Effects of Sterols on Permeability and Phase Transitions of Bilayers from Phosphatidylcholines Lacking Acyl Groups†

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ABSTRACT: Sonicated vesicles were prepared from synthetic phosphatidylcholines lacking acyl groups. The phospholipids used were diether phosphatidylcholines bearing 1,2-ditetradecyl, 1,2-dihexadecyl, and 1-*cis*-9′-octadecenyl and 2-hexadecyl chains and alkyl analogues such as (2-octadecyleicosyl)phosphorylcholine, which has no diacylglycerol or glycerol diether moiety. At temperatures above the lipid phase transition, cholesterol and ergosterol reduced the efflux rates of $^{86}Rb^+$ mediated by valinomycin, of $^{45}Ca^{2+}$ mediated by A23187, and of the water-soluble fluorescence probe, carboxyfluorescein. Incorporation of epicholesterol at 25 mol % had essentially no effect on the permeability of the vesicles. Since the effects of these sterols on the permeability of vesicles prepared from diether phosphatidylcholines were very similar to those found with vesicles from the corresponding diesters, it was concluded that there is no requirement for the carbonyl oxygens of PC for PC–sterol interaction to occur; thus, from the determination of lipid bilayer permeability behavior, hydrogen bonding of the type cholesterol-OH···O=C-phospholipid is not a primary force stabilizing this interaction at the polar interface of bilayers. Scanning calorimetry measurements indicated that the sizes of the cooperative units of the phospholipids in aqueous dispersions are similar, that diether phosphatidylcholines have slightly higher transition temperatures than those of the corresponding diesters, and that cholesterol and ergosterol diminished the total enthalpy changes of the main order–disorder transition of these phospholipids. The effect of increasing cholesterol concentrations on the thermotropic behavior of (2-octadecyleicosyl)-phosphorylcholine was not altered in its analogues having increased steric bulk in the polar head group region, suggesting that no direct or specific PC polar head group–cholesterol interaction occurs.

Ether-linked glycerophospholipids are natural components of membranes. Diether phospholipids comprise the major component of the polar lipids of extremely halophilic bacteria (Kates, 1972), and alkyl and alk-1-enyl ether-linked phospholipids occur in mammalian membranes of normal (Horrocks, 1972; Pugh et al., 1977) and (at higher levels) of cancer cells (Snyder, 1972; Friedberg & Halpert, 1978). Information concerning the structural differences occurring in membranes as a result of substitution of alkyl ether linkages for the corresponding diester linkages has been gathered by using calorimetric, spectroscopic, surface area, and permeability measurements. Diether phosphatidylcholines have slightly higher gel to liquid-crystalline phase transition temperatures than the corresponding diester phosphatidylcholines, but phosphatidylcholines bearing both an ether and ester chain have slightly lower transition temperatures than the corresponding diester compounds (Chupin et al., 1979; Lee & Fitzgerald, 1980). These transition temperature differences suggest that the absence of the carboxyl groups in the region of the glycerol backbone may result in a larger intermolecular attraction; thus, diether-PC[1] molecules may pack together in the bilayer into a more dense arrangement presumably because diether-PC effectively has an extra methylene residue in the chain.

The carbonyl region of the phospholipid is considered to be an interfacial region between the hydrocarbon chains and the polar head group (Huang, 1976). Since the 3β-hydroxyl group of cholesterol is positioned in the ester bond region of diacyl-PC bilayers (Franks, 1976; Worcester & Franks, 1976; deKruijff, 1978), a hypothetical model of hydrogen bonding between the cholesterol hydroxyl group and one of the carbonyl oxygens of PC seemed plausible to describe the polar interaction between lecithin and cholesterol (Brockerhoff, 1974; Huang, 1977). Experimental support for the involvement of the PC carbonyl oxygen in lecithin–cholesterol interaction has come from NMR (Yeagle et al., 1975; Yeagle & Martin, 1976; Chatterjie & Brockerhoff, 1978) and Raman (Brown & Bicknell-Brown, 1980) observations of cholesterol-induced shifts in the carbonyl bands of diacylphosphatidylcholines. The apparent partial specific volume and X-ray long spacing of diether-PC–cholesterol multilayers differed from those of diester-PC–cholesterol bilayers, although X-ray diffraction patterns of pure diether-PC and diester-PC bilayers were similar (Schwarz et al., 1976). The relationship between lipid structure and membrane properties was also investigated by using permeability measurements. Addition of cholesterol to diether-PC bilayers did not significantly reduce the rates of passive diffusion of Na^+ and Cl^- (Schwarz & Paltauf, 1977), and differences in the activation energies for glycerol and urea

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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-octadecyleicosyl)phosphoryl]choline (see structure in Figure 1); Me-OEPC, [(2-octadecyleicosyl)phosphoryl]methylcholine [i.e., an alkyl phosphorylcholine in which $-CH(CH_3)CH_2N^+(CH_3)_3$ replaces the $-CH_2CH_2N^+(CH_3)_3$ moiety of OEPC]; *N*-Et-*N*,*N*-Me_2-OEPC, 2-hydroxyethyl *N*-ethyl-*N*,*N*-dimethylammonium 2-octadecyleicosyl phosphate (i.e., an analogue of OEPC which has an *N*-ethyl group in place of one *N*-methyl group); CF, carboxyfluorescein; DSC, differential scanning calorimetry; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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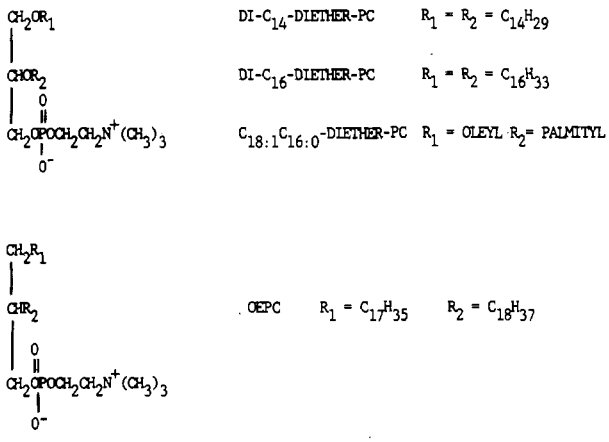

FIGURE 1: Structures of diether phosphatidylcholines and OEPC. The 1-oleyl-2-hexadecyl-3-phosphorylcholine, $C_{18:1}$ $C_{16:0}$ diether-PC, was racemic.

permeation into diether-PC–cholesterol liposomes compared with diester-PC–cholesterol liposomes were interpreted as providing support for participation of the carbonyl oxygen in lecithin–cholesterol interaction (Tirri et al., 1977). However, other nonelectrolyte permeability studies contradicted these findings by showing no requirement for hydrogen bonding as a stabilizing force in liposomes (Clejan et al., 1979). In addition, spin-label studies indicated that the order parameters of diether- and diester-PC bilayers were increased to the same extent by incorporation of cholesterol (Schwarz & Paltauf, 1977). Monolayer studies showed that absence of the carboxylate linkage at the 2 position of PC did not significantly influence the condensing effect of cholesterol (Paltauf et al., 1971; de Kruyff et al., 1973). Since maximum condensation of monolayers occurs from molecular packing that results in maximum cholesterol–acyl chain contact (Müller-Landau & Cadenhead, 1979), the geometric fit between phospholipid and cholesterol in the monolayer may not be influenced by the carbonyl groups of phospholipids. In the present paper we examine thermotropic transition data for aqueous dispersions of synthetic phosphatidylcholines and analogues that lack the ester carbonyl oxygens of lecithin and show that the rates of ionophore-mediated efflux of $^{45}Ca^{2+}$ and $^{86}Rb^{+}$ and of spontaneous diffusion of the water-soluble fluorescence probe, carboxyfluorescein, from unilamellar vesicles are similar in sterol-containing bilayers of diether and diester phosphatidylcholines. Our results do not support the notion that the carbonyl oxygens of PC are *required* for PC–sterol interaction.

Experimental Procedures

Materials

1,2-Diacyl-*sn*-glycero-3-phosphocholines (DMPC, DPPC, and DSPC) and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). *rac*-Glycerol-3-phosphorylcholine 1-*cis*-9′-octadecenyl 2-hexadecyl ether ($C_{18:1}$ $C_{16:0}$ diether-PC) was obtained from Serdary Research Laboratories (London, Ontario). 1,2-Ditetradecyl- and 1,2-dihexadecyl-*sn*-glycero-3-phosphocholines (structures in Figure 1) were prepared and purified according to the method of Chen & Barton (1971). The alkylphosphorylcholine, OEPC (Figure 1), which lacks both the ether oxygen and carbonyl oxygen atoms, was synthesized as described by Deroo et al. (1976). Analogues of OEPC having modified choline moieties were prepared by coupling of the desired quaternary ammonium alcohol tosylate salt with octadecyleicosylphosphoric acid, as described elsewhere (Deroo et al., 1976). All of the phospholipids migrated as single spots on silica gel G plates. Epicholesterol and ergosterol were supplied by Schwarz/Mann (Orangeburg, NY). The sterols were recrystallized twice from absolute ethanol. 6-Carboxyfluorescein was obtained from Eastman Kodak (Rochester, NY) and was recrystallized from aqueous ethanol as described by Blumenthal et al. (1978). Stock solutions of CF were prepared by titration with KOH to a pH of 7.4. Valinomycin and A23187 were obtained from Eli Lilly (Indianapolis, IN), and $^{45}Ca^{2+}$ and $^{86}Rb^{+}$ were purchased from New England Nuclear (Boston, MA).

Methods

Preparation of Phospholipid Suspensions for Scanning Calorimetry. Appropriate aliquots of stock solutions of phospholipid and sterol in chloroform were mixed in a 6 × 50 mm test tube. Chloroform was removed under a stream of nitrogen, and the tubes were dried in a vacuum desiccator for more than 4 h. To the dried film of the lipid was added 32 μL of buffer solution (100 mM KCl and 10 mM Tris, pH 7.8). Typically, the concentration of phospholipid in the mixture was 70 mM, and the proportion of cholesterol was varied from 0 to 50 mol %. The suspension was shaken on a Vortex mixer after a glass bead (3-mm diameter) had been added. The mixture was allowed to swell at 45–60 °C for 10 min, then vortexed again, and allowed to stand for at least 24 h at 2–4 °C. The mixture was shaken again at room temperature before calorimetric scanning.

Preparation of Vesicles Containing Trapped Ca^{2+}, Rb^{+}, or CF. Thin lipid films were prepared in vials from the desired amounts of PC and sterol. For loading of vesicles with Ca^{2+}, lipids were dispersed above the transition temperature in 10 mM imidazole, pH 7.0, containing 135 mM NaCl and 150 mM $CaCl_2$ (with $^{45}Ca^{2+}$). To entrap Rb^{+}, we dispersed the lipids in 10 mM imidazole, pH 7.0, containing 150 mM RbCl (with $^{86}Rb^{+}$). To entrap CF, we made dispersions in 50 mM Tris, pH 7.4, containing 200 mM CF. After the dispersions were shaken with a Vortex mixer, unilamellar vesicles were prepared by sonication for 40 min under nitrogen using a Heat Systems Model W375A sonicator (Plainview, NY) at power level 6 and 50% duty cycle. Sonication of the suspensions containing $^{86}Rb^{+}$ and $^{45}Ca^{2+}$ was conducted in a cup horn through which cold water was circulated. The vesicles were stored at 4 °C for several hours prior to gel filtration. Undispersed lipid and metal released from the probe were removed from the suspensions containing CF by centrifugation for 20 min at 10000 rpm in a SS34 rotor. The suspensions were passed through columns (1.5 × 30 cm) of Sephadex G-50 to remove untrapped Rb^{+}, Ca^{2+}, and CF. For studies of Rb^{+} efflux, elution was with 10 mM imidazole, pH 7.0, containing 150 mM choline chloride. Vesicles were eluted with 10 mM imidazole, pH 7.0, containing 135 mM NaCl for the Ca^{2+} efflux measurements, and with 50 mM Tris, pH 7.4, for assay of CF release. Gel filtration was done at room temperature for all vesicles except those prepared from $C_{18:1}$ $C_{16:0}$ diether-PC, with which the temperature was 4 °C. The final total lipid concentration was ~2 mM, except in vesicles from $C_{18:1}$ $C_{16:0}$ diether-PC and OEPC, in which the concentrations were about 2.4 and 3.0 mM, respectively. Phospholipid and sterol concentrations were determined as described previously (Wun & Bittman, 1977).

Assay of $^{86}Rb^{+}$ and $^{45}Ca^{2+}$ Efflux. Ionophore-mediated efflux was measured as described previously (Wun & Bittman, 1977). Stock solutions of valinomycin in dimethylformamide and A23187 in ethanol were prepared. A 10-μL aliquot of ionophore solution (final concentrations of 3.2 μM valinomycin and 52 nM A23187) or (for blanks) of dimethylformamide or ethanol was added to 1-mL portions of $^{86}Rb^{+}$- or $^{45}Ca^{2+}$-loaded vesicles. Vesicles were placed in Visking (1 cm

diameter) tubing, and the bags were knotted with air bubbles trapped to provide for mixing of the contents. The bags were placed in test tubes (1.7 × 3.5 cm) containing 4 mL of 10 mM imidazole, pH 7.0, containing either 150 mM choline chloride for the valinomycin-mediated Rb^+ efflux studies or 135 mM NaCl for the A23187-mediated Ca^{2+} efflux measurements. The tubes were shaken (giratory shaker, Model G-25, Precision Instruments) at the temperatures indicated under Results. Aliquots (100 μL) of the dialyzates at various times were placed in scintillation vials containing 10 mL of Bray's solution, and the radioactivity was measured in a Beckman scintillation spectrometer. An aliquot of the vesicles was taken after 18 h to determine the amount of ion still trapped in the vesicles. The rate of efflux was corrected for the spontaneous leakage of Rb^+ and Ca^{2+} in the presence of dimethylformamide or ethanol. Under the conditions used, the extent of passive diffusion of these ions was low (Wun & Bittman, 1977). The time course of ionophore-induced ion efflux was measured in triplicates from a given preparation of vesicles, and the percentage of efflux usually varied by <3%.

Fluorescence Assay of CF Release. Fluorescence measurements were recorded in a Hitachi Perkin-Elmer MPF-2A or an Aminco-Bowman spectrofluorometer by using excitation and emission wavelengths of 490 and 520 nm, respectively. Band-passes of 4 nm were used. Efflux of trapped CF was followed by transferring a 50-μL aliquot of vesicles to 2.5 mL of the buffer solution at the desired temperature. Temperatures were controlled by circulating water through a jacketed cuvette holder. The time course of CF efflux from DMPC vesicles did not vary when the osmolarity of the buffer solution was increased by addition of 170 mM NaCl, indicating that an osmolarity gradient did not significantly affect CF leakage into the incubation medium. The total fluorescence intensity was measured after the addition of Triton X-100 (0.2% final concentration), which released all of the trapped CF. The results are reported as the percentage of the total CF in the vesicles released as a function of time. At least two preparations of vesicles were made from each PC. The data presented are the average of at least four measurements. Most experiments agreed within 5%.

Calorimetric Measurements. Sixteen microliters of the aqueous dispersions in sealed aluminum sample pans were run on a Perkin-Elmer DSC 1B calorimeter operating at a sensitivity of 1 mcal/min and a scanning rate of 0.625 or 1.25 K/min. At these two scanning rates identical values of enthalpy, transition temperature, half-height width, and the height of the transition profile were obtained. Each sample was scanned at least 3 times. The instrument was calibrated with myristic acid, benzoic acid, and indium. The transition temperatures at the midpoint and at the base of the peak were measured directly. It was assumed that the base line over the interval of the transition could be well approximated by taking the average of the values of the beginning and the end of transition. The area under the transition profiles was measured by digitizing the trace with an Elograph digitizer attached to a PDP 11/10-G minicomputer. These integrals were used to calculate both the calorimetric (ΔH_{cal}) and van't Hoff (ΔH_{VH}) enthalpies of the transition; the size of the "cooperative unit" undergoing the transition was calculated from the height of the DSC transition by the method outlined by Hinz & Sturtevant (1972).

Results

Differential Scanning Calorimetry. The DSC profiles of the aqueous dispersions of the PC analogues were very similar in shape. As expected, the transition temperature increased by ~15 °C when the length of each chain was increased by two methylene groups (Table I). The transition temperatures of the di-C_{14} and di-C_{16} diether phosphatidylcholines are approximately 2.5 and 1.9 °C higher, respectively, than those of the corresponding diacyl compounds. The transition temperatures of *N*-Et-*N*,*N*-Me_2-OEPC [which has an ethyl group in place of one of the three choline methyl groups, i.e., $-CH_2CH_2N^+(C_2H_5)(CH_3)_2$] and of Me-OEPC [which has a methyl group α to the phosphorus in the choline moiety, i.e., $-CH(CH_3)CH_2N^+(CH_3)_3$] are depressed by ~2 °C compared with OEPC itself, indicating the influence of increased steric bulk in the polar head group. Table I also shows that the size of the cooperative unit was similar in each of the phospholipids investigated. The only exception is DMPC, which has previously been shown to have ~200 PC molecules in the cooperative unit (Hinz & Sturtevant, 1972). The size of the cooperative unit is, however, very sensitive to trace impurities (Albon & Sturtevant, 1978); therefore, this parameter cannot be meaningfully compared for samples prepared from different sources of fatty acids.

Table I: Thermotropic Transition Data for Various Phosphatidylcholine Analogues in Aqueous Dispersions[a]

phospholipid	transition temp (K)	ΔH_{VH}[b] (kcal mol^{-1})	ΔH_{cal} (kcal mol^{-1})	$\bar{n}$[c] (molecules)
DMPC	297.0	1320	6.6	200
DPPC[d]	314.7	774	9.1	85
DSPC	329.0	800	10.6	76
di-C_{14} diether-PC	300.5	500	7.5	66
di-C_{16} diether-PC	316.5	628	8.8	72
di-C_{18} diether-PC	328.6	506	9.7	52
OEPC	320.0	646	8.1	80
N-Et-*N*,*N*-Me_2-OEPC[e]	317.8	700	9.4	74
Me-OEPC[e]	317.9	606	9.3	65

[a] Transition temperatures were determined from the midpoint of transition; the error associated with each value is ±0.4 °C. [b] The van't Hoff enthalpy, ΔH_{VH}, was calculated from $4RT^2$ ($d\alpha/dT$), where $d\alpha/dT$ is the midpoint slope. [c] The size of the cooperative unit, $\bar{n}$, was calculated from the quotient $\Delta H_{VH}/\Delta H_{cal}$. [d] The DPPC used in the calorimetric study was racemic. [e] The structures of *N*-Et-*N*,*N*-Me_2-OEPC and Me-OEPC are shown in Clejan et al. (1979).

Increasing the cholesterol concentration in PC bilayers decreases the energy content of the gel to liquid-crystalline transition (Ladbrooke et al., 1968; de Kruyff et al., 1972). The thermotropic behavior of aqueous dispersions of the di-C_{14}, di-C_{16}, and di-C_{18} diether phosphatidylcholines and of OEPC mixed with increasing amounts of cholesterol or ergosterol (10–50 mol %) showed diminished total enthalpy changes of the main order–disorder transition and a marked asymmetry in the shape of the transition profile (data not shown). The phase transition enthalpy decreased from 8–10 kcal/mol of PC in the absence of sterol to 0 when cholesterol or ergosterol was incorporated at >35 mol %. The transitions of *N*-Et-*N*,*N*-Me_2-OEPC and of Me-OEPC were broadened as a function of sterol content essentially to the same extent as the transition of OEPC.

Efflux of $^{86}Rb^+$. The time course of valinomycin-mediated Rb^+ efflux from vesicles followed first-order kinetics (Figure 2). At temperatures above the phase transition, the rates of Rb^+ release mediated by valinomycin are markedly reduced by incorporation of 50 mol % cholesterol. This is consistent with the reduced motional freedom of the phospholipid paraffin chains by cholesterol incorporation, which diminishes the capacity of valinomycin to catalyze transmembrane Rb^+ movement (de Gier et al., 1970). The half-times of efflux

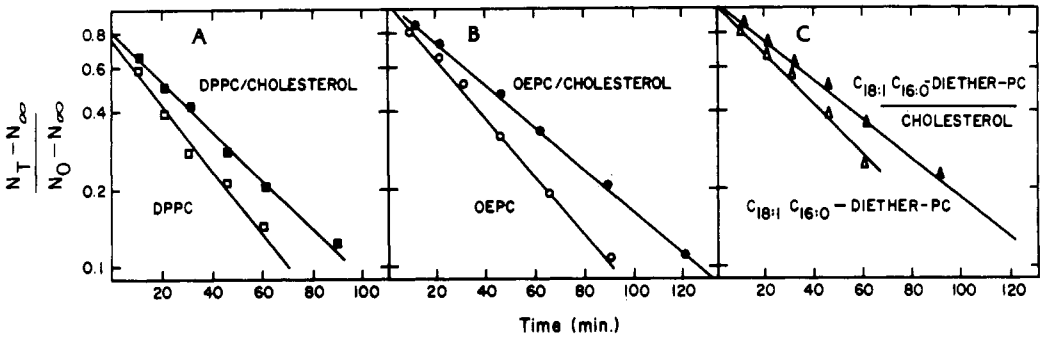

FIGURE 2: First-order plots of efflux kinetics of trapped $^{86}Rb^+$ from vesicles in the presence of valinomycin. Vesicles were formed in the absence or presence of 50 mol % cholesterol from (A) (□) DPPC and (■) DPPC and cholesterol, (B) (○) OEPC and (●) OEPC and cholesterol, and (C) (△) $C_{18:1}$ $C_{16:0}$ diether-PC and (▲) $C_{18:1}$ $C_{16:0}$ diether-PC and cholesterol. The conditions for the efflux measurements are described under Methods. The temperatures were (A) 46 °C, (B) 58 °C, and (C) 25 °C. N_0, N_t, and N_∞ are the radioactivities (cpm) trapped in the vesicles at time zero (time of addition of valinomycin), at time t, and at equilibrium, respectively. The value of N_∞ was determined from double-reciprocal plots of N_t^{-1} vs. time^{-1} at times approaching equilibrium.

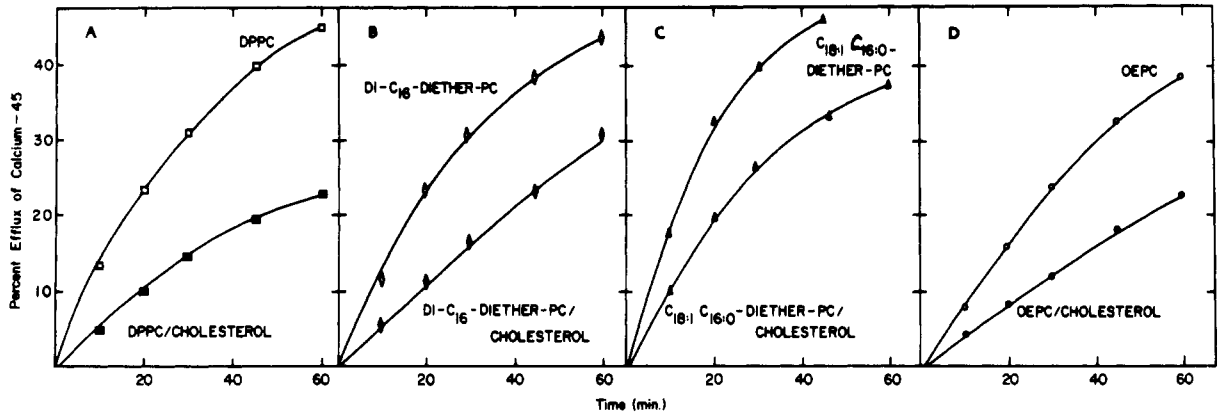

FIGURE 3: Time course of A23187-mediated efflux of $^{45}Ca^{2+}$ from vesicles. Vesicles were prepared in the absence or presence of 50 mol % cholesterol from (A) (□) DPPC and (■) DPPC and cholesterol, (B) (◊) di-C_{16} diether-PC and (♦) di-C_{16} diether-PC and cholesterol, (C) (△) $C_{18:1}$ $C_{16:0}$ diether-PC and (▲) $C_{18:1}$ $C_{16:0}$ diether-PC and cholesterol, and (D) (○) OEPC and (●) OEPC and cholesterol. The temperatures at which the measurements were made were (A and B) 45 °C, (C) 26 °C, and (D) 58 °C. The amount of Ca^{2+} trapped in the vesicles was 0.30–0.35 mmol/mol of lipid.

obtained from Figure 2 are as follows (the temperatures at which the efflux kinetics were measured are shown in parentheses): DPPC, 14 min, and DPPC–cholesterol, 21 min (42 °C); OEPC, 28.5 min, and OEPC–cholesterol, 40 min (58 °C); $C_{18:1}$ $C_{16:0}$ diether-PC, 32.8 min, and $C_{18:1}$ $C_{16:0}$ diether-PC–cholesterol, 43 min (24 °C). Thus, cholesterol incorporation reduced the rates of Rb^+ movement from vesicles of a diester-PC, a diether-PC bearing one unsaturated chain, and an alkyl analogue of PC having neither a diacylglycerol nor a glycerol diether moiety.

The effects of cholesterol on the first-order kinetics for $^{86}Rb^+$ efflux from diester-PC, diether-PC, and OEPC vesicles are summarized in Table II. In vesicles from DPPC and di-C_{16} diether-PC, cholesterol reduced the rate constant to the same extent. Although a quantitative difference exists between the effects of cholesterol on valinomycin-mediated Rb^+ efflux from DMPC and di-C_{14} diether-PC vesicles, it is obvious that the rate of $^{86}Rb^+$ release from each type of vesicle was decreased markedly by cholesterol. The experiments presented in Figure 2 and Table II do not agree with the results of Schwarz & Paltauf (1977), who found that cholesterol did not affect the rate of passive $^{22}Na^+$ diffusion from vesicles of a diunsaturated diether-PC at 4 °C.

Efflux of $^{45}Ca^{2+}$. As with valinomycin-induced $^{86}Rb^+$ release, cholesterol suppressed the rate and extent of A23187-mediated $^{45}Ca^{2+}$ efflux from vesicles at temperatures above the lipid phase transition (Figure 3). Although there are quantitative differences between DPPC and di-C_{16} diether-PC vesicles at longer times of dialysis (panels A and B), inhibition of Ca^{2+} efflux from diether-PC and OEPC vesicles (panels B, C, and D) shows that acyl linkages are not required in PC for cholesterol to modify the permeability properties of bilayers. Ergosterol (incorporated at 33 mol %) suppressed A23187-mediated $^{45}Ca^{2+}$ efflux at 32 °C from di-C_{14} diether-PC

Table II: Effect of Cholesterol on the Rate Constant of Valinomycin-Induced $^{86}Rb^+$ Efflux from Vesicles from Diester, Diether, and Alkyl Analogues of Phosphatidylcholines[a]

	$10^2 \times k$ (min^{-1})	temp (°C)	% decrease by cholesterol
DPPC	2.88	42	
DPPC–cholesterol (1:1)	2.19	42	24
di-C_{16} diether-PC	3.20	42	
di-C_{16} diether-PC–cholesterol (1:1)	2.48	42	23
DMPC	3.29	30	
DMPC–cholesterol (1:1)	2.04	30	38
di-C_{14} diether-PC	3.34	30	
di-C_{14} diether-PC–cholesterol (1:1)	2.56	30	23
$C_{18:1}$ $C_{16:0}$ diether-PC	2.19	24	
$C_{18:1}$ $C_{16:0}$ diether-PC–cholesterol (1:1)	1.75	24	20
OEPC	2.62	58	
OEPC–cholesterol (1:1)	1.87	58	29

[a] The first-order rate constants were calculated from plots of log $[(N_t - N_\infty)/(N_0 - N_\infty)]$ vs. time.

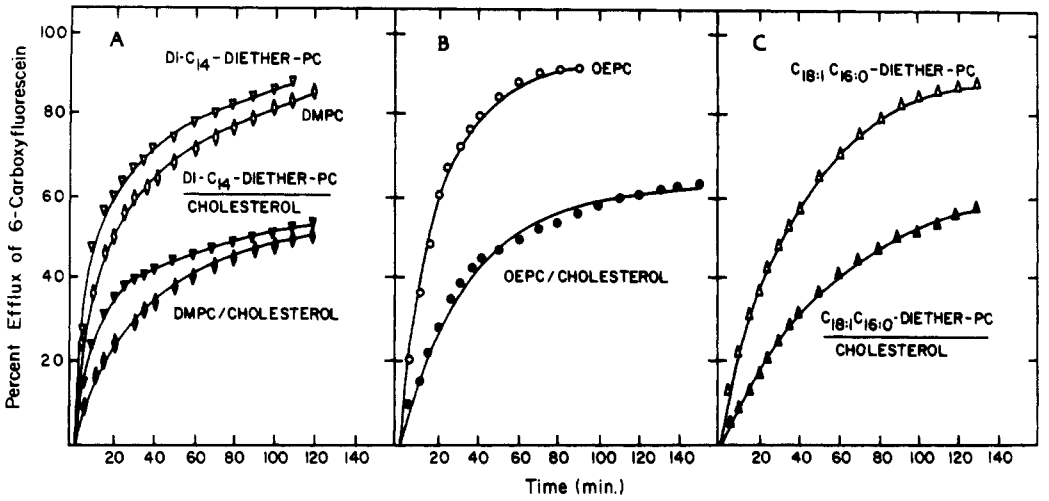

FIGURE 4: Effect of cholesterol on percent of CF released from diester-PC, diether-PC, and OEPC vesicles as a function of time. Vesicles were prepared in the absence and presence of 25 mol % cholesterol from (A) (▽) di-C_{14} diether-PC, (▼) di-C_{14} diether-PC and cholesterol, (◇) DMPC, and (◆) DMPC and cholesterol, (B) (○) OEPC and (●) OEPC and cholesterol, and (C) (△) $C_{18:1}$ $C_{16:0}$ diether-PC and (▲) $C_{18:1}$ $C_{16:0}$ diether-PC and cholesterol. The temperatures were (A) 35 °C, (B) 56 °C, and (C) 26 °C. The volumes of CF trapped per total lipid concentration, which were calculated by assuming an extinction coefficient at 490 nm of 6.1×10^4 M^{-1} cm^{-1} for CF in aqueous solution, were as follows: DMPC, 0.39 L/mol; DMPC–cholesterol, 0.50 L/mol; di-C_{14} diether-PC, 0.34 L/mol; di-C_{14} diether-PC–cholesterol, 0.40 L/mol; OEPC, 0.43 L/mol; OEPC–cholesterol, 0.40 L/mol; $C_{18:1}$ $C_{16:0}$ diether-PC, 0.13 L/mol; $C_{18:1}$ $C_{16:0}$ diether-PC–cholesterol, 0.11 L/mol.

vesicles to an extent similar to that of DMPC vesicles. In contrast, epicholesterol (at 25 mol %) had virtually no effect on the Ca^{2+} efflux kinetics from DMPC or di-C_{14} diether-PC vesicles (data not shown). This result is consistent with numerous other membrane permeability studies which have shown that a 3β-hydroxyl group is essential for PC-sterol interaction [e.g., Jain (1975) and Demel & de Kruyff (1976)].

Efflux of CF. The rate of increase of the fluorescence intensity of CF is a direct measure of CF efflux from vesicles, since vesicles containing 200 mM CF are self-quenched (R. Cohen, Y. Barenholz, and T. E. Thompson, unpublished experiments). Figure 4 shows that cholesterol (at 25 mol %) decreased the rate and extent of efflux of this water-soluble fluorescence probe. The reduction in CF permeability produced by cholesterol incorporation occurred independently of the presence of acyl linkages in PC. To further study the effects of sterols on CF efflux, we prepared vesicles from DMPC and epicholesterol, ergosterol, or varying concentrations of cholesterol. Epicholesterol (incorporated at 25 mol %) produced no significant alteration in CF permeability (Figure 4A vs. Figure 5). The inhibition by ergosterol (incorporated at 25 mol %) was very similar to that produced by the same concentration of cholesterol (Figure 5). Incorporation of 16.7, 25, and 50 mol % cholesterol caused increasing inhibition of CF efflux.

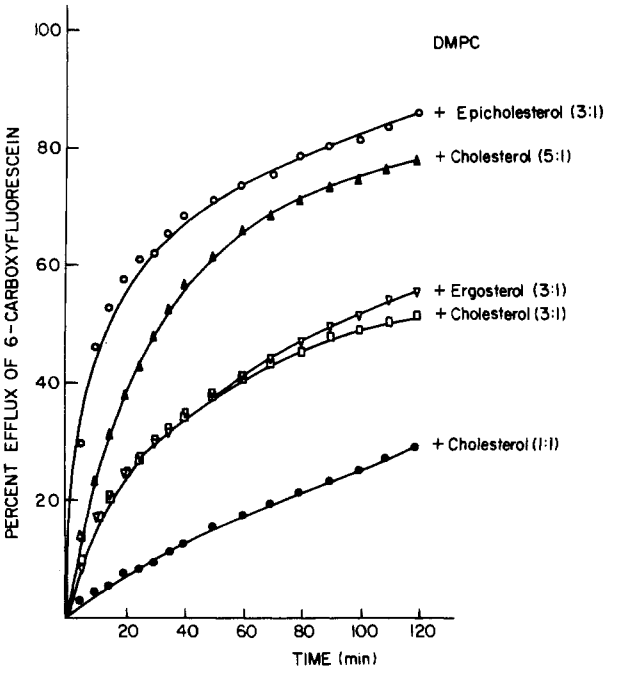

FIGURE 5: Effects of epicholesterol, cholesterol, and ergosterol on time course of CF efflux from DMPC vesicles. Epicholesterol (○) and ergosterol (▽) were incorporated at 25 mol %. The concentrations of cholesterol were (▲) 16.7, (□) 25, and (●) 50 mol %. The temperature was 35 °C.

Discussion

It is well documented that cholesterol reduces membrane permeability to cations, anions, and neutral solutes (Demel et al., 1972; de Kruyff et al., 1972; Papahadjopoulos et al., 1973; Bittman & Blau, 1972). We have concluded that the effects of cholesterol or ergosterol in PC bilayers occur without any specific requirement for acyl groups in the phospholipid. This conclusion arises from our observations that cholesterol and ergosterol reduced the rates of efflux of three water-soluble entrapped components from unilamellar vesicles prepared from phosphatidylcholines that lack the carbonyl group. The permeability measurements presented in this paper obtained by using sonicated vesicles provide confirmation of the conclusions based on studies of glycerol, urea, acetamide, and glucose permeability in liposomes (Clejan et al., 1979). The results conflict with the permeability studies of Schwarz & Paltauf (1977) and the conclusion of Tirri et al. (1977), whose investigations with diether-PC bilayers were taken to demonstrate that the carbonyl oxygens were necessary for lecithin–cholesterol interaction.

Aqueous dispersions of di-C_{14} and di-C_{16} diether phosphatidylcholines exhibited slightly higher phase transition temperatures than those of DMPC and DPPC (Table I), confirming previous studies in which phospholipids containing two ether linkages to glycerol had transitions at slightly higher temperatures than those of their diester analogues (Abramson, 1970; Chen & Barton, 1971; Vaughan & Keough, 1974; Chupin et al., 1979; Lee & Fitzgerald, 1980). The intermolecular attraction among diether-PC molecules may therefore

be somewhat greater than that among the analogous diesters. However, the results in Table I show that the number of PC molecules per cooperative unit is very similar; moreover, monolayer (Paltauf et al., 1971) and X-ray diffraction studies (Schwarz et al., 1976) showed that the organization of diether-PC and diester-PC bilayer membranes was similar. The permeability measurements reported here at temperatures above the lipid phase transition show patterns of ionophore-mediated $^{86}Rb^+$ and $^{45}Ca^{2+}$ efflux and spontaneous diffusion of CF to be similar. Thus, the bilayer structures are not likely to be markedly different.

The DSC studies also indicate that no specific or direct PC polar head group–cholesterol interaction occurs. Increasing the steric bulk of the polar head group region of OEPC (in *N*-Et-*N*,*N*-Me_2-OEPC and Me-OEPC) did not alter the thermotropic behavior as a function of sterol concentration compared with OEPC. These measurements agree with our recent studies of nonelectrolyte permeability in multilayered liposomes from OEPC analogues having modified choline moieties (Clejan et al., 1979). They also support NMR studies showing that the motion and orientation of several nuclei in the polar head group of PC are largely unaffected by incorporation of cholesterol [see references cited in Clejan et al. (1979)].

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