure by measuring its accessibility to phosphatidylcholine exchange protein purified from bovine liver. Our results show that the translocation of phosphatidylcholine across the membrane in these vesicles is a relatively fast process.

Materials and Methods

Isotopes. Cholesteryl [1-^{14}C]oleate (25.7 Ci/mol) was from the Radiochemical Centre, Amersham. [24-^{14}C] Cholic acid (47.5 Ci/mol) was obtained from the Centre d'Études Nucléaires de Saclay, France, and was recrystallized from acetone/water (4 : 1, v/v) before use. [9,10-^{3}H]Palmitic acid (500 Ci/mol) was from New England Nuclear. Radioactivity was counted with 5 ml of Aquasol, a xylene-based scintillation solution for aqueous samples from New England Nuclear. Appropriate spillover corrections were made to obtain the amounts of ^{3}H and ^{14}C in a given sample.

Phospholipids. Egg lecithin (grade I) was purchased from Lipid Products (South Nutfield, Surrey, U.K.) and used without further purification. [^{3}H]-Dipalmitoylphosphatidylcholine was synthesized from L-α-lysopalmitoylphosphatidylcholine (Sigma) and radioactive palmitic acid according to the method of Gupta et al. [12] (final specific radioactivity 110 Ci/mol). Phospholipids were stored in $CHCl_3/CH_3OH$ (1 : 1, v/v) under nitrogen at —20°C.

Erythrocyte membrane preparation. Erythrocyte ghosts were prepared by the method of Steck [13]. The membranes were suspended at concentrations of 2—5 mg of protein/ml in 5 mM sodium phosphate, pH 7.6, containing 0.02% sodium azide and stored at 0—4°C for no longer than 4 days prior to use for the exchange reactions.

Phospholipid exchange protein. The phosphatidylcholine-specific exchange protein was purified from bovine liver as described by Kamp and Wirtz [14], but omitting the last purification step (gel filtration). During purification exchange activity was determined with the ghost-vesicle assay as described below. 1 unit of exchange activity was defined as the transfer of 1% of the labeled phosphatidylcholine during 1 min under standard conditions. The

exchange protein was purified 710-fold over the pH 5.1 supernatant and had a specific activity of 533 units/mg in our ghost-vesicle assay. The protein was stored at −20°C in 50% glycerol and dialyzed against 1000 vol. of 5 mM sodium phosphate, pH 7.6, before use. The exchange activity was fully retained by this procedure.

Lipid vesicles. For exchange reactions sonicated phosphatidylcholine vesicles were prepared from 1 mg egg lecithin, 25 μCi [^{3}H]dipalmitoylphosphatidylcholine and 2 μCi cholesteryl[^{14}C]oleate. The organic solvents were evaporated at 20°C under a stream of nitrogen. The lipids were redissolved in diethyl ether and carefully dried again. The lipid mixture formed a thin film on the wall of the test tube. The lipids were dispersed in 200 μl of 100 mM NaCl/10 mM phosphate, pH 7.6 (buffer A) using a Vortex mixer. The tube was then immersed in a sonicating waterbath and was sonicated at 20°C for 30 min. The resulting clear suspension of lipid vesicles was fractionated on a Sepharose 4B column (0.6 × 60 cm, flow rate 2 ml/h, in buffer A) to obtain a completely homogeneous preparation, which is referred to as 'sized' vesicles. For standard assays during purification of the exchange protein, sonicated lipid vesicles were used without fractionation.

'Cholate' vesicles were prepared from 10 mg egg lecithin , 50 μCi [^{3}H]-dipalmitoylphosphatidylcholine and 4 μCi cholesteryl [^{14}C]oleate as follows. The lipids were dried under nitrogen at room temperature as described above and suspended in 450 μl of buffer A using a Vortex mixer. The lipids were then solubilized by adding 50 μl of 20% sodium cholate (Merck) in buffer A (ratio of phosphatidylcholine/cholate 1 : 1, w/w). The clear solution of mixed micelles was then applied on a Sephadex G-50 column (2.1 × 28 cm, flow rate 7 ml/h, in buffer A) to remove the detergent. The lipid vesicles which were eluted with the void volume of the column were pooled and used for exchange reactions.

For 'cholate-dialysis' vesicles, mixed micelles were prepared as described above. The cholate was then removed by dialysis against 1000 vol. of buffer A for 12 h. The resulting lipid vesicles were applied on a Sepharose 4B column (0.6 × 60 cm, flow rate 2 ml/h, in buffer A) to separate small unilamellar vesicles from large multilamellar aggregates. In order to determine residual cholate in vesicles prepared by either detergent-removing technique, mixed micelles were prepared in the presence of [^{14}C]cholate (omitting the non-exchangeable marker).

Exchange reactions. For standard exchange reactions the ghost vesicle assay described in Ref. 15 was used with sonicated lipid vesicles containing 1 μg egg lecithin, 0.05 μCi [^{3}H]dipalmitoylphosphatidylcholine and 0.06 μCi cholesteryl[^{14}C]oleate as phospholipid donor. For determination of phosphatidylcholine exchange between the various types of lipid vesicles and erythrocyte ghosts we used the following assay conditions. Lipid vesicles (14—34 μg of phosphatidylcholine containing 0.17—0.7 μCi of [^{3}H]dipalmitoylphosphatidylcholine and 0.014—0.06 μCi of cholesteryl [^{14}C]oleate) were incubated at 37°C with erythrocyte ghosts (2—5 mg of protein, ratio of ghost to vesicle phosphatidylcholine greater than 25) in the presence of exchange protein (10—15 units) in 5 ml of buffer A. At different times 0.5 ml of the incubation mixture was removed, of which 0.1 ml was transferred to a counting vial

and 0.4 ml was centrifuged at 4°C for 15 min in an Eppendorf 5412 centrifuge. 0.1 ml aliquots of the supernatant were transferred to counting vials. For long-term incubations the ghosts were pelleted by centrifugation after 1 h and replaced by fresh acceptor membranes. All experiments included control samples without exchange protein and incubations were carried out in duplicate.

Exchange calculations. Since cholesteryl [^{14}C]oleate serves as a non-exchangeable marker, the decrease in the $^{3}H/^{14}C$ ratio during incubation measures the fraction of labeled vesicle phosphatidylcholine transferred to ghosts. The extent of exchange was calculated as follows. In the supernatants the fraction of [^{3}H]dipalmitoylphosphatidylcholine remaining with the vesicles after the reaction was taken to be the supernatant $^{3}H/^{14}C$ ratio divided by the $^{3}H/^{14}C$ ratio for the starting vesicles. In the absence of exchange protein only a small fraction (less than 10% after 6 h of incubation) of the [^{3}H]dipalmitoylphosphatidylcholine was transferred to ghosts. The recovery of lipid vesicles in the supernatant was higher than 80% throughout the whole incubation as determined with recovery of non-exchangeable marker.

Analytical determinations. Protein was measured by the method of Lowry et al. [16] using bovine serum albumin as a standard. Phospholipid phosphorus was estimated by the method of Chen et al. [17].

Results

Formation and chromatography of different types of small unilamellar phosphatidylcholine vesicles

'Cholate' vesicles. A typical elution profile of [^{3}H]phosphatidylcholine vesicles containing cholesteryl [^{14}C]oleate as a non-exchangeable marker, which were prepared by solubilizing a lipid dispersion with sodium cholate and removing the detergent from the mixed phosphatidylcholine/cholate micelles by gel filtration on Sephadex G-50, is shown in Fig. 1. Careful characterization by Brunner et al. [1] has indicated that the vesicles eluting with the void volume of the column are single-walled and homogeneous by several criteria. We have measured the amount of cholate left associated with the lipid vesicles with [^{14}C]cholate and found that more than 99.8% of the added cholate was removed by chromatography on Sephadex G-50. This corresponds to a molar ratio of phosphatidylcholine/cholate of greater than 275, indicating that the number of cholate molecules per lipid vesicle is less than 20.

Sonicated vesicles. These were first fractionated on a column of Sepharose 4B. A typical elution profile for sonicated [^{3}H]phosphatidylcholine vesicles containing cholesteryl [^{14}C]oleate is shown in Fig. 2. The higher ratio of $^{14}C/^{3}H$ in the void-volume fractions as compared to the vesicle fractions may indicate either the presence of cholesteryl ester particles or the preference of the cholesterol ester for multilayers [7]. For exchange experiments with ghosts, vesicle fractions 14—16 were used.

'Cholate-dialysis' vesicles. [^{3}H]Phosphatidylcholine vesicles containing cholesteryl [^{14}C]oleate were prepared by removal of cholate from the detergent-solubilized lipid suspension according to the method of Racker and coworkers [18]. Using this technique 97.5% of the initial cholate was removed,

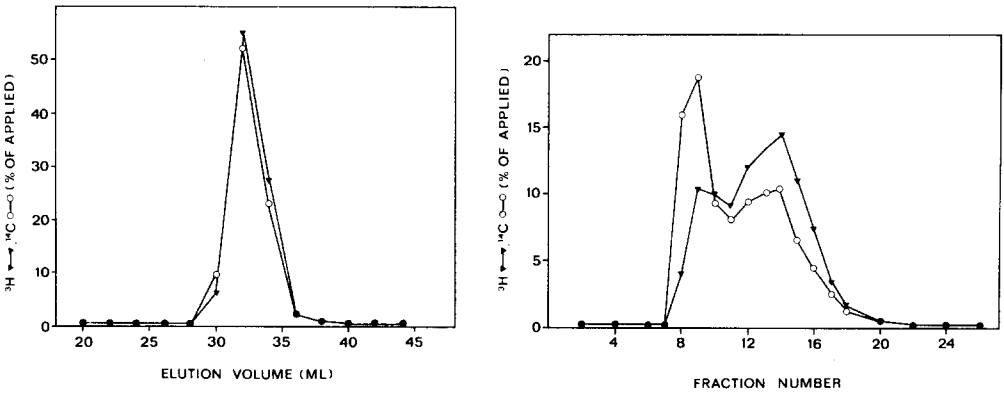

Fig. 1. Gel filtration of mixed ^{3}H-labeled phosphatidylcholine/cholate micelles on Sephadex G-50. The phosphatidylcholine dispersion containing [^{3}H]dipalmitoylphosphatidylcholine and cholesteryl[^{14}C]-oleate was solubilized with sodium cholate, then applied to a Sephadex G-50 column (2.1 X 28 cm, V_0 = 32 ml) and eluted with NaCl/phosphate buffer; 2-ml fractions were collected and analyzed for radioactivity. For details see Materials and Methods.

Fig. 2. Elution profile of sonicated ^{3}H-labeled phosphatidylcholine vesicles from Sepharose 4B. Unsized phosphatidylcholine vesicles containing [^{3}H]dipalmitoylphosphatidylcholine and cholesteryl[^{14}C]oleate were made in NaCl/phosphate buffer. The vesicles were applied to a Sepharose 4B column (0.6 X 60 cm, V_0 = 9 ml) and eluted with the same buffer. Fractions of 1 ml were collected and analyzed for radioactivity. For details see Materials and Methods.

as determined with [^{14}C]cholate. Consequently, the removal of cholate by dialysis is approx. one order of magnitude less effective as compared to removal by gel filtration. The 'cholate-dialysis' vesicles were then sized by chromatography on Sepharose 4B. The elution pattern (see Fig. 3) showed a major peak containing approx. 75% of the phosphatidylcholine and a minor peak in the void volume. By this procedure an additional 50% of the [^{14}C]-cholate was removed and the molar ratio of phosphatidylcholine/cholate in these vesicles was estimated to be greater than 50. We used only those fractions which eluted on the descending portion of the second peak (fractions 12—14) for our exchange experiments.

Exchange reactions

'Cholate' vesicles were incubated with an excess of erythrocyte ghosts (ghost to vesicle phosphatidylcholine ratio approx. 30 : 1) and exchange protein. The time course of the protein-mediated transfer of [^{3}H]dipalmitoylphosphatidylcholine from the vesicles to ghosts is shown in Fig. 4. Within 3 h of exchange 95% of the total ^{3}H radioactivity was transferred from the vesicles to the ghosts. In the absence of exchange protein very little phospholipid transfer occurred.

In order to validate the unexpectedly large fraction of exchangeable phosphatidylcholine in 'cholate' vesicles, we measured the exchange of [^{3}H]dipalmitoylphosphatidylcholine between sized sonicated vesicles and ghosts using the same incubation conditions. In agreement with previous studies [7—9,11] we found that approx. 70% of the [^{3}H]dipalmitoylphosphatidylcholine was

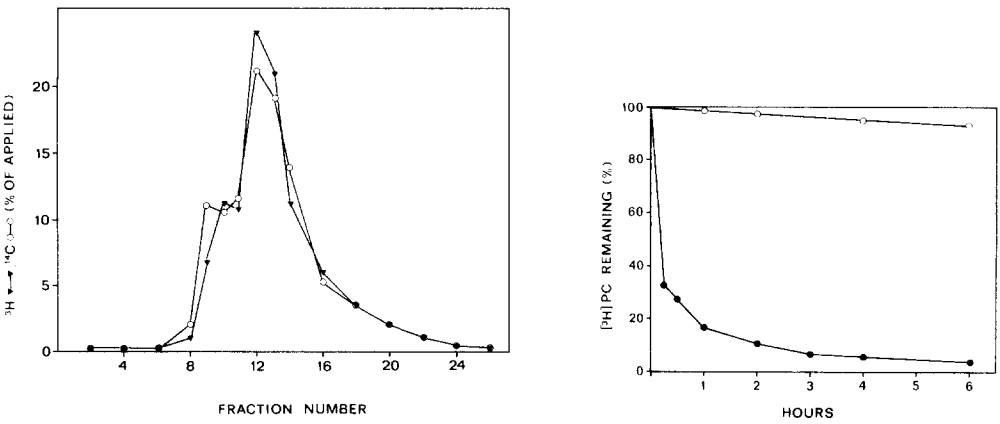

Fig. 3. Elution profile of 'cholate-dialysis' phosphatidylcholine vesicles from Sepharose 4B. The phosphatidylcholine dispersion containing [^{3}H]dipalmitoylphosphatidylcholine and cholesteryl[^{14}C]oleate was solubilized with sodium cholate in NaCl/phosphate buffer. The mixed micelles were extensively dialyzed against the same buffer and then applied on a Sepharose 4B column (0.6 X 60 cm, V_0 = 9 ml) and eluted with the same buffer. Fractions of 1 ml were collected and analyzed for radioactivity. For details see Materials and Methods.

Fig. 4. Transfer of [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]PC) from 'cholate' vesicles to erythrocyte ghosts. ^{3}H-Labeled 'cholate' vesicles were incubated with an excess of ghosts with (●——●) and without (○——○) phospholipid exchange protein as described under Materials and Methods. This figure is representative for three experiments and each point represents the average of two separate incubations.

directly available for exchange; whereas the remaining 30% were exchanged at a much slower rate (Fig. 5).

Because of the marked difference in exchangeable phosphatidylcholine of 'cholate' vesicles as compared to sonicated vesicles, we suspected the residual cholate (molar ratio phosphatidylcholine/cholate greater than 275) to be responsible for the almost total exchangeability of the phosphatidylcholine. We therefore attempted to further reduce the cholate content by subjecting the vesicles to a second gel filtration or to extensive dialysis. Although the

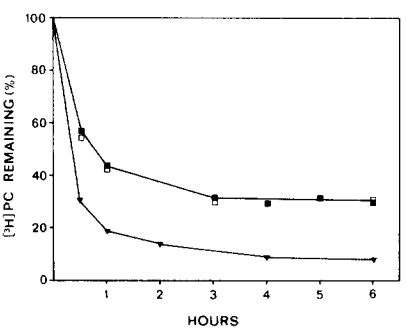

Fig. 5. Transfer of [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]PC) from sonicated (■——■) or sonicated, cholate-treated (□——□) or 'cholate-dialysis' vesicles (▼——▼) to erythrocyte ghosts. ^{3}H-Labeled sized phosphatidylcholine vesicles were incubated with an excess of ghosts in the presence of phospholipid exchange protein as described under Materials and Methods. The curves are representative for two experiments and each point represents the average of two separate incubations.

levels of remaining cholate after the second gel filtration or the dialysis were beyond detection (molar ratio of phosphatidylcholine/cholate greater than 500), no significant differences were found in the exchangeability of phosphatidylcholine. Thus, even after repeated gel filtration or extensive dialysis of the 'cholate' vesicles approx. 90% of the [^{3}H]dipalmitoylphosphatidylcholine was exchanged within 2 h of incubation with the exchange protein. In a further attempt to demonstrate an effect of cholate, we added increasing amounts of sodium cholate to sized sonicated vesicles (molar ratio of phosphatidylcholine to cholate equal to 250, 25 and 2.5). At molar ratios of phosphatidylcholine to cholate greater than 2 some of the detergent will be incorporated into the lipid bilayers without disrupting them [19]. Since little is known about how fast surfactant molecules move through the lipid bilayer [20], the cholate-containing suspension of lipid vesicles was briefly sonicated (10 min) to ensure a homogeneous distribution of cholate over the two monolayers of the membrane. Aliquots of these cholate-containing vesicles were then incubated with an excess of ghosts in the presence of exchange protein. The time course of protein-mediated exchange for all vesicle preparations was essentially the same as the one shown for sonicated vesicles (see Fig. 5). Thus, the readily available phosphatidylcholine pool remained unchanged in the presence of increasing cholate concentrations and amounted to approx. 70% for all cholate-containing vesicles.

In order to compare our results with those of other studies [21], we also examined the exchangeability of [^{3}H]dipalmitoylphosphatidylcholine from phosphatidylcholine vesicles prepared by the detergent dialysis procedure. Using identical conditions as described above, we found that the readily available pool of phosphatidylcholine in sized 'cholate-dialysis' vesicles was significantly higher as compared to sonicated vesicles (Fig. 5). The results were essentially the same as those presented for 'cholate' vesicles: approx. 90% of the labeled phosphatidylcholine from 'cholate-dialysis' vesicles was removed by exchange within 3 h. This is in contrast to the observations of Dicorleto and Zilversmit [21] showing that for phosphatidylcholine vesicles prepared by cholate dialysis only 65% of the total phosphatidylcholine is readily available for exchange, while the remaining 35% is exchangeable at a much slower rate. The discrepancy in findings might be explained by the fact that we used sized rather than unfractionationed 'cholate-dialysis' vesicles for our exchange experiments. Indeed, we have found (see Fig. 3) that phosphatidylcholine vesicles prepared by the cholate dialysis technique are partly excluded from Sepharose 4B, indicating that multilamellar structures might be present.

The formation of unilamellar phosphatidylcholine vesicles by the cholate removal techniques itself might lead to a non-equilibrium lipid distribution in the plane of the membrane or across the membrane. Similarly, the distribution of residual cholate molecules between the two monolayers of the vesicles prepared by detergent removal might not be uniform. In both cases, sonication of the 'cholate' or 'cholate-dialysis' vesicles should lead to a redistribution of the lipids or remaining detergent molecules and therefore restore the membrane bilayer stability. However, sonication of both 'cholate' and 'cholate-dialysis' vesicles had no influence on the availability of vesicle. [^{3}H]dipalmitoylphos-

phatidylcholine for exchange reactions. Thus, the exchangeable phosphatidylcholine pool after a 2 h incubation with exchange protein amounted to approx. 90% for both preparations before and after sonication.

Discussion

Different methods are available to prepare unilamellar lipid vesicles of reasonably homogeneous dimensions. These include sonication of aqueous lipid dispersions [22,23], injection of solutions of lipid in organic solvent into the aqueous medium [24,25] and removal of detergent from detergent-solubilized lipid suspensions [26—28]. The cholate procedure originally introduced by Racker and coworkers [29] was further developed by Brunner et al. [1]. The artificial membrane system obtained by this method consists of single-walled phosphatidylcholine vesicles having a somewhat larger average diameter than sonicated vesicles. Minor differences only were observed in the physicochemical properties of 'cholate' and sonicated vesicles [1].

We present here evidence that in small unilamellar vesicles formed by detergent removal phosphatidylcholine is nearly completely exchangeable. This result is in contrast to the limited exchangeability reported for unilamellar phosphatidylcholine vesicles prepared by sonication [7—9,11]. Our findings indicate that the transmembrane movement of phosphatidylcholine in vesicles prepared by detergent removal is greatly enhanced as compared to sonicated phosphatidylcholine vesicles. The following mechanism might be responsible for the rapid translocation of phosphatidylcholine across the membrane. First, residual cholate molecules (which were calculated to amount to approx. 20 or less per vesicle) might have the ability to trigger non-bilayer structures [30]. It would then be expected that the local occurrence of non-bilayer lipids would destabilize the bilayer structure leading to a redistribution of membrane lipid across the membrane. We have, however, no experimental evidence to support this hypothesis since all our attempts to induce transmembrane motion of phosphatidylcholine in sonicated vesicles by means of cholate failed.

Alternatively, it is possible that the structural arrangement of residual cholate molecules within the lipid bilayer of vesicles prepared by detergent removal is different from the one of cholate molecules bound to sonicated vesicles. The cholate molecule can be viewed as a rigid ring structure with a hydrophilic and a hydrophobic face to which is attached a mobile hydrophilic tail [31]. When phosphatidylcholine is dispersed in an excess of cholate (molar ratio of phosphatidylcholine/cholate less than 2), mixed phosphatidylcholine/cholate micelles form [32]. As proposed by Small and coworkers [33] these mixed micelles consist of a bimolecular disc of phosphatidylcholine, each layer of which is stabilized by a ring of cholate molecules covering the external hydrocarbon chains and presenting their hydrophilic face to water. Upon removal of cholate from the concentrated phosphatidylcholine/cholate solution (by gel filtration or dialysis) the size of the mixed micelles increases progressively with increasing phosphatidylcholine/cholate ratio until the phase transition from the mixed micellar state into the bilayer vesicles occurs, after which all the phosphatidylcholine will be converted to bilayer vesicles. Cholate molecules incorporated into lipid bilayers are thought to be present as

dimers, trimers or tetramers confined to one monolayer of the membrane, with the hydroxyl groups turned towards each other and the hydrophobic surface of the molecules turned outwards [32]. We propose that the cholate molecules which remain associated with the nascent phosphatidylcholine vesicles form bilamellar aggregates of four or more molecules having the hydrophilic parts in contact and exposing their hydrophobic parts to the phosphatidylcholine chains. These aggregates closely resemble the proposed structure for primary bile salt micelles consisting of 4—8 cholate molecules [34], but may be considered as 'inverted' micelles. Cholate micellar structures which are transmembrane, are thought to only be formed when phosphatidylcholine/cholate micelles are converted to closed bilayers and to not occur after binding of cholate to preformed phosphatidylcholine vesicles. It might then be further speculated that these bilayer-spanning inverted cholate micelles may facilitate the transmembrane exchange of phosphatidylcholine by providing an intramembrane surface with which the phospholipid headgroup can interact during passage through the membrane. It is also possible that the presence of the phospholipid exchange protein is required in order to enhance the transbilayer movement of phosphatidylcholine.

Similar findings have been reported for single bilayer vesicles prepared from rat liver microsomal lipids [35]. The availability of lysophosphatidylcholine for enzymatic hydrolysis by lysophospholipase was found to be higher in vesicles prepared by the cholate technique when compared with sonicated vesicles.

Other conditions have been described in which the lipid translocation across the membrane in small, unilamellar lipid vesicles is significantly increased. Thus, asymmetric perturbation of the bilayer [11,36], phase transitions [37] and incorporation of glycophorin, a transmembrane protein [38,39], greatly facilitate the transmembrane movement of phosphatidylcholine in sonicated vesicles. Recently, Ca^{2+}-induced rapid transbilayer movement of phosphatidylcholine has been described in phosphatidylcholine/cardiolipin bilayers [40]. The transbilayer cholate micelle may represent a very simple structure to allow rapid translocation of phosphatidylcholine through the membrane.

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