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Dilatometric Studies of the Subtransition in Dipalmitoylphosphatidylcholine†

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ABSTRACT: The phase transition between the newly discovered low-temperature subgel phase and the gel phase of dipalmitoylphosphatidylcholine has been studied by using dilatometry. Equilibrium measurements show that the subtransition upon heating is centered at 13.5 °C, has a dilatometric half-width of 0.6 °C, and comprises a specific volume change of 0.009 mL/g (about one-fourth the size of the main transition). When the gel phase is cooled, the subtransition does not occur until below 5 °C. The rate of formation as a function of incubation temperature for 1 °C < T_I < 6 °C was determined; it is not well fit by quantitative theories based upon homogeneous nucleation. However, some form of nucleation is present since temperature-jump studies show that once the subgel phase has started to form, it continues to grow in the range 6 °C < T_J < 12.8 °C. Thus, the true transition temperature lies between 12.8 and 13.5 °C, but nucleation of the subgel phase is severely retarded above 6 °C, leading to the large hysteresis observed upon cooling.

The polymorphism of fully hydrated phosphatidylcholines has been extensively studied, revealing the existence of a low-temperature gel phase, an intermediate phase, and a high-temperature "melted" phase. Recently, the transition into yet another phase has been reported by Chen et al. (1980) in hydrated phosphatidylcholine bilayers with alkyl chains with 16 (DPPC), 17, or 18 carbon atoms. The new transition, which these authors called the "subtransition", takes the gel phase into a new low-temperature phase, which we will call the subgel phase. This transition was observed to be extremely hysteretic in differential scanning calorimetry (DSC) experiments. When the phase was heated, it occurred between 15 and 19 °C, whereas when the gel phase was cooled, the subgel

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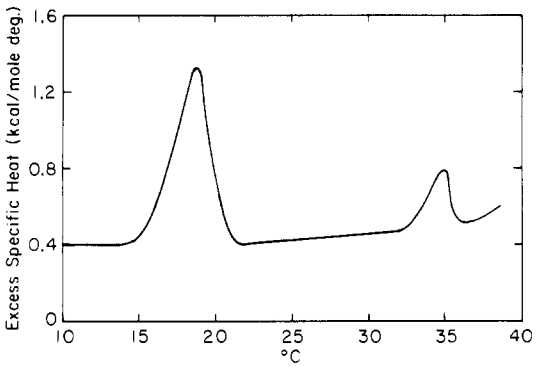

FIGURE 1: Smoothed DSC scan of DPPC (0.9 mg/mL) that was stored at 0–2 °C for 1 week. Both the subtransition and the lower transition are shown. The scan rate was 12.3 °C/h.

phase did not occur until below 6 °C. The formation of the subgel phase was observed to be extremely slow (half-time of tens of hours).

In this study, we focus on two questions that arose from the earlier work. First, what is the true transition temperature? Second, what is the source of the large hysteresis previously observed? Because of the slow processes involved in subgel phase formation, our dilatometer (Wilkinson & Nagle, 1978), which can be used for equilibrium measurements, is well suited to this type of investigation. Also, it is of interest in characterizing the thermodynamic properties of the transition to obtain the volume changes.

Materials and Methods

DPPC (L-α-dipalmitoylphosphatidylcholine) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. DSC scans of dilute samples displayed a main transition half-width of 0.25–0.3 °C, a value about 2 times larger than that of our best previous samples (Nagle & Wilkinson, 1978), but approximately the same as that of the commercial samples on which most of the earlier measurements were performed; the main transition half-widths of these commercial samples are still narrow enough to indicate high purity. The lipid was dispersed in water or buffer (20 mM phosphate, pH 6.8) by either vortexing or stirring it at temperatures higher than the main transition temperature (T_M). Calorimetric scans were performed with a Microcal MC-1 calorimeter (Microcal Inc., Amherst, MA) at a heating rate of 10–12 °C/h and with a lipid concentration of approximately 1 mg/mL. Samples were periodically shaken by hand during storage. The dilatometer has been previously described (Wilkinson & Nagle, 1978). For the dilatometric experiments, the lipid concentration was 31 mg/mL for sample I and 23 mg/mL for sample II. The samples were constantly stirred in the dilatometer. Since these experiments were of long duration (15 weeks for sample I), the lipid was analyzed for breakdown at the completion of the experiment. No traces of lysolecithin were detected on thin-layer chromatograms.

In temperature-jump experiments conducted with the dilatometer, only data taken after 30 min have been used so as to ensure that instrumental equilibration was established. Tests performed in the single-phase regions far removed from the phase transitions showed that the system had equilibrated approximately 15–20 min after the new temperature had been reached.

Results

Calorimetry. Figure 1 is a representative heating scan of a DPPC sample that was stored at 0–2 °C for 1 week. An endothermic peak centered at about 18 °C due to the subtransition is present. The enthalpy of the subtransition is 2.6 ± 0.3 kcal/mol. Both of these values are very similar to the results of Chen et al. (1980) on the subtransition of commercial DPPC. Also, in keeping with the earlier work, no calorimetric evidence for the subtransition could be found when DPPC samples were stored at $T > 6$ °C. However, if they were first stored at 0–2 °C for several days and then the temperature was raised above 6 °C but kept below 12 °C, the subtransition was still observed. This result is in agreement with the dilatometric data to be discussed in the next section.

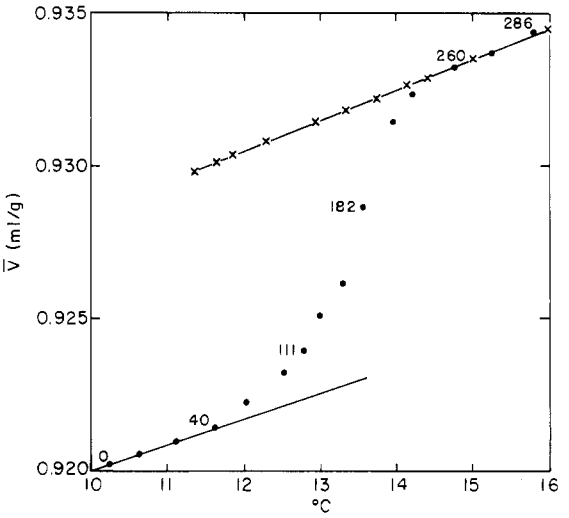

FIGURE 2: Temperature dependence of the specific volume of DPPC near the subtransition. The dots represent equilibrium heating points with the elapsed time in hours shown. The (×)'s are a cooling scan at 3 °C/h. The lowest point in the cooling run was allowed 12 h to equilibrate. The straight lines indicate the thermal expansion in the single-phase regions.

Nearly all of the experiments reported on here were done in the presence of added salt (sodium phosphate). A DPPC sample in water was stored at 2 °C for 4 days and then examined in the DSC in order to rule out the possibility that the salt caused the formation of the subgel phase. An endothermic peak at 17.5 °C was observed which indicates the presence of the subtransition.

Dilatometry. Figure 2 shows the apparent specific volume of sample I. Prior to the measurements shown in Figure 2, sample I had been converted into the subgel phase and then heated through the subtransition once. It was then incubated at 4 °C for 10 days and then gradually heated to 10.25 °C with a volume increase corresponding to an extension of the lower straight line in Figure 2. This gives a coefficient of expansion $\alpha = (1/V)\delta V/\delta T = (93 \pm 5) \times 10^{-5}$ deg^{-1} in the subgel phase as compared to $\alpha = (100 \pm 5) \times 10^{-5}$ deg^{-1} in the gel phase; the latter α is about 10% higher than we reported previously (Nagle & Wilkinson, 1978). The data represented by dots in Figure 2 were taken in the following way. After measuring V at temperature T_i (e.g., 12.8 °C in Figure 2) the temperature was then increased quickly (5–20 min) to the next higher T_{i+1} (13.0 °C in Figure 2). Well after V had stabilized to the value plotted in Figure 2, T_{i+1} was increased to T_{i+2}. The time between data points was typically 10–14 h, and the duration of the experiment was 286 h. Figure 2 shows a definite transition with a midpoint at 13.5 °C, more than 4 °C below the calorimetric transition in Figure 1 and 1.5 °C below the lowest transition temperature observed or extrapolated by Chen et al. (1980); this is reasonable since even the slowest calorimetric scanning rates are more than 100 times faster than this dilatometric scan. Nevertheless, the dilato-

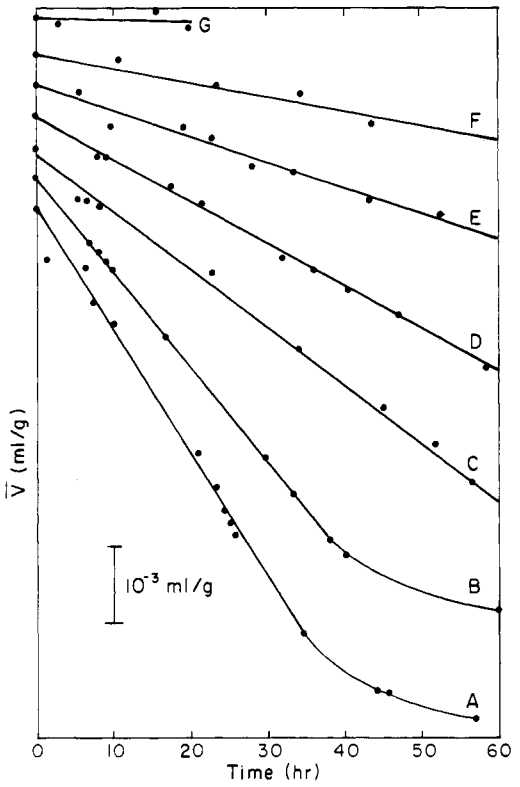

FIGURE 3: Relative specific volume as a function of time for DPPC suspensions at various incubation temperatures: (A) 1.1, (B) 1.9, (C) 3.0, (D) 4.0, (E) 4.25, (F) 4.85, (G) 5.3 °C. The curves have been displaced for ease of viewing.

metric data are *not* reversible. When the sample is cooled, $V(T)$ is shown by (×) in Figure 2, and the transition into the subgel phase is not observed until much lower temperatures.

Kinetic measurements of the transition into the subgel phase are shown in Figure 3. In these measurements sample I was first converted completely out of the subgel phase by heating to 20 °C or more. It was then cooled as quickly as possible (less than 3 h) to an incubation temperature, T_I, and measurements of $\Delta V(T_I,t)$ were taken as a function of time t and T_I. Curve A in Figure 3 for T_I = 1.1 °C shows a roughly linear decrease in V until about 80% of the gel phase is converted into subgel phase, followed by a slower completion of the conversion. Although the completion of the conversion is not shown for the higher temperatures in Figure 3, the same general pattern was followed. At the highest T_I = 5.3 °C in Figure 3, any conversion was too slow to be measured.

The initial rate of conversion, $R(T_I)$ found from the slopes of the linear portions of the curves in Figure 3 is plotted vs. the incubation temperature in Figure 4. The data in Figure 4 include additional measurements on sample I not shown in Figure 3 and two measurements on a fresh sample II. As sample I aged over 15 weeks, there was a slight systematic decrease in $R(T_I)$.

A set of experiments, to be called "incubated T-jump measurements", which help to explain many of the oddities of this subtransition, will now be described. The sample is incubated at T_I < 5 °C until about half of it is converted to the subgel phase. Then the temperature is jumped to T_J > 5 °C. Figure 5 shows the result of this experiment for T_I = 2.5 °C and T_J = 6.2 and 10 °C. The conversion into the subgel phase continues to completion at T_J, even though the pure gel phase does not begin to convert to subgel phase at the T_J temperatures. Also, the rate of conversion actually increases after the T-jump and is faster for T_J = 10 °C than for 6.2 °C.

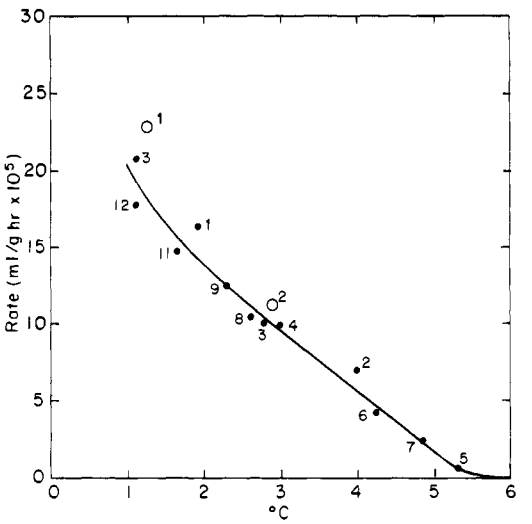

FIGURE 4: Initial rate of specific volume decrease $R(T_I)$ as a function of incubation temperature, T_I. The dots are for sample I and the open circles for sample II. The numbers give the order in which the data were obtained.

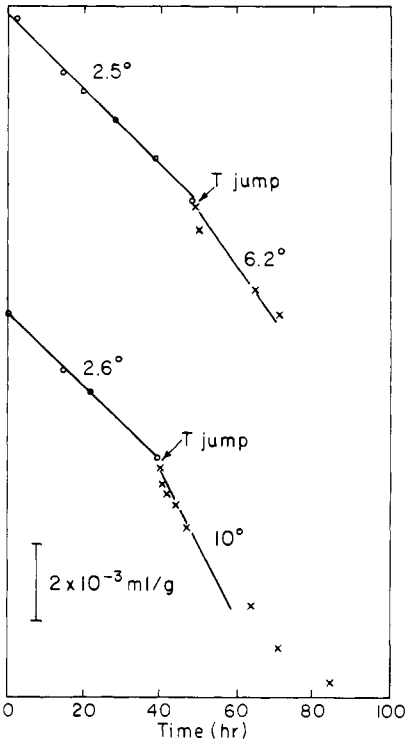

FIGURE 5: Relative specific volume as a function of time with temperature jumps from 2.5 to 6.2 °C at 48 h in (a) and from 2.6 to 10.0 °C at 40 h in (b).

In Figure 6a a sequence of T-jumps are shown. After an initial quick increase in V (this increase closely follows the normal expansion expected in either the gel phase or the subgel phase), V then decreases isothermally with time, corresponding to a continued conversion into the subgel phase. In the measurements reported in Figure 6a this continues to occur up to T_{J3} = 12.8 °C, fairly close to the transition temperature, T_S, measured in Figure 2. For T_{J4} = 13.5 °C the subgel phase melts into the gel phase. However, the results can be more complicated, as shown in Figure 6b, where the sign of ΔV may depend upon the past history as illustrated quite dramatically at T_{J3} = 12.6 °C = T_{J6}. Another set of experiments not shown here involves incubation at T_I = 13.5 °C. After about half the subgel phase has melted, a T-jump to a lower temperature

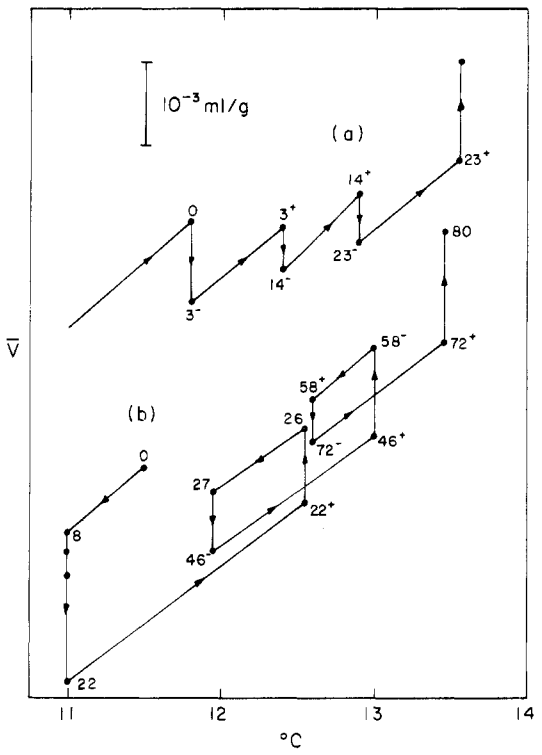

FIGURE 6: Temperature-jump experiments on two samples (a and b) which were initially approximately 50/50 mixtures of gel and subgel phases. The vertical arrows indicate the extent and direction of volume change at constant temperature, while the nonvertical arrows represent the changes during the relatively rapid temperature jumps. The time in hours is given beside each point. The specific volumes initially were nearly equal for the two samples but have been displaced for easy viewing.

is performed. In all cases V then proceeds to decrease isothermally, indicating reformation of the subgel phase. However, the conversion does not go to completion.

Discussion

The results of our calorimetric experiments are quite consistent with the pioneering work of Chen et al. (1980) which revealed that there is a new major phase transition in the lecithin DPPC. As is characteristic of many solid–solid transitions, including the lower transition in DPPC (Nagle & Wilkinson, 1978; Lentz et al., 1978), the subtransition is highly hysteretic. From the calorimetric measurements the true transition temperature, T_S, has only been determined to be between T_{S1} = 5 °C, which is the highest temperature at which the subphase forms upon cooling, and T_{S2} = 15 °C, which is the lowest (extrapolated) calorimetric transition temperature. In contrast our dilatometry measurements, performed more than 100 times more slowly than any calorimetry experiments, reduce T_{S2} to 13.5 °C (Figure 2). It may be remarked that this lowering of T_{S2} is not likely to be due to impurities because the main transition is just as sharp as observed by Chen et al. (1980), and the subtransition is considerably sharper. The dilatometric half-width, defined as the narrowest temperature interval in Figure 2 over which V changes by $\Delta/2$, is only 0.6 °C. In order to compare with calorimetric half-widths, one should take the derivative of Figure 2. This gives $\Delta T_{1/2}$ = 1.0 °C, but this is still much sharper than the calorimetric half-width in Figure 1 which is close to the half-width (2.6 °C) reported previously. It may also be noted that when the dilatometer was scanned at faster rates (6 °C/h), the midpoint of the transition and the half-width both increased to values consistent with those seen in calorimetry.

A standard theory of the process of phase transformation is that nuclei of a critical size of the new phase are formed spontaneously and then proceed to grow; nuclei of smaller than critical size shrink back to the old phase, even though the new phase is thermodynamically stable, because of excessive surface free energies. The rate of growth of a new phase is given by theory to be (Dunning, 1969)

$$R(T) = C \exp[-\Delta E/(kT)] \exp[-A/[T(\Delta T)^2]] \quad (1)$$

where $\Delta T = T_S - T$ is the undercooling temperature and ΔE is an activation energy for growth of nuclei and domains. For small values of ΔT the second exponential in eq 1 is most rapidly varying and can become so small that no appreciable rate of conversion into the new phase is observable. Thus, it is not surprising that T_{S1} = 5 °C is definitely lower than the true transition temperature, T_S. It was our initial hope that we could obtain T_S by finding the best fit of eq 1 to the rate of conversion data in Figure 4. We were not successful, perhaps because of the aging effect on the rates but more likely because eq 13 does not represent the kinetics of our system. In this latter regard we investigated replacing the factor $\exp[-A/[T(\Delta T)^2]]$ by $\exp[-B/(T\Delta T)]$ to model two-dimensional nucleation which might be more appropriate for individual bilayers, but the results were slightly poorer. In both cases the T_S which gave the best fit was about 6 °C and ΔE was negative.

Fortunately, the incubated T-jump measurements (Figures 5 and 6) answer the outstanding questions. After incubation and partial conversion to subgel phase at T_I and below T_{S1}, a jump to T_J between 6 and 12.8 °C results in complete conversion into the subgel phase. This proves that the true transition temperature, T_S, is at least as high as 12.8 °C, because once domains of both phases are present, the thermodynamically stable phase wins out. The reason for the lack of conversion of the pure gel phase into subgel phase between T_{S1} and T_S is due to severe retardation of nucleation due to the second factor in eq 1. This picture is also supported by the T-jump measurements from an incubation at T_I = 13.5 °C to lower T_J. The fact that conversion is not complete for such T-jump measurements is explained by the possibility that in some isolated macroscopic domains of the sample all the subgel phase nuclei are already melted.

We would also like to offer a speculative explanation for the rather more complicated T-jump measurements shown in Figure 6b. Subgel phase domains may grow in a dendritic pattern, and the maximum curvature of the domain boundaries will be larger for temperatures with more rapid growth. Although Figure 5 shows that the growth rate increases up to 10 °C, it must decrease for higher T_{J1}. When T_{J1} is jumped to a yet higher T_{J2}, the leading edges of the dendritic structure where the curvature is greatest may melt back, yielding a positive $\Delta V(t)$ for a period of time. According to this explanation ΔV should eventually reverse sign, and the subgel phase should grow with a dendritic structure of lesser curvature. The larger the ΔV which occurs at T_{J1}, the larger should be the initial positive ΔV at the higher T_{J2}. This accounts for the difference between the measurements in parts a and b of Figure 6. Consistent with this explanation it might be noted that $\Delta V(t)$ at T_{J3} = 12.8 °C in Figure 6a initially seemed to swing slightly positive before decreasing.

Although some details of the kinetics and nonequilibrium aspects of the subtransition may be worth exploring further, it seems clear that enough is now known to be able to obtain true equilibrium properties of the transition. Some of these quantities are presented in Table I along with the comparable properties for the lower and main transitions. The last row

Table I: Equilibrium Thermodynamic Phase Transition Properties of DPPC

	transitions		
	sub	lower (PRE)	main
$T_{midpoint}$	13–13.5	34[a]	41.4[a]
$\Delta T_{1/2}$	1.0[c]	2[a]	0.25[b]
ΔH (kcal/mol)	2.6 ± 0.4[b]	1.0 ± 0.2[b]	8.4 ± 0.3[b]
ΔV (μL/g)	9.0 ± 0.5	3.3 ± 0.5[a]	35 ± 1[a]
ΔU_{vdW} (kcal/mol)	1.5–1.8	0.5–0.6	5.5[a]

[a] Nagle & Wilkinson (1978). [b] This work, but see Chen et al. (1980) for range of values. [c] From the derivative of Figure 2 for comparison with calorimetric half-widths. The dilatometric half-width from Figure 2 is 0.6 °C.

of Table I gives estimates of the energy required to expand the hydrocarbon chain portion of the bilayer against internal van der Waals forces, assuming that the entire measured expansion occurs in the hydrocarbon chain region. The calculation essentially follows the one described in detail earlier (Nagle & Wilkinson, 1978) for the main transition. The range of values of ΔU_{vdW} presented in Table I (for both the sub-transition and the lower transition) arises from two different ways of treating the repulsive interactions. The lower values in Table I are derived by retaining the less accurate repulsive term in the formula of Salem (1962). However, if either of these transitions involves any appreciable orientational or stacking ordering of the hydrocarbon chains with respect to one another, then a more refined calculation is in order. Such ordering would appear to reduce the negative repulsive part of ΔU_{vdW}. The larger values of ΔU_{vdW} in Table I are calculated by ignoring the repulsive part of the interaction completely. In any case ΔU_{vdW} is safely smaller than, but still a significant fraction of, ΔH.

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Cholesterol–Phospholipid Interaction in Membranes. 1. Cholestane Spin-Label Studies of Phase Behavior of Cholesterol–Phospholipid Liposomes†

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ABSTRACT: The effect of cholesterol concentration on the thermotropic phase behavior of aqueous phospholipid multibilayers was monitored by means of electron spin resonance spectroscopy (ESR) of a cholestane spin-label (CSL). The spin-label itself induces an additional transition in several different phospholipids, which is attributed to local melting around the spin probe. In contrast, cholesterol prevents its neighboring phospholipids from undergoing fluidization. Small additions of cholesterol affect the position of the probe-induced lipid mobilization curve. The phospholipid main gel–liquid-crystal transition, which is also observed as a separate change in probe mobilization, is not affected by low concentrations of cholesterol. These observations indicate the presence of two phases, a cholesterol-rich phase and a pure phospholipid phase, and indicate that CSL preferentially enters the cholesterol-rich phase. Addition of more than 20 mol % cholesterol abolishes the bulk phospholipid phase. This is evidenced by the disappearance of the gel–liquid-crystal transition as observed by ESR. However, the CSL-induced transition is present at all concentrations of cholesterol and CSL. The behavioral differences between the two sterols caution against using this probe as a direct substitute for cholesterol. However, it remains a useful tool for monitoring the phase behavior of cholesterol–phospholipid bilayer systems.

Cholesterol is an important component of all animal membranes, both cellular and intracellular. This ubiquitous amphiphilic sterol may constitute up to 50% of the total lipid in some biological membranes. However, its exact role in the structure and function of lipid bilayer membranes has remained elusive, despite extensive study of cholesterol's effects on model and natural membrane systems during the past decade. Various models proposed for cholesterol–lipid interactions have included suggestions of complex formation at 20 (Copeland & McConnell, 1980), at $33^1/_3$ (Engelman & Rothman, 1972) or at 50 (Phillips & Finer, 1974) mol % of the sterol. Different experimental techniques appear to yield contradictory indications of the association stoichiometry. Also, the molecular interaction that gives rise to cholesterol–lipid complex associations has remained obscure. Hydrogen bonding (Brockerhoff, 1974), van der Waals interactions (Yeagle et al., 1977), and molecular length (Suckling et al., 1979) have all been proposed as the essential feature in sterol–lipid interaction. Much work has also been devoted to studying the phase behavior of aqueous cholesterol–lipid bilayer systems, with equally confusing interpretations. There have been suggestions for construction of phase diagrams with

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