Evidence for Transbilayer, Tail-to-Tail Cholesterol Dimers in Dipalmitoylglycerophosphocholine Liposomes

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Received June 20, 1994; Revised Manuscript Received January 10, 1995[⊗]

ABSTRACT: The behavior of multilamellar liposomes of 2,3-dipalmitoyl-*sn*-glycero-1-phosphocholine (DPPC) was studied by differential scanning calorimetry (DSC) in the presence of ≤5 mol % of the amphiphilic solutes methyl oleate, cholesterol, pregnenolone, and dehydroandrosterone. The DSC thermograms indicate that the solutes are miscible only with the liquid-disordered (l_d) phase, and not with the solid-ordered (s_o) phase. The slopes of the T_m vs solute concentration curves confirm this conclusion: It appears that the s_o-l_d phase transition of DPPC, which corresponds to the melting of the phospholipid chains, can be treated as a simple melting process and, thus, could be used as a cryoscopic system. In that case, its melting point depression constant, K_f, can be calculated *a priori* from the experimentally measured heat of fusion per gram of DPPC, l_f, and the temperature of the phase transition of pure DPPC, T_o, by the equation $K_f = RT_o^2/(1000l_f) = 12.3 \pm 0.9$ K g M^{-1} cm^3. With methyl oleate as the solute, the T_m vs methyl oleate concentration plot is linear, and from the slope we calculate $K_f = 12.9 \pm 0.8$ K g M^{-1} cm^3. Thus, methyl oleate appears to form an ideal cryoscopic system with dipalmitoyllecithin liposomes: It is fully miscible with the l_d phase but is apparently insoluble in the s_o phase. Pregnenolone and dehydroandrosterone also form ideal cryoscopic systems with dipalmitoyllecithin liposomes: The T_m vs solute concentration plots are linear and yield the correct MWs for these solutes. In contrast, the T_m vs concentration plot of cholesterol is curved; the slope below 2 mol % solute corresponds to the monomeric MW, but above 3 mol % it approaches that of a dimer. The full curve is consistent with a simple equilibrium dimerization of the solute in the l_d phase. Thus, cholesterol appears to be insoluble in the s_o phase, and it readily forms a dimer in the l_d phase. Space-filling considerations indicate that the dimer is a transbilayer one. Such a dimer of cholesterol would be consistent with a variety of observations reviewed by Sankaram and Thompson [Sankaram, M. B., & Thompson, T. E. (1990) *Biochemistry 29,* 10676−10684]. Since cholesterol is insoluble in the s_o phase, it must form below T_m a separate phase or at least separate domains. The domains, however, should not be too small since the relatively large boundary region of the microdomains would affect the thermodynamic stability of the solid phase.

Cholesterol induces large changes in the physical properties of membranes, especially in the structure of the phospholipid bilayer, and at extreme concentrations it may even lead to phase separation within the lipid compartment. Cholesterol-induced changes are also manifest during the gel−liquid crystal phase transitions in artificial phospholipid bilayer membranes, such as dipalmitoylphosphatidylcholine (DPPC)[1] multibilayer liposomes. These events have been probed by a variety of techniques including NMR (Vist & Davis, 1990; Ipsen *et al.*, 1987; Presti & Chan, 1982), fluorescence polarization (Recktenwald & McConnell, 1981; Alecio *et al.*, 1982; Smutzer & Yeagle, 1985), and differential scanning calorimetry (DSC) (Van Ginkel *et al.*, 1986; Ladbrooke *et al.*, 1968; Mabrey *et al.*, 1978; Melchior *et al.*, 1980). These studies ultimately yielded a rather complex phase diagram resulting from the interactions of cholesterol with organized phospholipids at the molecular level. In brief, MLVs of DPPC undergo a sharp phase transition around 41 °C; the two phases involved in the transition have been variously called the solid-ordered (s_o), or gel (G), phase on the one hand and the liquid-disordered (l_d), or fluid, or liquid crystalline, or L_α, phase on the other. Cholesterol progressively lowers the temperature of this phase transition (Sankaram & Thompson, 1990, 1991), and it is assumed that up to about 10 mol % cholesterol is fully miscible with both the s_o and the l_d phase. When the composition of the bilayer reaches a critical mole fraction of cholesterol, a new phase appears; this is the liquid-ordered (l_o), or β, phase, which consists of DPPC containing a constant, high concentration of cholesterol. The cholesterol-poor and cholesterol-rich phases are in equilibrium with each other until the cholesterol concentration of the mixture equals that of the liquid-ordered phase, approximately 30 mol %. Within this cholesterol concentration range, therefore, there is no change in the transition temperature. At higher cholesterol concentrations no phase separation is observed; only an enrichment of the fluid phase in solubilized cholesterol is seen.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1995.
[1] Abbreviations: DPPC, dipalmitoylglycerophosphocholine; pregnenolone, 3β-hydroxy-5-pregnen-20-one; dehydroandrosterone, 3β-hydroxy-5-androsten-17-one; MLVs, multibilayer liposomes; DSC, differential scanning calorimetry; l_d, liquid-disordered phase; l_o, liquid-ordered phase; s_o, solid-ordered phase; T_m, melting temperature; NMR, nuclear magnetic resonance.

0006-2960/95/0434-3851$09.00/0

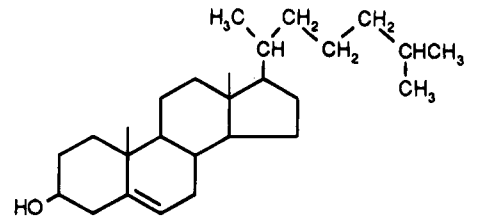
It had been assumed that the transition between the s_o and l_d phases is described by the *solidus* and *liquidus* lines representing the continuously changing composition of both phases during the transition, but the existence of these two lines is rather poorly documented. In the course of using the DPPC−cholesterol system for calibrating our differential scanning calorimeter, we observed a sharpening of the phase transition by cholesterol at low concentrations instead of the broadening expected if the chain melting were accompanied by continuously changing compositions of the two phases. This then prompted us to investigate in some detail the behavior of the DPPC−cholesterol system at low cholesterol concentrations. DPPC was used in all our experiments, because the range of transition temperatures of DPPC bilayers is ideal from the experimental point of view, even though the transition temperatures of DPPC bilayers are much higher than those of cell membrane bilayers. Nevertheless, the results obtained with DPPC are directly relevant to cell membranes, since "PC:cholesterol membrane phase diagrams have a universal form which is relatively independent of the precise chemical structure of the PC molecule" (Thewalt & Bloom, 1992).

In the present report, we propose to show by the use of methyl oleate as the reference solute that DPPC liposomes behave as an ideal cryoscopic solvent system for a variety of steroids. More importantly, the cryoscopic system indicates that cholesterol forms monodisperse solutions only at very low concentrations, and at concentrations higher than 2 mol % it forms a dimeric structure within the bilayer. We propose that cholesterol forms a tail-to-tail dimer when dissolved in the l_d phase. Our results are consistent with observations and conclusions made previously by Sankaram and Thompson (Sankaram & Thompson, 1990, 1991) who postulated that monomers of cholesterol span the nonpolar core of the two leaflets of the bilayer.

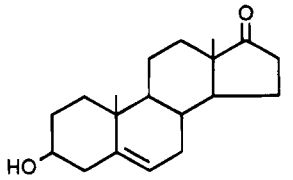
MATERIALS AND METHODS

Chemicals. 2,3-Dipalmitoyl-*sn*-glycero-1-phosphocholine, grade ≈ 99%, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, grade ≈ 99%, and cholesterol, grade 99+%, were obtained from Sigma, and methyl oleate was a product of NuChek Prep, Inc.; these compounds were used without further purification. 3β-Hydroxy-5-pregnen-20-one (pregnenolone, or U-2865) and 3β-hydroxy-5-androsten-17-one (dehydroandrosterone, or U-4614) were obtained from the Biological Screening Office at the Upjohn Company. The structures of these compounds are shown in Figure 1. Other chemicals were reagent grade or better. Water was purified with a Corning Mega-Pure all-glass distillation apparatus; for HPLC, we used water from Burdick and Jackson.

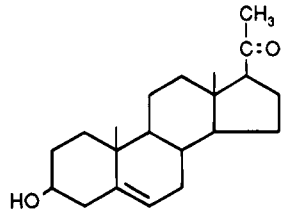
Calorimetry. Lipid/sterol liposomes were made by first dissolving in chloroform the components in 2-mL volumetric tubes that had been washed, rinsed with nitric acid, and dried at 110 °C. The chloroform was removed under a stream of nitrogen, and the samples were vacuum-dried for 1−3 h to remove all traces of solvent. The dried films were stored at −20 °C. In all our experiments we used a 10 mM Tris-HCl buffer containing 150 mM NaCl, pH 7.40. Before use, a sample containing 0.36 mg of lipid was warmed to room temperature; then 2 mL of buffer, preheated to 50−55 °C, was added, and the mixture was vigorously vortexed for 1 min. The tube was sealed, placed in a heating block at 50−55 °C, and vortexed for 1 min every 15 min. Approximately



Cholesterol



Dehydroisandrosterone



Pregnenolone

FIGURE 1: Structures of sterols used in this study.

45 min after the buffer had been added, the liposome sample was transferred to the sample cell of the calorimeter, where it was equilibrated at 25 °C for 5−15 min.

The instrument used for our measurements was a Microcal MC-2 scanning calorimeter interfaced to an IBM-PC. Instrument operation and data analysis were performed using the software program DA-2, provided by the manufacturer. The instrument settings were the same in all experiments reported: filtering constant = 10 s; scan rate = 30 deg/h; sensitivity = 1×. The direct experimental results, in mcal/min versus temperature, were transformed to mcal/deg versus temperature and normalized to the number of moles of lipid (phospholipid + cholesterol) in the cell, in order to obtain data in the form of mcal deg^{-1} $mmol^{-1}$ versus temperature.

Data Analysis. Differential scanning calorimetry, DSC, measures ΔC_p, the apparent specific heat capacity of the solute plus solvent minus that of the solvent, as a function of temperature. For a phase transition the area under the ΔC_p versus temperature peak measures the experimental molar transition enthalpy ($\Delta H°$):

$$\Delta H° = M\int_{T_1}^{T_2}\Delta C_p\,dT \qquad (1)$$

where M is the molar mass in units of g/mol. The experimental $\Delta H°$ measures directly the enthalpy change associated with the process whether or not it is a thermodynamic equilibrium and whether or not it is reversible. If the process is an ideal, first-order equilibrium, then the symmetry of the curve allows one to calculate from the area, the height of the peak, and the T_m (Privalov & Potekhin, 1986) the true thermodynamic enthalpy change, ΔH^*, called the van't Hoff enthalpy, corresponding to that equilibrium (Freire & Biltonen, 1978).

The phase transition from liquid to solid phase is most often a cooperative process resulting from the simultaneous

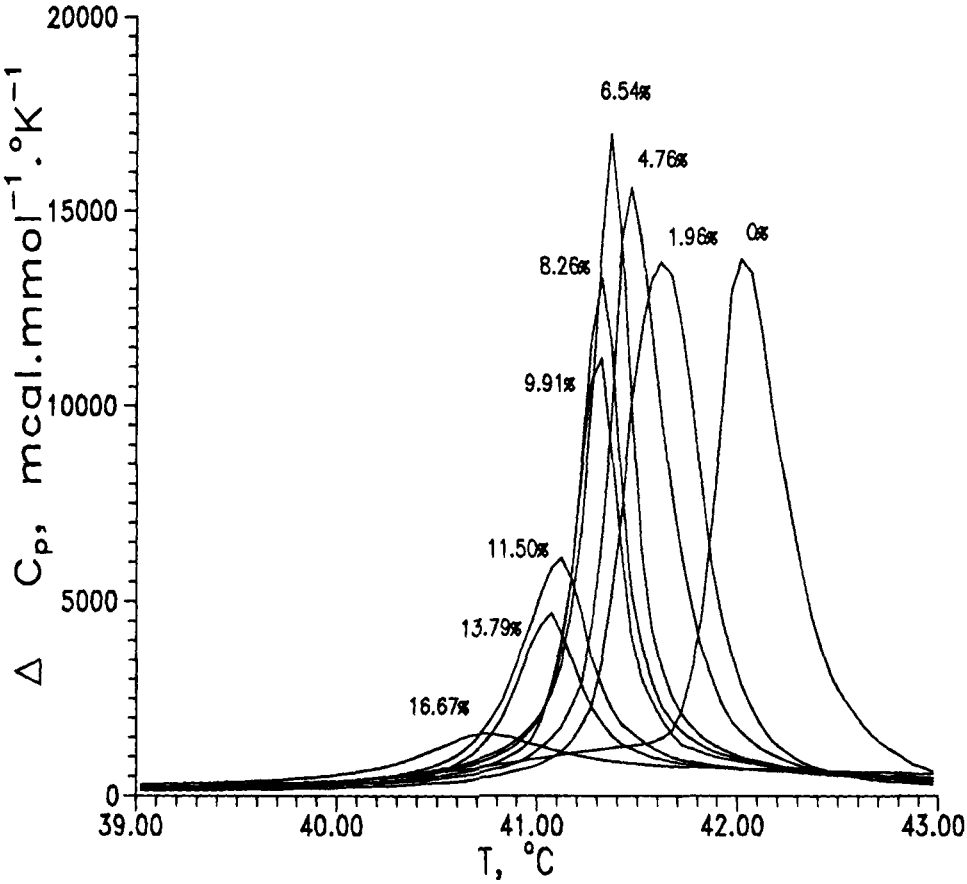


FIGURE 2: Representative DSC scans of DPPC MLVs in the presence of the indicated mol % of cholesterol. DSC scans of DPPC–cholesterol MLVs were recorded as described in Methods.

formation of noncovalent bonds between n molecules. The value of n, the number of molecules in the "cooperative unit", can be calculated from the ratio $\Delta H^*/\Delta H^\circ$, or, in terms of experimental variables, from the equation (Mountcastle *et al.* 1978)

$$n = 4RT_m^2 \frac{\Delta C_p \max}{\Delta H^{\circ 2}} \quad (2)$$

RESULTS

DPPC MLVs were chosen as our model membrane because this system is well characterized from the point of view of its phases and miscibility with cholesterol. The lipid bilayers in MLVs are planar for all practical purposes, and therefore the phase transition is sharp. The 2,3-dipalmitoyl-*sn*-glycero-1-phosphocholine was chosen for most of our experiments because of its resistance to possible trace contaminant phospholipases and/or lipases. The behavior of the above isomer toward cholesterol should be experimentally indistinguishable from that of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Guyer & Bloch, 1983; Davis *et al.*, 1981). Our preliminary experiments confirmed this conclusion (data not shown).

We first validated our experimental technique and established the limits of its accuracy by remeasuring the DSC behavior of DPPC–cholesterol mixed multilamellar vesicles (DPPC MLVs). Figure 2 shows representative scans at various concentrations of cholesterol. We found all of our measurements to be in reasonable agreement with earlier reports (Vist & Davis, 1990). The transition in our experiments is comparable to that reported previously; for example, in Figure 2 the width at half-height of the transition of pure DPPC is about 0.4 °C as compared to about 0.3 °C (Mabrey *et al.*, 1978) and about 0.5 °C (Estep *et al.*, 1978). The T_m in our experiments is slightly higher than in previous reports, about 42.1 vs 41.4 (Mabrey *et al.*, 1978) and 41.3 °C (Estep *et al.*, 1978). Since all of our analyses depend only on relative changes in T_m, we did not investigate this discrepancy further. From our data we constructed the phase diagram in Figure 3; the major features of this graph are also in full agreement with earlier observations. Since increases in cholesterol content between 6.5 and 20 mol % alter only the relative amounts of the s_o and l_d phases, the only result in DSC is the progressive decrease in peak size, without any change in T_m. Therefore, the discontinuity at 20 mol % manifests itself only as the vanishing of the transition peak, a rather elusive phenomenon. The discontinuities may be defined more accurately from a plot of the van't Hoff enthalpies, ΔH^*, as a function of the mole percent cholesterol. As shown in Figure 4, this plot yields two straight lines, whose intersection defines the lower boundary of the l_o phase. The intersection of the straight line from 10 to 20 mol % cholesterol with the abscissa yields the upper boundary of the s_o phase. Such an extrapolation is valid since the enthalpy should be proportional to the quantity of the s_o phase, which in turn is linearly related to the mol percent cholesterol. Finally, the enthalpies in the region of 0–6.5 mol % cholesterol appear to increase linearly with increasing cholesterol concentrations.

An increase in the cooperative unit, n—the number of molecules that form or break noncovalent bonds in concert during a phase transition—reflects stabilization of the bilayer, whereas a decrease indicates destabilization. A decrease in

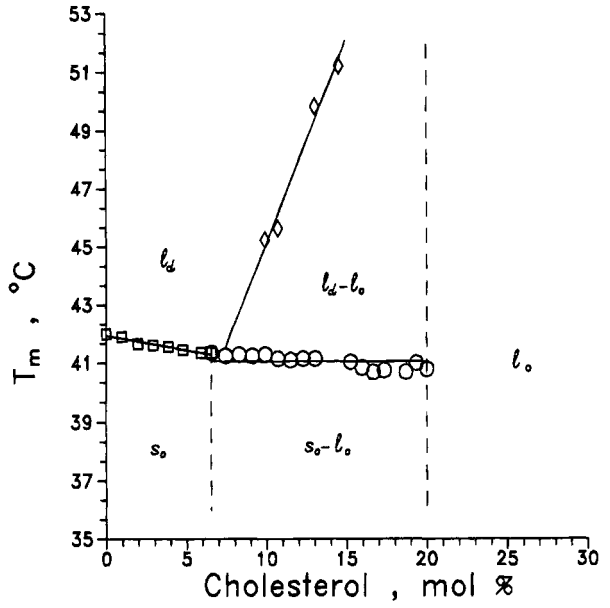


FIGURE 3: Phase Diagram of DPPC−cholesterol mixed MLVs as derived from DSC. The phase diagram for MLVs of cholesterol and DPPC was constructed from the T_m vs cholesterol data analyzed as described in Materials and Methods.

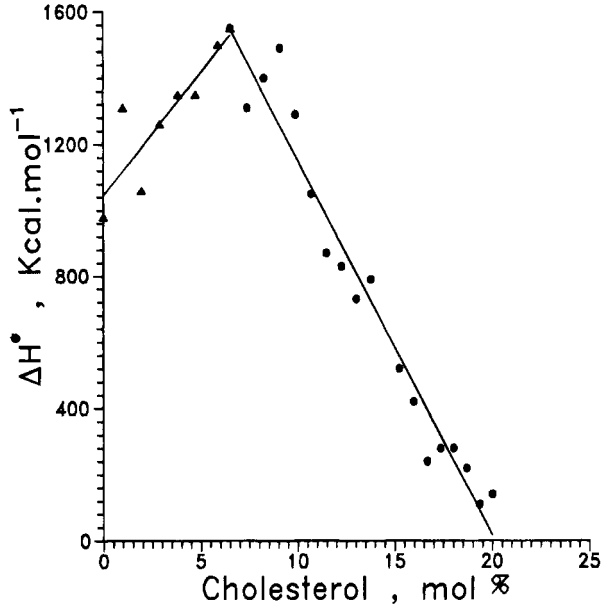


FIGURE 4: van't Hoff enthalpies of DPPC−cholesterol mixed MLVs. van't Hoff enthalpies of the s_o−l_d reversible phase transition of DPPC−cholesterol mixtures were calculated as described (Privalov & Potekhin, 1986). The solid lines represent the individual linear regression fits to the ascending limb (experimental points used in these calculations are indicated by ▲) and the descending limb (experimental points used in these calculations are indicated by ●).

the size of the cooperative unit can be recognized from the broadening of the transition peak. Addition of cholesterol to DPPC MLVs, as shown in Figure 5, first causes an increase in the size of the cooperative unit until the liposomes consist of approximately 5−10 mol % cholesterol. From that point on, any further increase in cholesterol concentration causes a decrease in the cooperative unit. The composition at the discontinuity between the two domains corresponds directly to that observed with the enthalpies of transition, and we feel that these two plots (Figures 4 and 5) yield an experimentally more readily accessible definition of the s_o−

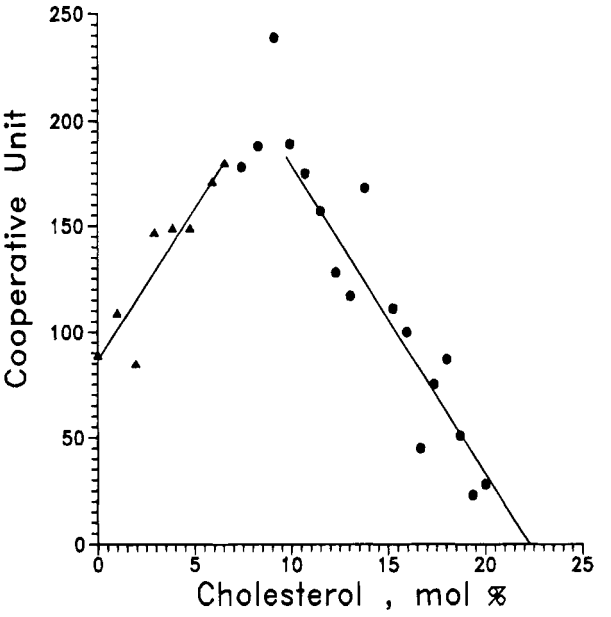


FIGURE 5: Cooperative unit of DPPC−cholesterol mixed MLVs. The cooperative unit for the s_o−l_d reversible phase transition of DPPC−cholesterol mixtures at each cholesterol concentration was calculated by using eq 3. The solid lines represent the individual linear regression fits to the ascending limb (experimental points used in these calculations are indicated by ▲) and the descending limb (experimental points used in these calculations are indicated by ●).

l_o boundary than the plot of T_m versus cholesterol (Figure 2).

Cholesterol−DPPC interactions were further explored by analyzing the lowering of the transition temperature at low solute concentrations. Although it is generally accepted that most lipophilic solutes, especially cholesterol, are soluble, *i.e.*, miscible at the molecular level, in the solid phase of DPPC bilayers, this conclusion is based, in fact, on tenuous experimental evidence (Vist & Davis, 1990). Since in two-dimensional mixtures immiscibility can create not only separate surface phases but also microdomains (Gaines, 1966), apparent solubility may just reflect this type of microdomain immiscibility. To relieve this ambiguity, we investigated the behavior of the cholesterol−DPPC system at low solute concentrations. For an ideal cryoscopic system, i.e., one in which the solute is only soluble in the liquid but not in the solid phase of the solvent, the melting point depression is a purely colligative property. If the cholesterol−DPPC liposomes constitute, in fact, such a system, then the molal depression constant, K_f, can be calculated from the heat of fusion per gram of DPPC, l_f, and the phase transition temperature, the "melting point", of pure DPPC, $T_{m,o}$, according to the equation (Glasstone, 1946; Klotz & Rosenberg, 1950)

$$K_f = \frac{RT^2_{m,o}}{1000 l_f} \quad (3)$$

For the heat of phase transition of DPPC liposomes in our system, integration of the peak yielded 11.7 ± 0.9 kcal/mol, i.e., 16.0 ± 1.2 cal/g. These results are somewhat at variance with those of earlier reports which center around 8.5−9 kcal/mol. The reason for this discrepancy is not immediately apparent; it may be due to different experimental conditions, to the method of integration, or perhaps to the absence of any broad transitions associated with the sharp transitions

Table 1: Molecular Weights (M_r) of Solutes by Freezing Point Depression

compd	concn range (mol %)	app M_r	M_r	aggregation state
cholesterol	3–7	715 ± 119	386.6	dimer
dehydroandrosterone	0–2	355 ± 85	288.4	monomer
	0–10	266 ± 38	288.4	monomer
pregnenolone	0–2	257 ± 38	316.5	monomer
	0–10	305 ± 31	316.5	monomer

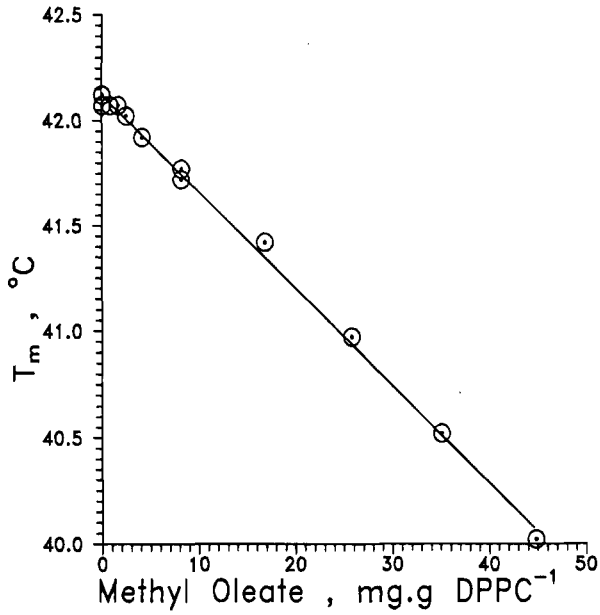


FIGURE 6: Effect of methyl oleate on the T_m of DPPC MLVs. Phase transition temperatures of methyl oleate–DPPC mixtures were determined as described in Materials and Methods. From eq 4 we calculate a cryoscopic constant of 12.9 ± 0.9 K g m^3 for DPPC. The solid line represents the linear regression fit to the experimental points.

in our scans. Using our experimental l_f and eq 3, we calculate, for DPPC, K_f = 12.3 ± 0.9 K g cm^3.

In an ideal cryoscopic system, K_f can be used to calculate the molecular weight of the solute, MW, from the melting point depression of the solvent with the equation

$$T_{m,o} - T_m = \frac{1000\,K_f}{MW} wf \qquad (4)$$

where T_m is the melting temperature in the presence of the solute and wf is the weight fraction of the solute. In order to determine whether DPPC is a cryoscopic solvent for lipid solutes, we first used methyl oleate as a representative solute. This choice was based on the fact that the single chain of this molecule should be fully solvated by DPPC. Also, the length and polarity of this molecule are quite comparable to those of DPPC, and if the s_o phase of DPPC is able to form a true solution with any lipid, then it certainly should do so with methyl oleate. The thermograms of methyl oleate–DPPC did not show any change in peak shape that would indicate compositional changes during phase transition (data not shown), and as shown in Figure 6, the lowering of the phase transition temperature of DPPC liposomes by methyl oleate does yield a straight line. From the slope of this line, we calculate an experimental K_f value of 12.9 ± 0.8 K g cm^3. Thus, DPPC appears to be a cryoscopic solvent for methyl oleate: The latter is fully miscible with the l_d phase of the phospholipid but appears to be insoluble in the s_o phase.

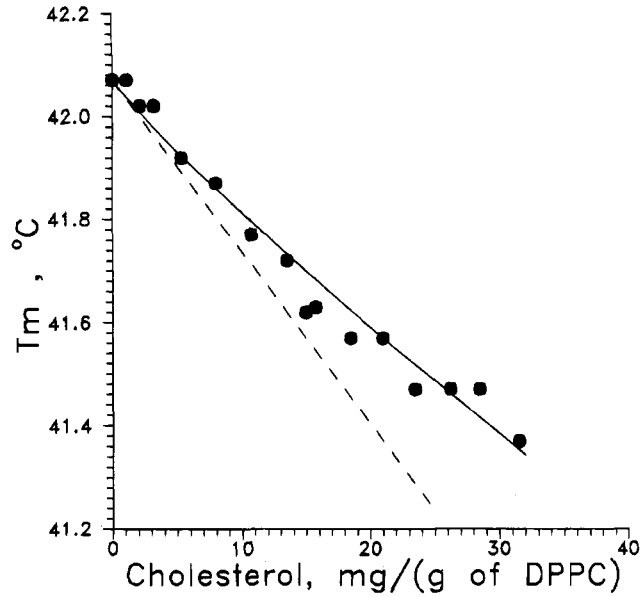


FIGURE 7: Experimental T_m vs solute curve of cholesterol/DPPC MLVs and the theoretical curve calculated from eq 7. The slopes of the T_m vs cholesterol concentration curve were first analyzed by a nonlinear least squares method using eq 7; the analysis yielded the parameters K_f = 12.2 K g m^3 and K_d = 22.5 mg of cholesterol/g of DPPC (~4.4 mol %). These parameters were then substituted into eq 7, and second-order Runge–Kutta integration was performed to generate the theoretical curve shown as the solid line. The dashed line is the theoretical curve for a purely monomeric cholesterol solute; the theoretical curve for a dimeric solute would be represented by a line with one-half of the slope of this line. Since the purpose of this plot is to determine MW, the abscissa is given in units of mg of cholesterol/g of DPPC. These units may be converted into cholesterol monomer mole percent with the conversion factor of 1 mol % = 5.1 mg/g.

In contrast to the results with methyl oleate, the concentration dependence of the lowering of the phase transition temperature produced by cholesterol is a curve, as shown in Figure 7. The slope to the points above 15 mg of cholesterol/g of DPPC (approximately 3 mol %) indicates that the apparent molecular weight increases toward that of a dimer, whereas the slopes at low solute concentrations gradually approach that corresponding to the molecular weight of the monomer. The deviations from simple behavior are not due to the appearance of the liquid-ordered phase, l_o, since that phase only begins to form at concentrations > 30 mg of cholesterol/g of DPPC (~6 mol %), as shown in Figures 4 and 5. The shapes of the thermograms, shown in Figure 2, do not indicate the presence of a *liquidus* and a *solidus* line: The thermograms remain symmetrical in the presence of cholesterol, and in fact, the cooperative unit increases with increasing cholesterol concentration. From these considerations we conclude that cholesterol is insoluble in the s_o phase, and it is progressively dimerized when dissolved in the l_d phase.

If cholesterol dimerizes in DPPC bilayers, then the curve in Figure 7 should be analyzable in terms of a reversible dimerization equilibrium. For a system of reversible dimerization, according to the reaction scheme

$$2[M] \overset{K_d}{\rightleftharpoons} [D] \qquad (5)$$

where [M] and [D] are the monomer and dimer concentrations, respectively, the dissociation constant for dimer formation, K_d, is given by

$$K_d = \frac{[M]^2}{[D]} \quad (6)$$

We show in the Appendix that for a cryoscopic system where the solute undergoes reversible dimerization, the slope of T_m versus x, the cholesterol concentration of the lipid phase in grams per liter, is given by the equation

$$-\frac{dT_m}{dx} = \frac{K_f}{387}\left[\frac{1}{2} + \frac{1}{1+\sqrt{1+\alpha x}}\right] - \frac{\alpha K_f x}{(2)(387)[2+2\alpha x+(2+\alpha x)\sqrt{1+\alpha x}]} \quad (7)$$

Since this equation is not readily integrable, local slopes were first calculated by the method of chords from the experimental T_m vs x data set. These slopes were then analyzed using eq 7 and a nonlinear least squares fitting program. We found that the data, although considerably scattered, were consistent with q 7, and the analysis yielded the parameters K_f = 12.2 K g m^3 and K_d = 22.5 mg of cholesterol/g of DPPC (~4.4 mol %). This calculated value of K_f is in good agreement with the theoretical value of 12.3 K g m^3. The consistency of the experimental data with this equation was further tested using the calculated K_d and K_f values and eq 7 to perform a second-order Runge–Kutta numerical integration. The theoretical curve thus obtained, shown in Figure 7, is in reasonable agreement with the experimental points and supports the concept of cholesterol dimerization.

In sharp contrast to the behavior of cholesterol in DPPC liposomes, the monolayers of mixtures of DPPC and cholesterol at the air/water interface indicate by the sharpness of the beginning of the transition region that cholesterol is not miscible with DPPC monolayers, even in the liquid-expanded state (Albrecht *et al.*, 1981). Thus, dimer formation of the solute appears to be a property of bilayers only. This then suggests that the dimer is formed between one molecule from each lamella of the bilayer, by hydrophobic interaction of the lipophilic tails of the molecules, as illustrated in Figure 8. Such an interaction is reasonable in view of the fact that the hydrophobic tails of the DPPC molecules themselves intertwine because of the unequal penetration of the *sn*-1 and *sn*-2 acyl chains into the lipid core. The translamellar dimer model of cholesterol is consistent with a variety of earlier observations that led Sankaram and Thompson to postulate that monomers of cholesterol span the nonpolar core of two leaflets of the bilayer (Sankaram & Thompson, 1990, 1991).

If cholesterol forms transbilayer dimers by tail-to-tail interactions, then sterols without isoprenyl side chains should not be able to dimerize in phospholipid liposomes. In order to test this corollary, we measured the cryoscopic behavior of two steroids, pregnenolone (U-2865) and dehydroandrosterone (U-4614), two molecules structurally similar to cholesterol but without the isoprenyl side chain. As shown in Figure 9, both compounds reduced the T_m of the DPPC liposomes, and the decrease was linear with concentration. Also, at low concentrations, they decreased the size of the cooperative units (data not shown). The apparent molecular weights of these compounds were calculated from these data using methyl oleate as a molecular weight standard. In the region of 0–2 mol % solute—where cholesterol is monomeric—the data were somewhat scattered, but they nevertheless yielded MW's of 257 ± 38 for pregnenolone and 355 ± 85 for dehydroandrosterone. Thus, within the rather large experimental error, these sterols form ideal cryoscopic systems with DPPC, and they form monodisperse solutions with the l_d phase. Unlike cholesterol, these two sterols remained monomeric at least up to 10 mol %: In the range of 0–10 mol % solute, pregnenolone yielded 266 ± 38 (actual = 316.46) and androsterone gave 305 ± 31 (actual = 288.43). These findings are thus consistent with the proposal that cholesterol molecules form transbilayer dimers in the l_d phase of DPPC multilayers via interactions of their hydrophobic alkyl chains.

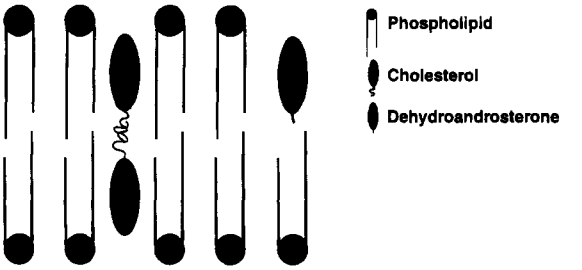


FIGURE 8: Schematic illustration of the proposed tail-to-tail dimers of cholesterol in the l_d phase of DPPC MLVs.

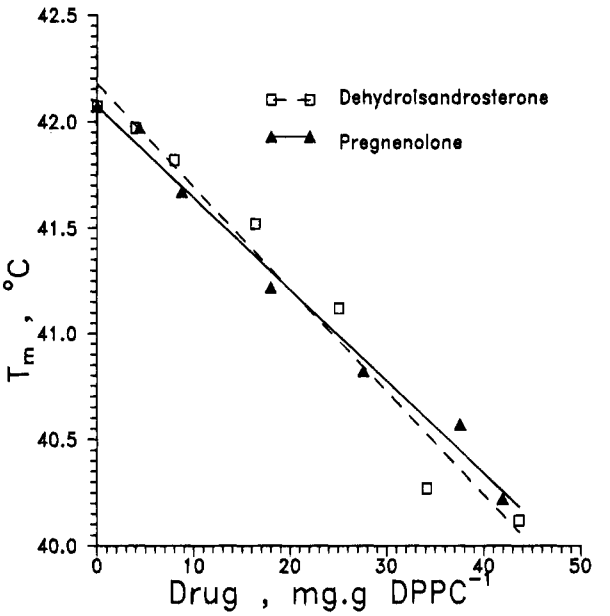


FIGURE 9: Lowering of the phase transition temperature of DPPC MLVs by dehydroandrosterone and by pregnenolone. The depression of the melting point of DPPC MLVs produced by these two sterols was determined as described in Materials and Methods: □, dehydroandrosterone; ▲, pregnenolone. The solid and dashed lines represent the linear regression fits to the experimental points.

DISCUSSION

The depression of the freezing point of DPPC liposomes produced by methyl oleate is consistent with the proposal that DPPC liposomes constitute an ideal cryoscopic solvent for lipids and that methyl oleate forms a true, homogeneous, monodisperse solution with the l_d phase of DPPC. The most important inference drawn from our DSC measurements is that cholesterol, contrary to previous assumptions, may exist in DPPC bilayers as an interlamellar tail-to-tail dimer, and it does not form a homogeneous solution with the s_o phase. Rather, solid cholesterol, as separate domains, may be

interspersed with s_o domains. The proposed model is consistent with the finding that compounds which do not have the isoprenyl tail do not behave as a dimerizing system.

According to this hypothesis, cholesterol and other interlamellar solutes are in the fully extended state, and as a result, their hydrophobic tails are in contact with each other. Such an interaction should increase the stability of the bilayer. Also, molecules of the appropriate shape may perhaps form transbilayer heterodimers with cholesterol, and thus, cholesterol may promote the dissolution of such lipids in the phospholipid bilayer. The formation of interleaflet dimers should also contribute to the ability of cholesterol to inhibit the insertion of external lipophilic compounds into biological membranes.

ACKNOWLEDGMENT

We thank the Biological Screening Office at the Upjohn Company for providing the solutes used in this study. We also thank Dr. M. B. Sankaram for helpful discussions. Special thanks go to Dr. T. E. Thompson from the University of Virginia Health Sciences Center, Charlottesville, VA, for his valuable comments and contributions.

APPENDIX

The dissociation constant of a reversible homodimer, K_d, is defined as

$$2M \overset{K_d}{\rightleftharpoons} D; \quad K_d = \frac{[M]^2}{[D]} \quad \text{and} \quad [D] = \frac{[M]^2}{K_d} \tag{8}$$

where [M] and [D] are the monomer and dimer concentrations, respectively. The number average molecular weight, MW_{NA}, of the monomer−dimer mixture at equilibrium is defined as

$$MW_{NA} = \frac{387[M] + (2)(387)[D]}{[M] + [D]} = 387\left[1 + \frac{[D]}{[M] + [D]}\right] = 387\left[1 + \frac{1}{1 + \frac{K_d}{[M]}}\right] \tag{9}$$

Defining the analytical monomer concentration as [A], we have $[A] = [M] + 2[D] = [M] + 2([M]^2/K_d)$. Thus, [M] as a function of [A] is

$$[M] = \frac{K_d}{4}\left(-1 + \sqrt{1 + \frac{8}{K_d}[A]}\right) \tag{10}$$

Defining x = [cholesterol] in g/L = 1000 wf, we can express [A] as $[A] = x/387$. Defining $\alpha = 8/(387K_d)$, we obtain

$$[M] = \frac{K_d}{4}(-1 + \sqrt{1 + \alpha x}) \tag{11}$$

Substituting this value into eq 9, we have

$$MW_{NA} = (2)(387)\left[\frac{1 + \sqrt{1 + \alpha x}}{3 + \sqrt{1 + \alpha x}}\right] \tag{12}$$

For a system where the MW_{NA} is a function of x, the change in T_m as a function of x can be derived from eq 4 as

$$-\frac{dT_m}{dx} = \frac{K_f}{MW_{NA}} - \frac{K_f x}{(MW_{NA})^2}\frac{d(MW_{NA})}{dx} \tag{13}$$

The derivative of eq 12 is

$$\frac{d(MW_{NA})}{dx} = \frac{(2)\alpha(387)}{\sqrt{1 + \alpha x}(3 + \sqrt{1 + \alpha x})^2} \tag{14}$$

Substituting eqs 12 and 14 into eq 13, we obtain the equation relating the changes in T_m to changes in x:

$$-\frac{dT_m}{dx} = \frac{K_f}{387}\left[\frac{1}{2} + \frac{1}{1 + \sqrt{1 + \alpha x}}\right] - \frac{\alpha K_f x}{(2)(387)[2 + 2\alpha x + (2 + \alpha x)\sqrt{1 + \alpha x}]} \tag{15}$$

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BI941356O