Permeability Properties of Sterol-Containing Liposomes from Analogues of Phosphatidylcholine Lacking Acyl Groups†

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ABSTRACT: The effect of sterols on the rates of nonelectrolyte movement in multilayered liposomal membranes formed from various phosphatidylcholine (PC) analogues was studied. We measured the temperature dependence of the permeation of small, polar solutes through lipid bilayers in the liquid-crystalline state of the following saturated phosphatidylcholines: diesters containing 14, 16, or 18 carbon atoms in each acyl chain; the corresponding diethers, which lack the carbonyl groups; and alkyl analogues, such as 2-octadecyleicosylphosphorylcholine (OEPC), which have no diacylglycerol or glycerol diether moiety. In addition, a diether-PC having one saturated and one unsaturated chain was used, and analogues of OEPC were synthesized in which the choline group is modified. At temperatures above the gel to liquid-crystalline phase transition of these diester, diether, and alkyl analogues of PC, the 3β-hydroxysteroids cholesterol and ergosterol decreased the permeability of glycerol, urea, acetamide, and glucose, whereas epicholesterol and lanosterol exerted little effect on the reflection coefficient for acetamide penetration. Cholesterol reduced solute permeability in liposomes from OEPC analogues having increased separation between the phosphoryl and quaternary ammonium groups of the phosphorylcholine moiety or increased steric bulk in the choline moiety and in the quaternary ammonium group. The activation energy and activation entropy for osmotic swelling of liposomes by glycerol and urea permeation and for fluorescence change of a liposome-associated merocyanine dye upon acetamide permeation were not altered significantly by cholesterol incorporation (48 mol %) into bilayers with the diester or diether phosphatidylcholines we examined. The similarities between the effects of sterols on the initial rates and reflection coefficients of polar nonelectrolyte diffusion in diester-PC bilayers and those from our phospholipids lacking acyl groups (diether-PC and OEPC analogues) indicate that carbonyl oxygens of the fatty acyl groups in the 1 and 2 positions of PC are not essential to obtain interaction with cholesterol. Thus, there is no specific requirement for hydrogen bonding between the hydrogen of the sterol hydroxyl group and a carbonyl oxygen of diester-PC. The studies with OEPC analogues suggest that no rigid structural specificity in the choline moiety of phosphorylcholine must be met for lecithin–cholesterol interaction.

The most extensively investigated lipid–lipid interaction in membranes is that of sterols with phospholipids. Sterols that have a planar steroid nucleus, a nonpolar side chain at C-17, and a 3β-hydroxyl group restrict the mobility of phospholipids in bilayers when the phospholipid is in the liquid-crystalline phase, resulting in a decrease in the permeability of the membrane [see review articles by Jain (1975), Chapman (1975), and Demel & de Kruyff (1976)]. The importance of van der Waals forces in stabilizing the phospholipid–sterol interaction must be stressed, since X-ray and neutron diffraction measurements in hydrated multilayers indicate that the steroid nucleus penetrates into the fatty acid region of the bilayer (Lecuyer & Dervichian, 1969; Franks, 1976; Worcester & Franks, 1976). Since 3α-hydroxysteroids exert diminished effects on the permeability properties of liposomes and *Acholeplasma laidlawii* cell membranes (de Kruyff et al., 1972; Bittman & Blau, 1972) in comparison with cholesterol, the stereochemical orientation of the sterol hydroxyl group at the aqueous–lipid interface must be considered in addition to the apolar forces between the steroid nucleus and the phospholipid fatty acyl chains. Studies with isosteric and nonisosteric analogues of phosphatidylcholine (PC)[1] bearing C–P (phosphonate) and C–P–C (phosphinate) linkages indicated that strict demands at the PC polar head group near the phosphorus–oxygen ester bonds must be met for PC–cholesterol interaction to occur (Bittman & Blau, 1972).

The motions of the choline methyl groups of PC and of the phosphorus atom are largely unaffected by incorporation of cholesterol into PC bilayers, as judged by C-13 (Keough et al., 1973), deuterium (Stockton et al., 1974; Gally et al., 1976), and phosphorus-31 NMR studies (Yeagle et al., 1975; Cullis et al., 1976; Brown & Seelig, 1978). These studies suggest that no direct interactions occur between cholesterol and the phosphocholine group in PC–cholesterol bilayers. Cholesterol appears to act as a spacer molecule, increasing the separation between PC head groups (Yeagle et al., 1977; Oldfield et al., 1978; Yeagle, 1978). It should be noted, however, that a direct interaction between cholesterol and the phosphate oxygen of PC has been proposed on the basis of infrared dichroism measurements in oriented multibilayers (Verma & Wallach, 1973) and NMR data obtained from aqueous dispersions (Darke et al., 1972). On the other hand, from measurements of force–area curves in monolayers and of calorimetric properties of liposomes from phospholipid analogues, de Kruyff et al. (1973) concluded that cholesterol does not form hydrogen bonds with any parts of the phospholipid molecule.

Neutron and X-ray diffraction data have been interpreted to give the location of the cholesterol hydroxyl group in egg

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[1] Abbreviations used: PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine; di-C_{14} diether-PC, glycerol-3-*sn*-phosphorylcholine 1,2-bis(tetradecyl ether); di-C_{16} diether-PC, glycerol-3-*sn*-phosphorylcholine 1,2-bis(hexadecyl ether); di-C_{18} diether-PC, glycerol-3-*sn*-phosphorylcholine 1,2-bis(octadecyl ether); $C_{18:1}$ $C_{16:0}$ diether-PC, *rac*-glycerol-3-phosphorylcholine 1-*cis*-9′-octadecenyl 2-hexadecyl ether; OEPC, (2-octadecyleicosylphosphoryl)choline; Me-OEPC, (2-octadecyleicosylphosphoryl)methylcholine; *N*-Et,*N*,*N*-diMe-OEPC, 2-hydroxyethyl *N*-ethyl-*N*,*N*-dimethylammonium 2-octadecyleicosyl phosphate; propyl-OEP, 3-hydroxypropyl *N*,*N*,*N*-trimethylammonium 2-octadecyleicosyl phosphate.

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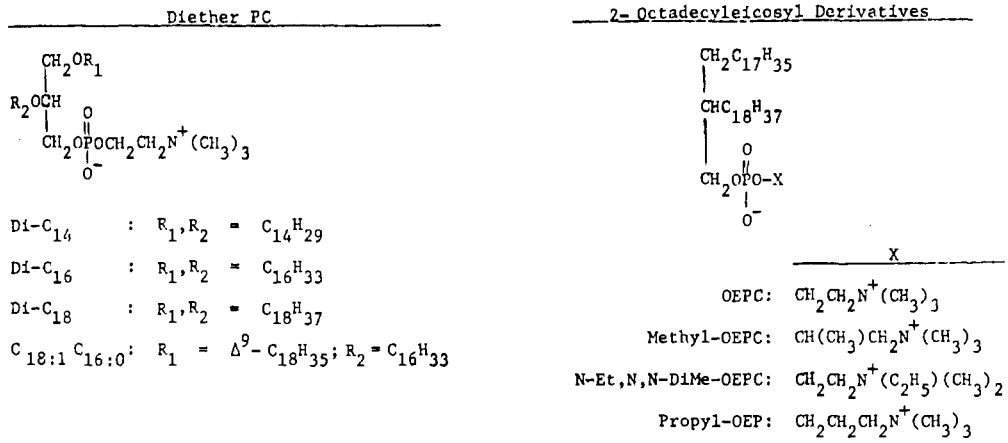

FIGURE 1: Structures of the synthetic phosphatidylcholines. The 1,2-dialkoxy-*sn*-glycero-3-phosphorylcholines (diether-PC) used are as follows: ditetradecyl, di-C_{14}; dihexadecyl, di-C_{16}; and dioctadecyl, di-C_{18}. The 1-oleyl-2-hexadecyl-3-phosphorylcholine, $C_{18:1}$ $C_{16:0}$ diether-PC, was racemic. The 2-octadecyleicosyl derivatives are as follows: (2-octadecyleicosylphosphoryl)choline, OEPC; (2-octadecyleicosylphosphoryl)-methylcholine, Me-OEPC; 2-hydroxyethyl *N*-ethyl-*N*,*N*-dimethylammonium 2-octadecyleicosyl phosphate, *N*-Et,*N*,*N*-diMe-OEPC; 3-hydroxypropyl *N*,*N*,*N*-trimethylammonium 2-octadecyleicosyl phosphate, propyl-OEP.

PC bilayers near the water interface, in the vicinity of the phospholipid carbonyl groups (Worcester & Franks, 1976; Franks, 1976). To minimize the contact of the steroid nucleus with the polar aqueous phase, hydrogen bonding between the cholesterol hydroxyl group and a PC carbonyl oxygen atom has been postulated (Brockerhoff, 1974; Huang, 1976, 1977; Yeagle & Martin, 1976). Differences in the extent of broadening of the NMR line widths of the fatty acyl methylene protons of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 2,3-dipalmitoyl-*sn*-glycero-1-phosphocholine upon addition of cholesterol were suggested to provide support for a model involving hydrogen bonding between the cholesterol hydroxyl group and the carbonyl oxygen at the 2 position of PC (Chatterjie & Brockerhoff, 1978). The model proposed by Huang (1976, 1977) considers the complementarity of packing between the phospholipid and sterol to be diminished when the orientation of the hydroxyl group is 3α rather than 3β.

The purpose of the present investigation was to examine whether this postulated hydrogen bond is an important stabilizing force between PC and cholesterol in bilayers. We tested the effects of sterols as a function of temperature on the permeability properties of liposomes prepared from synthetic PC analogues that lack the ester carbonyl oxygens of lecithin. The permeation rates of acetamide, glycerol, urea, and glucose through lipid bilayers of liposomes prepared from saturated diester-PC, diether-PC, and alkyl-PC analogues were measured in the presence and absence of sterols. We also made liposomes from a diether-PC bearing one unsaturated and one saturated chain and from alkyl-PC analogues in which the choline moiety is altered.

Experimental Section

Materials

1,2-Diacyl-*sn*-glycero-3-phosphorylcholines were purchased from Sigma Chemical Co. 1,2-Dialkoxy-3-*sn*-phosphatidylcholines were synthesized as described by Chen & Barton (1971). *rac*-1-Oleyl-2-hexadecylphosphatidylcholine ($C_{18:1}$ $C_{16:0}$ diether-PC) was purchased from Serdary Research Laboratories, London, Ontario. The synthesis of the alkylphosphorylcholine, OEPC, was described previously (Deroo et al., 1976). Analogues of OEPC in which the choline moiety was replaced (Figure 1) were synthesized by coupling of the desired quaternary ammonium alcohol tosylate salt with octadecyleicosylphosphoric acid, as described by Deroo et al. (1976). Dicetylphosphoric acid and cholesterol were purchased from Sigma Chemical Co. Epicholesterol and ergosterol were obtained from Schwarz/Mann. These sterols were recrystallized twice from ethanol. Lanosterol was a gift of Dr. W. R. Nes and was purified (to about 95% purity) by the methods of Bloch & Urech (1958) and Rees et al. (1966). The other lipids were found to be chromatographically pure on thin-layer analysis by using silica gel G plates. Stock solutions of lipids in chloroform were stored at −20 °C. Merocyanine 540 dye was obtained from Eastman Kodak. Phospholipase D from cabbage was purchased from Sigma Chemical Co., cholesterol oxidase (*Bacillus cereus*) was from Beckman Instruments, and horseradish peroxidase was from Worthington Biochemicals. [^{14}C]Glucose was purchased from New England Nuclear Corp. Acetamide, urea, and glycerol were reagent grade. The filipin complex was purified and dissolved as described elsewhere (Blau & Bittman, 1977).

Methods

Preparation of Liposomes. The appropriate amounts of PC, sterol, and dicetylphosphoric acid were added to a vial, and the chloroform solvent was removed under nitrogen and vacuum. The thin film of lipid that coated the walls of the vial was dispersed in aqueous medium by agitation with a Vortex mixer in the presence of glass beads. Multilamellar liposomes were prepared at temperatures several degrees above the lipid phase transition. (The transition temperatures were recorded by Jain et al., unpublished experiments.) Rates of solute permeation were also measured above the phase transition temperature. To ascertain the phospholipid and sterol content of the liposomes, liposomes were pelleted at 4 °C by centrifugation at 20 000 rpm for 30 min in a Sorvall SS 34 rotor. The assay of phospholipid and sterol, by using methods cited previously (Blau & Bittman, 1977), showed that liposomal concentrations were equal to the concentrations prior to centrifugation.

Swelling of Liposomes by Glycerol and Urea Permeation. A 50-μL aliquot of liposomes dispersed in 60 mM KCl was added to a 1-cm path length cuvette containing 0.6 mL of 0.4 M aqueous glycerol or urea solution. The final total lipid concentration was 0.5 mM, except in some experiments where 1.0 mM was used. Liposomes contained 4 mol % of dicetylphosphoric acid. After the liposome suspension was mixed rapidly with the hypertonic glycerol or urea solution, the initial rapid decrease in light scattering from liposome shrinking and

then the slow increase in light scattering from swelling were monitored on a Perkin-Elmer Hitachi MPF-2A spectrofluorometer. Both monochromators were set to 450 nm. The temperature was controlled by circulating water through the cuvette holder. The initial rate of liposome swelling was measured from the tangent to the light-scattering increase. The relative intensity at which the swelling phase was completed was also measured, allowing calculation of the rate constant, k, from the quotient of the initial rate/total amplitude of the light-scattering change. Plots of the logarithm of the amplitude of the change in light scattering against time were linear, demonstrating that the swelling process followed first-order kinetics. At least four measurements were made at each temperature. At temperatures above 35 °C, glycerol and urea solutions were saturated with helium to minimize air bubbles. The activation energy was calculated according to the equation $k = A \exp(-E_a/RT)$; semilogarithmic plots of the rate constants vs. the reciprocal of the absolute temperature were made by the method of least squares with a Tektronix 8200 plotter coupled to a Sigma 7 computer. The value of $\Delta S^{\ddagger}$ was obtained from the relationship $\Delta S^{\ddagger} = 4.576 (\log k - 10.753 - \log T) + E_a/T$, where k is in units of s^{-1}. It was assumed that $\Delta S^{\ddagger}$ is independent of temperature over the temperature range used.

Efflux of Trapped [^{14}C]Glucose. A trace of [1-^{14}C]glucose (2×10^7 cpm), specific activity 48.2 Ci/mol, in 0.5 mL of 75 mM KCl and 75 mM NaCl was added to a thin, dry film of the desired concentration of lipid coated on the walls of a conical centrifuge tube. The concentration of PC was 25 mM. Dicetylphosphoric acid was added to a concentration of 10 mol %. Liposomes were formed above 60 °C. To remove untrapped glucose, the liposomes were placed in a dialysis sac and dialyzed extensively (about 5 h) at room temperature against large volumes (1 L) of 75 mM KCl and 75 mM NaCl solution, with frequent changes of the dialysate. To monitor the time course of efflux of trapped glucose, the liposome suspension was transferred to a small test tube containing 3 mL of 75 mM KCl and 75 mM NaCl solution and shaken in a water bath at 55 °C. At intervals of 30 min, 100-μL aliquots were taken from the test tube for radioactive counting in a Beckman liquid scintillation counter. An aliquot was taken after 3 h from the dialysis bag to measure the amount of trapped glucose remaining in the liposome suspension.

Reflection Coefficients. Reflection coefficients for the rapidly permeant molecule, acetamide, were determined in liposomes prepared from PC analogues in the presence and absence of sterols with a Durrum stopped-flow spectrophotometer (Durrum-Dionex Corp., Sunnyvale, CA). The light transmitted at 180° was monitored at 450 nm. Changes in liposome volumes arising from shrinking and swelling were measured beginning at 150 ms after mixing. Measurements at shorter times were precluded by an artifact discussed elsewhere [e.g., Bittman et al. (1976) and Owen & Eyring (1975)]. The transmittance (T) following the 150-ms disturbance period was linear with respect to time, and the oscilloscope traces were similar to those shown previously for osmotic shrinking and swelling (Bittman & Blau, 1972). The initial absorbance was calculated from the initial value of T. The initial rate of absorbance change, dA/dt, was obtained from the measured dT/dt and then converted to $d(1/A)/dt$ by using the relationship $(A_{initial})^{-2} dA/dt = -d(1/A)/dt$. The latter quantity is proportional to relative changes in liposome volume arising from changes in osmolarity (Bangham et al., 1967). The reflection coefficient, σ, was obtained from the ratio C_{iso}/C_s (Goldstein & Solomon, 1960), where C_{iso} is the concentration of the isosmolar electrolyte (30 mM KCl) and C_s is the concentration of the permeant molecule (acetamide) at which no volume change occurs. The latter value was determined by the "zero-time" method (Goldstein & Solomon, 1960; Lelievre & Rich, 1973). The $d(1/A)/dt$ values were plotted vs. the acetamide concentrations, and C_s was determined by extrapolation to zero volume change.

Treatment of Liposomes with Filipin or with Enzymes. Aliquots of filipin in dimethylformamide were added to liposomes. The filipin concentration was determined spectrophotometrically at 358 nm by using the extinction coefficient cited previously (Blau & Bittman, 1977). The concentration of dimethylformamide did not exceed 1.0% (v/v). In experiments involving phospholipase D and cholesterol oxidase, liposomes containing 50 mol % cholesterol (1 mM total lipid concentration) were first incubated for 1 h at 37 °C with phospholipase D (10 μg/mL of liposome suspension) in 0.5 mM Tris-HCl buffer containing 50 mM $CaCl_2$, pH 5.6. Immediately after the phospholipase treatment, liposomes were incubated for 10 min at room temperature with cholesterol oxidase (0.2 unit/mL); horseradish peroxidase (50 units/mL) was added to decompose hydrogen peroxide generated upon oxidation of cholesterol. Then the reflection coefficients were measured in the stopped-flow apparatus. For assay of enzymatic action, incubations were stopped by adding chloroform–methanol (2:1 v/v), and the fraction of PC cleaved was assayed by thin-layer chromatography in a procedure similar to that of Martin et al. (1975). The cholesterol content was assayed colorimetrically.

Fluorescence Change of Liposome-Associated Merocyanine Dye. Merocyanine 540 dye dissolved in ethanol was added to liposomes containing 4 mol % dicetylphosphoric acid. The final dye concentration was 2.5 mM. The ethanol concentration in the liposome suspension never exceeded 0.5%. Liposomes (0.5 mM total lipid concentration) were prepared from the PC analogues in the presence or absence of cholesterol and were dispersed in 60 mM KCl solution. The fluorescence of the merocyanine dye was monitored at 580 nm (with excitation at 558 nm) with a Perkin-Elmer Hitachi MPF-2A spectrofluorometer. The fluorescence intensity of the dye was lower in the presence of liposomes than in aqueous solution; a steady level of fluorescence was reached within about 2 min after addition of dye to liposomes stirred in a cuvette. Addition of a small volume (e.g., 25 μL) of an aqueous stock solution of acetamide (final concentration of 57 mM) to liposomes (0.5 mL) that had reached the steady level of fluorescence caused an initial rapid drop in fluorescence intensity of approximately 8–12 %/s. A new steady level of fluorescence was reached several minutes after addition of acetamide. The initial rate of fluorescence decrease and the steady-state fluorescence intensity were temperature dependent; incorporation of cholesterol into PC bilayers reduced the initial rates. The rate constant was calculated at various temperatures between 30 and 55 °C, and activation parameters were obtained as stated above for nonelectrolyte permeation. No significant change was found in the wavelength of the emission peak upon swelling of the liposomes by acetamide addition. The decrease in fluorescence intensity of the dye observed on liposome swelling may arise from an increase in the amount of liposome-associated dye relative to the amount in the external medium, from permeation of the dye into the liposome bilayer, or from formation of dye aggregates.

Results

Osmotic Behavior of Liposomes from PC Analogues. The volume change experienced by an ideal osmometer exposed

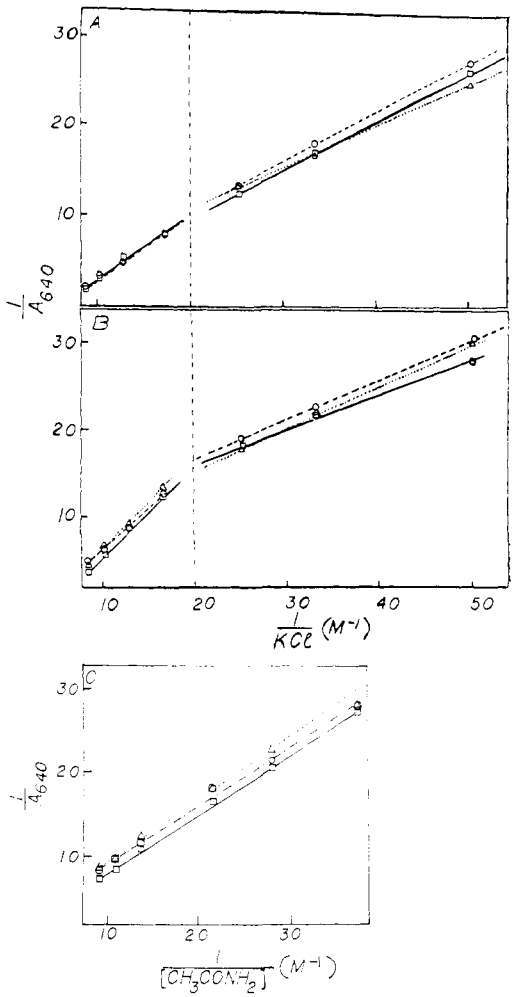

FIGURE 2: Boyle–van't Hoff plots of the reciprocal of the absorbance at 640 nm of liposomal suspensions vs. the final concentration of KCl or acetamide at equilibrium after osmotic shrinking or swelling. Liposomes containing 4 mol % dicetylphosphoric acid and 2 mM final total lipid concentration were dispersed in 60 mM KCl solution. The absorbance was measured on a Cary 14 spectrophotometer before and after liposomes were subjected to varying concentrations of KCl (panels A and B) or acetamide (panel C). Lipid bilayers were formed from the following phospholipids in the absence (panels A and C) and in the presence (panel B) of 48 mol % cholesterol: (○) di-C_{14} diether-PC and di-C_{16} diether-PC, (△) DMPC, and (□) OEPC and propyl-OEP. Osmotic behavior was examined at the following temperatures: 30 °C for DMPC and di-C_{14} diether-PC, 45 °C for di-C_{16} diether-PC, and 58 °C for OEPC and propyl-OEP. Swelling and shrinking conditions appear on the right and left sides, respectively, of the vertical dashed line in panels A and B.

to a change in the osmotic strength of the suspending medium is given by the Boyle–van't Hoff law, $V = K(1/c) + b$, where V is the volume change (volume is inversely proportional to absorbance for liposomes), K is a constant independent of wavelength and osmometer concentration, c is the osmotic pressure gradient across the membrane in terms of concentration, and b is the osmotic dead space (Bangham et al., 1967). Liposomes prepared in KCl solution above the phase transition temperatures of the diester-PC, diether-PC, and alkyl-PC analogues gave linear Boyle–van't Hoff plots when exposed to an osmotic pressure gradient of KCl (Figure 2A,B) or acetamide (Figure 2C). This indicates that liposomes formed from these PC analogues are ideal osmometers under these conditions. Negative-staining electron microscopy of OEPC liposomes showed the usual multilamellar appearance (O. R. Anderson, personal communication). When liposomes were mixed with 0.025–0.3 M acetamide solutions in the stopped-flow apparatus, the initial rates of swelling and shrinking were found to vary with the osmotic gradient in a similar manner in bilayers of diester-PC, diether-PC, and alkyl-PC analogues (Figure 3). Figure 3 also shows that the initial rate of volume change is markedly reduced in the presence of cholesterol.

Glycerol and Urea Permeation. The initial rates of glycerol and urea permeation into liposomes are decreased upon incorporation of cholesterol at various temperatures above the PC gel to liquid-crystalline phase transition. Since cholesterol decreases hydrocarbon chain mobility, reduced rates of solute permeation are expected. Figure 4 shows the temperature

Table I: Effect of Cholesterol on the Activation Parameters for Glycerol Permeation in Liposomes from Diester and Diether Phosphatidylcholines[a]

	without cholesterol		with cholesterol	
	E_a (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})	E_a (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})
DMPC	14.5 ± 1.8	−19.3 ± 4.5	15.5 ± 1.5	−18.4 ± 4.4
di-C_{14} diether-PC	15.1 ± 1.1	−18.0 ± 3.7	13.9 ± 1.1	−24.0 ± 3.8
DPPC	15.2 ± 1.9	−19.4 ± 5.0	14.6 ± 1.6	−21.0 ± 4.9
di-C_{16} diether-PC	14.4 ± 1.3	−23.5 ± 4.4	11.6 ± 1.5	−28.6 ± 5.0
$C_{18:1}$ $C_{16:0}$ diether-PC	14.6 ± 1.6	−18.6 ± 4.5	11.9 ± 1.9	−26.5 ± 4.5

[a] Liposomes were subjected to hypertonic glycerol solution as described under Methods. The temperature ranges were 35–55 °C for liposomes prepared from DMPC and di-C_{14} diether-PC, 42–58 °C for DPPC and di-C_{16} diether-PC, and 25–52 °C for $C_{18:1}$ $C_{16:0}$ diether-PC. Three separate liposome preparations were made from DMPC and di-C_{14} diether-PC, and two preparations were made from the other lipids. The data represent the average of at least four analyses at each temperature with each preparation. The errors represent the standard error of the mean. $\Delta S^{\ddagger}$ is reported at 318 K for DMPC and di-C_{14} diether-PC liposomes, 323 K for DPPC and di-C_{16} diether-PC, and 312 K for $C_{18:1}$ $C_{16:0}$ diether-PC. The molar ratio of PC to cholesterol was 1:1. The following are examples of the initial rates of liposome swelling (in arbitrary fluorescence intensity units per second) in the absence and (in parentheses) presence of cholesterol: DMPC at 318 K, 2.74 ± 0.07 (2.30 ± 0.01); di-C_{14} diether-PC at 318 K, 2.63 ± 0.03 (2.41 ± 0.03); DPPC at 323 K, 2.92 ± 0.05 (2.52 ± 0.02); di-C_{16} diether-PC at 323 K, 2.84 ± 0.05 (2.63 ± 0.05); and $C_{18:1}$ $C_{16:0}$ diether-PC at 312 K, 2.39 ± 0.04 (1.72 ± 0.03).

Table II: Effect of Cholesterol on the Activation Parameters for Urea Permeation in Liposomes from Diester and Diether Phosphatidylcholines[a]

	without cholesterol		with cholesterol	
	E_a (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})	E_a (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})
DPPC	15.3 ± 1.4	−17.8 ± 5.0	14.6 ± 1.7	−20.2 ± 5.2
di-C_{16} diether-PC	13.4 ± 1.4	−22.2 ± 4.5	13.0 ± 1.5	−24.9 ± 4.6
$C_{18:1}$ $C_{16:0}$ diether-PC	12.8 ± 2.1	−24.5 ± 4.9	11.5 ± 2.0	−26.9 ± 4.2

[a] The temperature dependence of urea permeation was examined over the ranges of 42–58 °C for DPPC and di-C_{16} diether-PC liposomes and of 25–52 °C for $C_{18:1}$ $C_{16:0}$ diether-PC liposomes. Two liposome preparations were made from each phospholipid, with or without cholesterol. $\Delta S^{\ddagger}$ is reported at 323 K for DPPC and di-C_{16} diether-PC liposomes and at 312 K for $C_{18:1}$ $C_{16:0}$ diether-PC liposomes. The molar ratio of PC to cholesterol was 1:1. The following are examples of the initial rates of liposome swelling (in arbitrary fluorescence intensity units per second) in the absence and (in parentheses) presence of cholesterol: DPPC at 323 K, 2.75 ± 0.01 (2.34 ± 0.05); di-C_{16} diether-PC at 323 K, 2.81 ± 0.02 (2.42 ± 0.05); and $C_{18:1}$ $C_{16:0}$ diether-PC at 312 K, 2.20 ± 0.03 (1.63 ± 0.04).

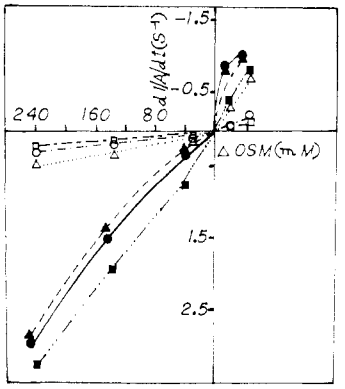

FIGURE 3: Plots of the initial rates of shrinking and swelling, $d(1/A)/dt$, vs. the change in osmolarity upon mixing of liposomes in the stopped-flow apparatus with an equal volume of acetamide solution (25–300 mM). Liposomes containing 4 mol % dicetylphosphoric acid and 2 mM final total lipid concentration were dispersed in 30 mM KCl solution. The transmittance arising from light scattering was measured at 450 nm. The change in reciprocal absorbance units per second was calculated as described under Methods. Liposomes were formed from (▲) DSPC, (△) DSPC and cholesterol (1:1), (●) di-C_{18} diether-PC, (○) di-C_{18} diether-PC and cholesterol (1:1), (■) OEPC, and (□) OEPC and cholesterol (1:1). The temperature was 58 °C. Similar traces were obtained with liposomes from di-C_{14} and di-C_{16} diether-PC, DMPC, DPPC, and propyl-OEP.

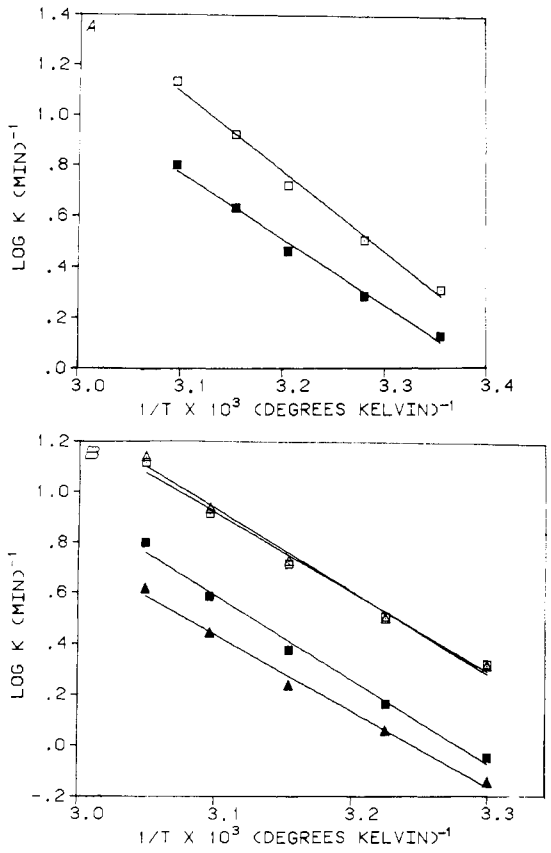

FIGURE 4: Plots of the logarithm of the rate constant for glycerol permeation into liposomes vs. the reciprocal of the absolute temperature. Liposomes were prepared from (A) (□) $C_{18:1}$ $C_{16:0}$ diether-PC and (■) $C_{18:1}$ $C_{16:0}$ diether-PC and cholesterol (1:1 molar ratio) and (B) (□) DMPC, (■) DMPC and cholesterol (1:1), (△) di-C_{14} diether-PC, and (▲) di-C_{14} diether-PC and cholesterol (1:1).

dependence of the rate constant for liposome swelling by glycerol permeation. A similar dependence was observed for urea permeation. Tables I and II present the activation parameters obtained from the Arrhenius plots for liposome swelling by glycerol and urea permeation. The activation

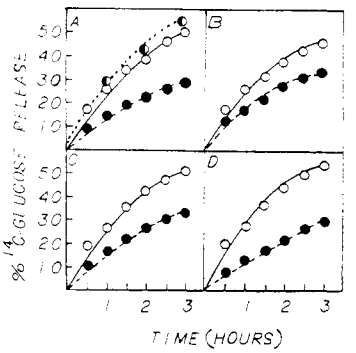

FIGURE 5: Time course of efflux of trapped [^{14}C]glucose from liposomes. Liposomes were formed in the absence or presence of 30 mol % sterol from (A) (○) OEPC, (○) OEPC and epicholesterol, and (●) OEPC and cholesterol; (B) (○) Me-OEPC and (●) Me-OEPC and cholesterol; (C) (○) *N*-Et,*N*,*N*-diMe-OEPC and (●) *N*-Et,-*N*,*N*-diMe-OEPC and cholesterol; and (D) (○) di-C_{16} diether-PC and (●) di-C_{16} diether-PC and cholesterol. The conditions are described under Methods. The temperature was 55 °C.

energy for solute permeation through diester-PC bilayers was similar to that of the corresponding diether-PC bilayers, but the initial rates were lower in diether-PC bilayers. Cholesterol incorporation to 48 mol % did not cause a significant change in the activation energies and entropies. In view of the errors associated with the activation energies and entropies, it is not possible to ascribe the difference in rate constant to either parameter.

Leakage of [^{14}C]*Glucose.* Cholesterol decreased the rate and extent of release of trapped glucose from OEPC liposomes, whereas epicholesterol did not (Figure 5A). This indicates that the 3β-hydroxyl group of cholesterol is required for reduction of glucose permeability of bilayers of these PC analogues. Cholesterol also reduced the rate and extent of leakage of glucose from liposomes formed from analogues of 2-octadecyleicosyl phosphate. Despite the substitution of a methyl group α to the phosphorus in the choline moiety (Me-OEPC, Figure 5B) and of a *N*-ethyl group in place of one *N*-methyl group (*N*-Et,*N*,*N*-diMe-OEPC, Figure 5C), the increase in steric bulk at the head-group region did not hinder the ability of the phospholipid to interact with cholesterol. Similar results were obtained with propyl-OEP liposomes. Figure 5D shows that cholesterol affects glucose permeation across di-C_{16} diether-PC bilayers in a time course similar to that observed in bilayers formed from phospholipids lacking oxygen atoms at the 1 and 2 positions.

Effect of Sterols on Reflection Coefficients. The reflection coefficient, σ, is an indication of the ability of a membrane to discriminate between solvent and solute molecules. The typical range of σ is between 0 (for solutes that are as permeable as water) and 1.0 (for solutes that are impermeant); however, if the solute is more permeant than the solvent, σ can be negative (Grim, 1953). The ability of cholesterol and ergosterol to increase the rigidity of bilayers above the lipid phase transition temperature results in an increase in σ of a permeant solute (acetamide in the present experiments) relative to the cholesterol-free bilayers (Figure 6). Lanosterol and epicholesterol failed to affect σ significantly compared to sterol-free PC bilayers.

Addition of the polyene antibiotic, filipin, to PC bilayers containing 50 mol % cholesterol resulted in the reappearance of the PC gel to liquid-crystalline phase transition; this calorimetric behavior is compatible with a filipin-induced, reversible withdrawal of cholesterol from the PC (Norman et al., 1972). We sought to use low doses of filipin to perturb the interaction of the various PC analogues with cholesterol,

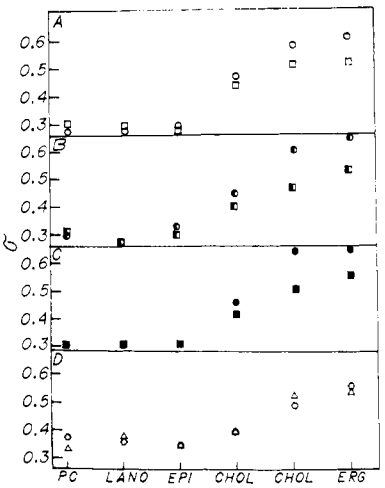

FIGURE 6: Effect of incorporation of sterols on the reflection coefficient, σ, for acetamide penetration into liposomes. Liposomes were formed from (A) (□) DMPC and (○) di-C_{14} diether-PC; (B) (◧) DPPC and (◐) di-C_{16} diether-PC; (C) (■) DSPC and (●) di-C_{18} diether-PC; and (D) (△) OEPC and (○) propyl-OEP. Reflection coefficients were measured in the absence of sterol (PC) and in the presence of 32 mol % of lanosterol, epicholesterol, and cholesterol and of 48 mol % of cholesterol and ergosterol. The following temperatures were used: (A) 30, (B) 45, (C) 58, and (D) 58 °C.

Table III: Reflection Coefficients for Acetamide Penetration into Liposomes from Diester, Diether, and Dialkyl Phosphatidylcholines[a] and the Effect of Filipin Treatment

	without cholesterol	with cholesterol[b]	
		without filipin	with filipin
DMPC	0.30	0.51	0.31
di-C_{14} diether-PC	0.20	0.60	0.38
DPPC	0.31	0.46	0.30
di-C_{16} diether-PC	0.29	0.57	0.38
DSPC	0.30	0.49	0.30
di-C_{18} diether-PC	0.31	0.60	0.36
OEPC	0.34	0.52	0.32
propyl-OEP	0.37	0.49	0.30
$C_{18:1}$ $C_{16:0}$ diether-PC	0.27	0.48	0.28

[a] Liposomes containing 4 mol % dicetylphosphoric acid and a total lipid concentration of 2 mM were dispersed in 30 mM KCl solution and mixed with an equal volume of acetamide solution. The initial rates of shrinking or swelling were measured from the stopped-flow oscilloscope traces, allowing calculation of reflection coefficients from the zero-time tangents as described under Experimental Section. The average error in σ was about ±0.03 or less. Experiments were conducted at the following temperatures: 30 °C for DMPC and di-C_{14} diether-PC liposomes; 45 °C for DPPC and di-C_{16} diether-PC liposomes; 58 °C for DSPC, di-C_{18} diether-PC, OEPC, and propyl-OEP liposomes; and 25 °C for $C_{18:1}$ $C_{16:0}$ diether-PC liposomes. [b] The molar ratio of PC to cholesterol was 1:1. Liposomes were treated with filipin for 10 min at room temperature in the dark at a molar ratio of cholesterol to filipin of 8:1. Addition of dimethylformamide alone (<1% v/v) did not affect σ. Addition of filipin (0.12 mM) to pure PC liposomes did not affect σ.

as indicated by the σ values for acetamide penetration. The data in Table III show that, under conditions where filipin causes no alteration of the σ value of pure PC bilayers, σ of cholesterol-containing PC bilayers is reduced by 35–40%. Interaction of filipin with cholesterol in liposomes from each PC we examined reversed the increase observed in σ in PC–cholesterol bilayers relative to pure PC bilayers. An additional type of experiment to examine the effect on σ of a specific perturbation of the bilayer was to subject liposomes to the action of phospholipase D and cholesterol oxidase prior to measurement of the rate of acetamide penetration. (In the absence of pretreatment with phospholipase D, little or no cholesterol was oxidized by cholesterol oxidase.) The decrease in σ upon oxidation of about 15% of the cholesterol (after phospholipase D action) in DPPC and di-C_{16} diether-PC liposomes was similar (about 25–30%). The response of σ to perturbation by the polyene antibiotic or cholesterol oxidase suggests that the PC analogues we examined interact with cholesterol in a qualitatively similar manner.

Table IV: Effect of Cholesterol on the Activation Parameters for Fluorescence Decrease of Liposome-Associated Merocyanine Dye on Acetamide Permeation[a]

	without cholesterol		with cholesterol	
	E_a (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal deg^{-1} mol^{-1})	E_a (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal deg^{-1} mol^{-1})
DMPC	17.2 ± 1.6	−11.6 ± 3.5	17.0 ± 2.2	−14.2 ± 4.4
di-C_{14} diether-PC	16.8 ± 1.6	−13.4 ± 3.8	16.4 ± 1.7	−16.1 ± 3.5
$C_{18:1}$ $C_{16:0}$ diether-PC	16.5 ± 2.0	−16.6 ± 5.0	16.1 ± 1.6	−20.4 ± 4.6

[a] The temperature dependence of the fluorescence decrease of merocyanine 540 dye was examined over the ranges of 30–55 °C for DMPC and di-C_{14} diether-PC liposomes and of 25–52 °C for $C_{18:1}$ $C_{16:0}$ diether-PC liposomes. Four liposome preparations were made from DMPC and di-C_{14} diether-PC, and two were made from $C_{18:1}$ $C_{16:0}$ diether-PC, with or without cholesterol. $\Delta S^{\ddagger}$ is reported at 310 K for DMPC and di-C_{14} diether-PC liposomes and at 312 K for $C_{18:1}$ $C_{16:0}$ diether-PC liposomes. The molar ratio of PC to cholesterol was 1:1. The following are examples of the initial rates of fluorescence decrease (in arbitrary fluorescence intensity units per second) in the absence and (in parentheses) presence of cholesterol: DMPC at 310 K, 2.04 ± 0.03 (1.54 ± 0.05); di-C_{14} diether-PC at 310 K, 1.89 ± 0.05 (1.56 ± 0.07); and $C_{18:1}$ $C_{16:0}$ diether-PC at 312 K, 1.94 ± 0.05 (1.52 ± 0.04).

Merocyanine Fluorescence. The activation parameters for the rate of fluorescence decrease of merocyanine on acetamide permeation are identical, within experimental error, in cholesterol-free and cholesterol-containing liposomes for the three phosphatidylcholines tested (Table IV). The incorporation of cholesterol to 48 mol % in the PC bilayers depressed the initial rate of fluorescence change by about 20–25% at each temperature. The activation data in Table IV show that the rate-limiting step in the fluorescence change arising from acetamide permeation is not influenced by the presence of (a) cholesterol, (b) a carbonyl oxygen atom in the PC, or (c) unsaturation in the alkoxy chain of the PC.

Discussion

Our studies of nonelectrolyte permeability in liposomes from PC analogues that lack the carbonyl group show that hydrogen bonding of the type cholesterol-OH···O=C-phospholipid is not the determining force stabilizing this interaction at the polar interface of the bilayers. The current studies corroborate the calorimetric data of Jain et al. (unpublished experiments), who observed a decrease in the enthalpy of the phase transition and lateral phase separation in these diether-PC and alkyl-PC analogues and in the corresponding diester phosphatidylcholines at cholesterol or ergosterol mole proportions up to approximately 25%. Our observation that epicholesterol failed to cause marked changes in the reflection coefficient for acetamide permeation and in glucose efflux across bilayers of diester phosphatidylcholines and PC analogues (Figures 5 and 6) agrees with other measurements of nonelectrolyte permeability in diester-PC bilayers (Demel et al., 1972). At the high mole proportions of sterol we used (30–32 mol %), it is likely that rates of solute permeability reflect the average mobility of the fatty acyl chains in the bilayer, since lateral

phase separations appear to be absent and the size of the cooperative unit is small. In contrast to the significant difference between cholesterol and epicholesterol in permeation processes, differential scanning calorimetry measurements of Jain et al. (unpublished experiments) showed that low mole proportions of these sterols induced a lateral phase separation in bilayers of diester phosphatidylcholines and PC analogues. The decrease in the enthalpy of the phase transition of the PC-rich phase caused by cholesterol incorporation was similar to that caused by epicholesterol. The extent of steric contact between PC and sterol molecules in the sterol-rich phase may be sufficiently great so as to minimize the dependence of lipid–sterol interaction on sterol structure relative to regions where more steric freedom exists.

Our results contrast with those of Tirri et al. (1977), who calculated a reduction of about 5 kcal mol^{-1} in activation energy and −16 cal mol^{-1} deg^{-1} in activation entropy for glycerol and urea permeation into diether-PC bilayers upon incorporation of cholesterol. Although the condensation effect of cholesterol was observed in monolayers of PC analogues lacking the carbonyl group (Fong et al., 1977), it was speculated that hydrogen bonding between the phospholipid carbonyl oxygen atom and the hydrogen of the cholesterol hydroxyl group is important in bilayers (Tirri et al., 1977). Schwarz & Paltauf (1977) reported that the rates of diffusion of Na^+, Cl^-, and glucose across vesicles prepared from a diether-PC were not affected by incorporation of cholesterol to 30 mol % and suggested that the carbonyl oxygens may be required for PC–cholesterol interaction; however, cholesterol reduced the freedom of motion of a fatty acid spin probe to a similar extent in liposomes formed from each PC (Schwarz & Paltauf, 1977). X-ray diffraction patterns for the organization of diether-PC and diester-PC bilayers were similar (Schwarz & Paltauf, 1976), but the apparent partial specific volume and the X-ray long spacing of multilamellar mixtures of diester-PC and cholesterol differed significantly from those of diether-PC and cholesterol.

The lower initial rates of nonelectrolyte permeation we observed in diether-PC liposomes compared with diester-PC liposomes (e.g., Figure 4) indicate that van der Waals interactions between hydrocarbon chains are stronger in diether-PC than in the corresponding diester-PC. This is also reflected in the slightly higher phase transition temperatures of the diethers (Jain et al., unpublished experiments).

The activation energies we report for glycerol penetration of diester-PC and diether-PC bilayers (Table I) are in the middle of the range reported previously for glycerol permeation across diester-PC bilayers [19 kcal mol^{-1} by de Gier et al. (1971); 18 kcal mol^{-1} by McElhaney et al. (1973); 11 kcal mol^{-1} by Cohen (1975); 16 kcal mol^{-1} by Tirri et al. (1977)]. Our finding of no significant change in activation energy upon incorporation of cholesterol to 48 mol % (Tables I, II, and IV) agrees with studies of glycerol permeation in diester-PC liposomes (de Gier et al., 1971; McElhaney et al., 1973; Tirri et al., 1977), although there are reports of small increases in activation energy on addition of cholesterol (Cohen, 1975; Blok et al., 1977). Small, polar solutes are believed to penetrate through bilayer membranes as single, anhydrous species [e.g., Stein (1967), de Gier et al. (1971), and McElhaney et al. (1973)]. The rate-limiting step is thought to involve dehydration of the hydrophilic solute at the interface region; part of the activation process may also involve solute movement from the polar interface toward the hydrophobic phase of the membrane, where conformational isomerizations in the hydrocarbon chains may be considered (Blok et al., 1977). The data we present suggest that the transition-state structure corresponding to the rate-limiting step in liposome swelling from the passage of small, polar solutes across bilayers of phosphatidylcholines bearing diacyl, dialkoxy, and dialkyl chains is not altered significantly upon addition of cholesterol. Permeation of the anhydrous solutes through the hydrophobic interior of the membrane is slower in the presence of cholesterol than in the presence of epicholesterol, where intermolecular packing between neighboring PC and sterol molecules is apparently weaker and the increase in lateral ordering of the paraffin phase is not so high.

Our conclusion that in unsonicated liposomes the ester groups of PC do not represent a primary site for interaction with cholesterol supports the study of de Kruyff et al. (1973), in which the effects of cholesterol on the force–area characteristics of phospholipid monolayers and on the calorimetric properties of liposomes were found to occur without any specific requirement for acyl groups in the phospholipid. It remains to be determined whether in other bilayers systems (e.g., those containing charged lipids, membrane proteins, or low sterol to phospholipid ratios) the phospholipid–sterol interaction may be stabilized by associations involving the acyl carbonyl group.

Acknowledgments

We are grateful to Robert J. Hickey and Dr. Daniel Sverdlik for providing the computer program for use with the plotter.

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