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Phospholipids containing nitrogen- and sulfur-linked chains: kinetics of cholesterol exchange between vesicles

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We have examined the kinetics of [^{14}C]cholesterol exchange between unilamellar vesicles formed from the following synthetic glycerophosphatidylcholines: (a) those having acyl (OC(O)R), acylamino (NHC(O)R), carbamoyl (NHC(O)OR), and acylthio (SC(O)R) chains at the *sn*-2 position, and (b) those having alkyl (OR) and thioalkyl (SR) chains at the *sn*-1 position. Replacement of the glycerol oxygen atom at the *sn*-2 position of PC with a NH group did not affect the rate of cholesterol exchange to a significant extent, suggesting that the amide group of sphingomyelin is not primarily responsible for the very slow rate of exchange of cholesterol observed from sphingomyelin vesicles. Replacement of the glycerol oxygen at the *sn*-2 position of phosphatidylcholine with a sulfur atom caused the rate of spontaneous cholesterol exchange to increase by a factor of 1.6. Substitution of an *O*-alkyl chain for the acyl chain at the *sn*-1 position of 2-acylthiophosphatidylcholine or substitution of a thioalkyl chain for the *O*-alkyl *sn*-1 chain of 1-alkyl-2-acylaminodeoxyphosphatidylcholine also did not result in a marked difference in cholesterol exchange rate. The data suggest that interactions other than intermolecular hydrogen bonding are involved in determining the rates of intermembrane cholesterol exchange. Significantly, these kinetic studies also lend support to the continued use in model membranes of synthetic sulfur- and nitrogen-substituted phosphatidylcholines, which have been employed to study properties of lipolytic enzymes, since synthetic acylamino- and acylthio-phospholipids form vesicles that give cholesterol exchange rates that closely resemble those found in vesicles prepared with diester-phosphatidylcholines.

Introduction

Sulfur- and nitrogen-substituted phosphatidylcholines are synthetic phospholipid analogues that have attracted a great deal of attention recently because of their interesting biophysical and pharmacological properties [1,2]. These lipids have acyl or alkyl chains linked to the glycerol backbone via a nitrogen or sulfur atom instead of an oxygen atom. Acylthio phospholipids provide chromogenic phospholipase substrates that permit spectrophotometric assay of lipolytic enzymes [2–5]. Phospholipids that have a *sn*-1 thioether or a hexadecanamido chain in place of the *sn*-1 *O*-alkyl chain and a *sn*-2 *O*-methyl group have antitumor properties against various leukemic cells [6,7]. Acylaminodeoxy phospholipids that have a RCONH group at the *sn*-2 position are resistant to phospholipase A_2 catalyzed degradation and are potential phospholipase inhibitors and potential drug carriers [8–11]. In the present paper, we present the first report of the kinetics of cholesterol exchange between vesicles prepared with derivatives of phosphatidylcholine (PC) in which an oxygen atom has been substituted by a sulfur or nitrogen atom. Our experiments are aimed at elucidating the role of interlipid interactions (i.e., hydrogen bonding and van der Waals interactions) in sterol desorption and at an evaluation of the possible alterations in physical behavior that may be introduced in vesicles by introducing *N*-and *S*-linked phospholipids in place of endogenous phospholipids.

Previous studies have demonstrated that the rate of spontaneous cholesterol movement between membranes decreases as the sphingomyelin (SPM) content

Abbreviations: BSA, bovine serum albumin; DCP, dicetyl phosphoric acid; DPPC, dipalmitoyl-PC; PC, phosphatidylcholine; SPM, sphingomyelin.

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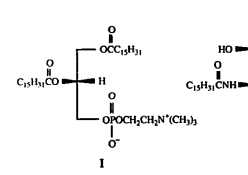

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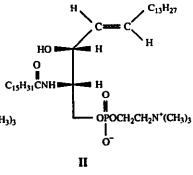

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of the bilayer of the donor species increases [12–20]. Since the rate-determining step of transfer of cholesterol from the donor to excess acceptor vesicles is the desorption from the donor particles (for recent reviews, see Refs. 21–23), it appears that the decrease in the rate of cholesterol transfer arises from SPM-induced tightening of lipid–lipid interactions at the interface of donor particles.

A variety of physical techniques have provided indications of a preferential interaction of cholesterol with SPM compared with glycerophospholipids in noncocrystallizing lipid mixtures (for a review, see Ref. 24). Increased van der Waals attractive interactions have been considered to account for some of the differences found in molecular packing of cholesterol-containing SPM and PC systems [17]. Intermolecular hydrogen bonding has also been considered to contribute to the higher affinity of cholesterol for SPM compared with glycerophospholipids [25,26]. Although the crystal structures of sphingolipids indicate that intermolecular hydrogen bonding takes place in the crystalline state [27–29], there is no direct evidence for a greater extent of hydrogen bonding between cholesterol and SPM vs. glycerophospholipids in membranes. A comparison of the structures of PC and SPM (I and II, respectively) reveals that there are increased opportunities for intermolecular hydrogen bonding in SPM; the allylic hydroxy group and amide group of SPM are likely sites at which hydrogen-bonding interactions may take place between SPM molecules and between SPM and other lipids, making SPM-containing bilayers more ordered than bilayers formed from PCs with similar chain composition. It was pointed out a long time ago [30] that SPM has both hydrogen-bond donor and acceptor sites but PC has only hydrogen-bond acceptor sites; intermolecular hydrogen bonding involving the amide and hydroxy groups of sphingolipids has been proposed from infrared, spin label, and X-ray diffraction studies [31–33]. In order to probe the role of the amide group of SPM in reducing the rate of cholesterol exchange between SPM vesicles relative to PC vesicles, we prepared vesicles from two PC analogues having a *sn*-2 acylaminodeoxy chain in place of the ester chain (compounds **1** and **2**, see structures in Table II). We also measured the kinetics of intervesicle cholesterol exchange using vesicles of carbamoyl-PC (compound **3**), which is another nitrogen-bearing PC analogue; the ROCONH group of **3** replaces the RCONH group of the amido-PCs **1** and **2**. Analogs **1–3** may be considered as glycerolipid analogues of SPM, which possesses a NHCOR group.

To further assess the possible role of interlipid hydrogen-bonding capability on the rate of intervesicle cholesterol exchange we have measured the rates of cholesterol movement between vesicles prepared from thio-containing PCs. Since sulfur is less electronegative than oxygen, such analogues permit another estimate of the importance of hydrogen-bonding groups in PCs on the rate of cholesterol transfer between bilayers. We present data on the effects of replacing the oxygen atom at the *sn*-2 position of DPPC with sulfur (compound **4**) on the rate of spontaneous cholesterol exchange between unilamellar vesicles prepared with diacyl-PCs. To estimate the difference between an acylthio vs. an aminoacyl chain at the *sn*-2 position in PCs having an ether-linked chain at the *sn*-1 position, we have measured cholesterol exchange rates from vesicles prepared with compound **5**, which has a *sn*-2 thiopalmitoyl chain and a *sn*-1 *O*-hexadecyl chain.

Studies of spontaneous lipid transfer kinetics have provided information about the lateral organization of lipids in membranes [23]. A network of 'hydrogen-bonded belts' has been proposed to be present at the bilayer surface of various phospholipids [34,35] including acylamido-PCs [36]. This stabilizing hydrogen-bonded network, which may become loosened on addition of cholesterol, has been postulated to influence cholesterol transfer between membranes [37]; however, this hypothesis has not yet been thoroughly tested. The present study addresses the topic of the influence of modifications in the hydrogen-bonding capability of PCs in the donor vesicles on the rates of spontaneous cholesterol desorption from the vesicle surface.

Materials and Methods

Materials

Dipalmitoyl-PC (DPPC), *N*-palmitoyl-SPM, cholesterol, dicetyl phosphoric acid (DCP), bovine serum albumin (BSA), and DEAE-Sepharose CL-6B were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic PC analogues were prepared as described in the following publications: compound **1**, Ref. 38; compounds **2** and **3**, Ref. 39; compound **4**, Ref. 40; compound **5**, Ref. 41. [4-^{14}C]Cholesterol (specific activity 57.5 mCi/mmol) and [9,10(n)-^{3}H]glycerol trioleate (specific activity 15.4 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The purities of cholesterol and PCs were assayed by thin-layer chromatography on silica gel G plates (Analtech, Newark,

DE) using solvent systems as described previously [41,42]. The solvent system for *N*-palmitoyl-SPM was chloroform/methanol/ammonium hydroxide/water (65:30:3:3, v/v). The PCs, cholesterol, and *N*-palmitoyl-SPM migrated as single spots on TLC when visualized by spraying with 10% sulfuric acid in ethanol followed by charring on a hot plate. The purity of [^{14}C]cholesterol was $\geq$ 99% as determined by TLC.

Preparation of vesicles

The vesicles were prepared as described previously [13] with a slight modification. Donor (1.0 mM total lipid) and acceptor vesicles (10.0 mM total lipid) contained 6 mol% of cholesterol. DCP (15 mol%) and a trace (0.08 μCi) of [^{14}C]cholesterol were incorporated in the donor vesicles. A trace (0.046 μCi) of [^{3}H]triolein was incorporated in the acceptor vesicles as a nonexchangeable marker. The lipid film was dispersed in the desired volume of 20 mM sodium phosphate buffer with 1 mM EDTA and 0.05% (w/v) sodium azide (pH 6) and incubated at 50°C for 10 min. After the lipid film was dispersed completely by vortexing, the liposome suspensions were sonicated at room temperature in a Heat Systems Ultrasonics (Farmingdale, NY) Model W375 sonicator. The aqueous dispersion of the donor species was sonicated using a cup-horn for 1 h continuously, and the aqueous dispersion of the acceptor species was sonicated using a microtip at 50% duty cycle for 1 h. The vesicle suspensions were then centrifuged for 10 min at 10000 rpm in a DuPont Sorvall centrifuge with a SS-34 rotor. The supernatant was taken for the experiments. Examination of the donor vesicles prepared from compounds **1–5** by negative-staining electron microscopy revealed that they were entirely unilamellar. The sizes and homogeneities of the donor vesicles were measured by photon correlation spectroscopy at 50°C and 90° scattering angle as described previously [18] or at 20°C and 90° scattering angle according to a previous procedure [43]. The diameters of the donor vesicles were in the range of 128–227 nm (index of polydispersity, 0.18–0.23).

[^{14}C]Cholesterol exchange between vesicles

Exchange of [^{14}C]cholesterol was initiated by mixing and vortexing equal volumes of donor and acceptor vesicles after each had been preincubated separately for 10 min at 50°C in a Labline shaking water bath. Exchange was followed at 50°C which is above the transition temperatures of the phospholipids we used. BSA (2% w/v) was present in the incubation medium. Aliquots (200 μl) of the incubation medium were transferred to DEAE-Sepharose CL-6B columns (0.6 × 3 cm). After the aliquots had entered completely into the column, 300 μl of buffer was added immediately to wash the column and to begin the collection of the eluate. The column was washed by applying another 1 ml of the buffer, and the eluate was collected in the same vial. To the eluate was added 8 ml of Scintiverse II (Fisher Scientific Co.) or 5 ml of Ecoscint scintillation cocktail. Vials were vortexed for about 30 s and then counted in a Packard 2000CA liquid scintillation counter for 10 min. The ^{14}C- and ^{3}H-dpm were corrected for spillover. The recovery of the neutral acceptor vesicles in the eluate was about 90%, and < 0.5% of the donor vesicles were eluted; this is consistent with previous results of this laboratory [13].

Thermotropic properties

It should be noted that the preparation of the aqueous dispersions and the kinetic measurements were carried out at 50°C in order to obtain the PCs in the liquid-crystalline phase. Differential-scanning calorimetry studies showed that the main gel-to-liquid-crystalline phase transition (T_m) of compounds **1–5** is in the range of 38–41°C. Furthermore, DPPC and *N*-palmitoyl-SPM have T_m values of approx. 40–42°C. The T_m values of the phospholipids we used were determined in multilamellar suspensions using the following calorimeters: DASM-4 (NPO Biopribor, Puschino, U.S.S.R.) [45,46], MC-2 (Microcal, Northampton, MA), or Hart Model 7707 (Pleasant Grove, UT). Scanning rates of 6 C° to 15 C° per hour were used. The following T_m values were obtained: **1**, 39.8°C; **2**, 38.0°C; **3**, 39.7°C; **4**, 40.8°C; **5**, 39.4°C; DPPC, 41.7°C; *N*-palmitoyl-SPM, 39.7°C.

Calculation of half-times

The fraction of [^{14}C]cholesterol in the acceptor vesicles is defined as $\alpha_t = [^{14}C/^{3}H]_t/[^{14}C/^{3}H]_{mix}$, where $[^{14}C/^{3}H]_t$ and $[^{14}C/^{3}H]_{mix}$ represent the ratio of [^{14}C]cholesterol to [^{3}H]triolein in the eluate at time t and in the donor-acceptor vesicles without separation. The initial value at time zero and the infinity value are α_0 and α_∞, respectively. To estimate the values of α_0, α_∞, and rate constant k, all the kinetic data (α_t) were fit to a function of time, $F(t)$, where $F(t) = \alpha_\infty + [(\alpha_0 - \alpha_\infty)\exp(\text{slope} \times t)]$. The pseudo first-order rate constant k was calculated from the relationship $k = -\text{slope}/1.1$ (for a 10-fold excess of acceptor species) as described previously [47]. The half-times were determined from the relationship $t_{\frac{1}{2}} = \ln 2/k$. The size of the exchangeable pool of [^{14}C]cholesterol is calculated from $\{[(\alpha_\infty - \alpha_0)/(1 - \alpha_0)] \times 1.1\} \times 100\%$.

Results and Discussion

The half-time for cholesterol exchange from donor *N*-palmitoyl-SPM unilamellar vesicles is about 9-fold higher than that from donor DPPC unilamellar vesicles at 50°C (Table I). This rate difference does not arise from a difference in donor vesicle size since the two

TABLE I

Comparison of half-times for [^{14}C]cholesterol exchange from DPPC and N-palmitoyl-SPM vesicles at 50 °C

Donor vesicles contained 6 mol% cholesterol, 15 mol% DCP, and 79 mol% DPPC or *N*-palmitoyl-SPM (total lipid concentration, 1.0 mM). Acceptor vesicles contained 6 mol% cholesterol and 94 mol% DPPC (total lipid concentration, 10.0 mM). Kinetic measurements were carried out in the presence of BSA (2% w/v). The number in parentheses indicates the number of different vesicle preparations used. The size of the exchangeable pool of [^{14}C]cholesterol was 98% in DPPC and 83% in *N*-palmitoyl-SPM donor vesicles, respectively.

Phospholipid in donor vesicles	$t_{1/2}$ (min)
DPPC	185 ± 20 (3)
N-Palmitoyl-SPM	1626 ± 48 (2)

donor vesicles have similar diameters (128 and 175 nm for DPPC and *N*-palmitoyl-SPM vesicles, respectively). A slower rate of cholesterol desorption from SPM-containing vesicles reflects the tighter association of cholesterol with the vesicle surface. It has been postulated that the amide and/or allylic hydroxy group of SPM interact with the 3β-hydroxy group of cholesterol via hydrogen bonding (see Ref. 24). In order to test the role of the amide group of SPM in the interaction with cholesterol, we studied cholesterol exchange rates from donor vesicles prepared using PCs with a *sn*-2 *N*-linked chain. Fig. 1 shows the time course of exchange of [^{14}C]cholesterol between donor vesicles prepared from the amido- and carbamoyl-PCs, i.e., compounds **1**, **2**, and **3**, and acceptor DPPC vesicles. The kinetic measurements were taken until the fraction of [^{14}C]-cholesterol in the acceptor vesicles increased only slightly with time, i.e. until the exchange process approached equilibrium. [^{14}C]Cholesterol was fully exchangeable in donors prepared from compounds **2** and **3**, and about 80% of the total [^{14}C]cholesterol underwent exchange from donor vesicles prepared with compound **1**. The semi-log plots (inset, Fig. 1) indicate that only one kinetic pool of [^{14}C]cholesterol was present in the donor vesicles. The half-times for cholesterol exchange from vesicles of these PCs are not very different from the half-time obtained using DPPC vesicles ($t_{\frac{1}{2}}$, 217.7, 270.5, and 175.6 min for vesicles from compounds **1**, **2**, and **3**, respectively (Table II) vs. $t_{\frac{1}{2}}$ of 185 min for DPPC vesicles (Table I)). The sizes of these donor vesicles are similar to each other (diameters of 163, 227, and 178 nm for vesicles from compounds **1**, **2**, and **3**, respectively). The difference in rates of cholesterol exchange between vesicles from DPPC and from compounds **1–3** is much less marked than that between DPPC and *N*-palmitoyl-SPM vesicles. Since the NHCO group is present in *N*-palmitoyl-SPM and in compounds **1–3**, the kinetic results suggest that neither the weak hydrogen-donating property of the NH group nor the strong hydrogen-accepting property of the C = O group is the primary source of the very slow desorption of cholesterol molecules from the surface of SPM particles.

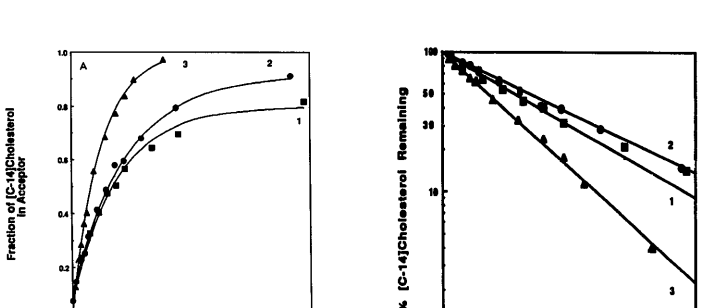
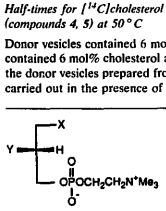


Fig. 1. (A) Time course of [^{14}C]cholesterol exchange between PC/cholesterol unilamellar vesicles at 50°C in the presence of BSA (2% w/v). The vesicles contained 6 mol% cholesterol. The host phospholipid in the acceptor vesicles was DPPC. (B) First-order plot of the exchange data, i.e. $\log[(F(t)-\alpha_\infty)/(\alpha_0-\alpha_\infty)]$ vs. t. The *N*-linked PCs in the donor vesicles are: compound **1**, 1-ether-2-amide PC; compound **2**, 1-thioether-2-amide PC; compound **3**, 1-thioether-2-carbamoyl PC. The solid lines in B are obtained by exponential curve fit of a y vs. t plot, where $y = \alpha_\infty + [(\alpha_0 - \alpha_\infty)\exp(\text{slope} \times t)]$.

TABLE II

Half-times for [^{14}C]cholesterol exchange from vesicles prepared from PCs with a sn-2 N-linked chain (compounds 1–3) or a sn-2 S-linked chain (compounds 4, 5) at 50 °C

Donor vesicles contained 6 mol% cholesterol, 15 mol% DCP, and 79 mol% PC analogue (total lipid concentration, 1.0 mM). Acceptor vesicles contained 6 mol% cholesterol and 94 mol% DPPC (total lipid concentration, 10.0 mM). The sizes of the exchangeable pool of [^{14}C]cholesterol in the donor vesicles prepared from compounds **1**, **2**, **3**, **4**, and **5** were 78%, 100%, 100%, 100%, and 99%, respectively. Kinetic measurements were carried out in the presence of BSA (2% w/v). The number in parentheses indicates the number of different vesicle preparations used.



Compound	X	Y	$t_{1/2}$ (min)
1 1-ether-2-amide-PC	$OC_{16}H_{33}$	$NHCOC_{15}H_{31}$	218±29 (2)
2 1-thioether-2-amide-PC	$SC_{16}H_{33}$	$NHCOC_{15}H_{31}$	271±20 (3)
3 1-thioether-2-carbamoyl-PC	$SC_{16}H_{33}$	$NHC(O)OC_{14}H_{29}$	176±22 (3)
4 1-ester-2-thioester-PC	$OC(O)C_{15}H_{31}$	$SC(O)C_{15}H_{31}$	115±20 (2)
5 1-ether-2-thioester-PC	$OC_{16}H_{33}$	$SC(O)C_{15}H_{31}$	112± 1 (2)

To further analyze whether hydrogen-bonding groups in PC have a significant influence on the rate of cholesterol exchange, vesicles were prepared from thio-containing PCs. Fig. 2 shows the time course of exchange of [^{14}C]cholesterol from donor vesicles containing PCs with a *S*-linked chain at the *sn*-2 position. The exchange of [^{14}C]cholesterol reached equilibrium after about 6 h of incubation. Again, [^{14}C]cholesterol in the donor vesicles was completely exchangeable and only one kinetic pool was found in the donor vesicles (inset, Fig. 2). Table II shows that the half-times of cholesterol exchange from vesicles prepared with compounds **4** and **5** are very similar to each other ($t_{\frac{1}{2}}$, 115 and 112 min for vesicles from compounds **4** and **5**, respectively). The rates of cholesterol exchange between donor vesicles prepared from compounds **4** and **5** and acceptor DPPC vesicles are faster than that between DPPC vesicles by a factor of 1.6. This modest difference in rates does not arise from a difference in donor vesicle size, since the diameters are similar (183

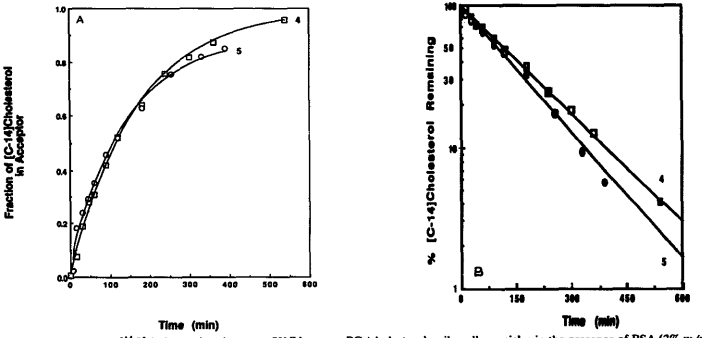


Fig. 2. (A) Time course of [^{14}C]cholesterol exchange at 50°C between PC/cholesterol unilamellar vesicles in the presence of BSA (2% w/v). The vesicles contained 6 mol% cholesterol. The host phospholipid in the acceptor vesicles was DPPC. (B) First-order plot of the exchange data. The *S*-linked PCs in the donor vesicles are: compound **4**, 1-ester-2-thioester PC; compound **5**, 1-ether-2-thioester PC.

and 189 nm for vesicles from compounds **4** and **5**, respectively; diameter of DPPC vesicles, 128 nm).

The data summarized in Tables I and II indicate that the replacement of the glyceryl oxygen atom at the *sn*-2 position of DPPC with sulfur results in only a relatively small increase in the rate of cholesterol exchange. In fact, a comparison of the half-times of cholesterol exchange from vesicles prepared from compounds **1** and **5** (which have the largest difference in hydrogen-bonding capability of the five synthetic nitrogen- and sulfur-substituted PCs we studied) indicates that only a 1.9-fold difference in rate exists ($t_{\frac{1}{2}}$ values of 218 ± 29 vs. 112 ± 1 min, respectively). Thus, the hydrogen belts postulated to be formed by amide groups of phospholipids [9,34–37] do not appear to exert a critical influence on the cholesterol desorption rate from the vesicle surface.

In conclusion, our results suggest that hydrogen bonding between cholesterol and PC does not play an important role with respect to the rate of spontaneous movement of cholesterol between vesicles. The data we present with PCs containing *N*- and *S*-linked chains indicate that the much slower rate of cholesterol exchange from vesicles containing SPM does not arise because the -NHCOR group of SPM causes its carbonyl oxygen to be more electron-rich than the corresponding carbonyl in acyl-PC or because the NH group is capable of stronger hydrogen-bonding interactions than the glyceryl-linked oxygen of the acyl group of PC. Hence, interlipid hydrogen bonding between the 3β-hydroxy group of cholesterol and the NH group of SPM does not appear to exert a large influence on the rate of cholesterol desorption from the SPM vesicle surface. Moreover, we have recently studied the role of the allylic hydroxy group of SPM on the kinetics of cholesterol exchange; cholesterol desorption rates from vesicles prepared with synthetic SPM analogues having a hydrogen atom or a methoxy group at the 3 position are similar, indicating that the slower rate of exchange of cholesterol from SPM vesicles does *not* arise because of contributions from hydrogen bonding involving the hydroxy group at the 3 position of SPM [48]. Therefore, other lipid–lipid interactions appear to be more important than hydrogen bonding in determining the rates of intervesicle cholesterol exchange. For example, van der Waals interactions may exert a significant role; small decreases in molecular area can lead to large increases in lateral cohesion energies [17], resulting in a large increase in the half-time of cholesterol exchange.

Since synthetic PCs **1–5** formed vesicles that showed cholesterol exchange kinetic behavior similar to that found in DPPC vesicles (Table II), it is suggested that these compounds can be incorporated into well-defined model membrane systems in place of endogenous diester-PCs without significantly affecting the physical properties of the bilayer. In contrast, some of the detailed patterns of phase transition characteristics of acylamino- and acylthio-PCs differ from those of the corresponding diester-PCs, indicating that some differences may exist with respect to conversions between phases formed by aqueous dispersions of these compounds [9,36,45,46,49]. Further studies employing different biophysical methods will be required to assess how closely nitrogen- and sulfur-substituted PC analogues resemble the behavior of endogenous oxygen-substituted lipids in vesicles and cellular systems.

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