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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phase boundaries at 20 mol % (Shimshick & McConnell, 1973; Melchior et al., 1980), at $33^1/_3$ mol % (Gershfeld, 1978; Lentz et al., 1980), and at 40 mol % cholesterol (Kroon et al., 1975).

Whereas many previous investigators have examined the often rather indirect effects of cholesterol on overall membrane properties, this work attempts to determine the local influence of cholesterol molecules on the dynamical properties of those lipid molecules in their immediate environment. Electron spin resonance spectroscopy (ESR)[1] was employed to monitor the behavior of 3-nitroxide cholestane (CSL), a cholesterol-like spin-label molecule, incorporated into various phospholipid–cholesterol multibilayers. Being more cholesterol-like than lipid-like, the cholestane spin-label is expected to provide information about cholesterol-rich regions of the bilayer, which phospholipid spin probes may not even enter. Also, behavioral differences between the two sterols have proved valuable in elucidating cholesterol's mode of operation. Although there have been previous studies with this label in oriented multibilayer systems (Hemminga, 1975; Shimoyama et al., 1978), this is the first thorough, systematic investigation by ESR of the thermal behavior of phospholipid multibilayers dispersed in excess water.

Experimental Procedures

All phospholipids used in this study were purchased from Calbiochem. Cholesterol was obtained from Sigma (Sigma grade), and spin probes were obtained from Syva. All materials were found to be chromatographically pure by thin-layer chromatography and used without further purification.

For preparation of multibilayer samples, lipids, cholesterol, and spin-label were weighed out in the desired proportions on a Cahn 25 electrobalance and dissolved in spectroscopic-grade chloroform. Samples were dried first under prepurified nitrogen gas and then under vacuum for at least 4 h. They were hydrated with deionized, Millipore-filtered water. A glass bead was added to the 10-mL round-bottom flask containing the sample, which was vortexed at maximum speed for a minimum of 20 min while being heated above the lipid phase transition temperature. Samples were hydrated just to the point where they were fluid enough to be manipulated with a syringe into 1.5-mm capillary tubes. Generally, 50–70 wt % water was added to the lipid mixtures, to make sample concentrations of 300–500 mg/mL. For calorimetry studies, samples were diluted to 50–60 mg/mL.

ESR spectra were recorded on a Varian E-line Century Series spectrometer. Temperature was regulated by means of a Varian V4540 variable-temperature controller, in which nitrogen gas is cooled by passage of the transfer line through liquid nitrogen. The gas is heated by a heater–thermistor element just before entering the cavity via a double-walled, evacuated quartz Dewar. Temperature was monitored continually by means of a copper–constantan thermocouple, placed directly into the sample tube but not projecting into the ESR cavity. Nearly all temperature scans were performed by heating samples from a low temperature, well below any transitions. However, the transitions were shown to be reversible and without hysteresis by conducting several cooling scans, which produced results identical with those of the heating scans. Samples were incubated for at least 20 min at the starting temperature and for at least 5 min before scanning at each new temperature. Temperature increments of 2.5 °C were usual.

The microwave frequency was about 9.1 GHz, while the magnetic field was set at about 3245 G. All spectra were recorded at a modulation frequency of 100 kHz and microwave power of ≤10 mW. The modulation amplitude was usually less than one-tenth, and never more than one-third, of the peak-to-peak line width of the narrowest line.

All calorimetry scans were performed with a Privalov calorimeter (Privalov et al., 1975) in collaboration with Professor J. M. Sturtevant. The reference was deionized water.

Results

CSL is a rigid sterol molecule, whose nitroxide radical "head group" is firmly attached to the steroid ring system. Therefore, this probe group can rotate only if the molecule as a whole undergoes rotational motion. Since the sterol is intercalated between the hydrocarbon chains of the lipid molecules, conclusions about the motion of this molecule can be correlated directly with the environmental viscosity. At low temperature, when the lipids are immobile, this spin-label produces a powder spectrum of three lines, whose outermost hyperfine maxima are produced by molecules whose $2p_\pi$ orbitals are parallel or nearly parallel to the external magnetic field (hyperfine z axis; McConnell & McFarland, 1970). At higher temperature, when the lipids melt, the rigid sterol probe becomes free to rotate and perhaps wobble within the more fluid lipid matrix. Cylindrical rotation of the spin-label about its long molecular axis leads to averaging of the nuclear hyperfine splitting components that are perpendicular to that axis (x and z components). The outer extrema move in to a rapid-motion limit determined by the average of these components (Presti, 1981). This limit may be reduced even further by onset of wobble, in which tilting of the long molecular axis causes some y component to be averaged in as well. Thus, the separation between the two outer extrema ($2A_\perp$) is monitored as the indicator for fluidity of the lipids around the spin-label.

Figure 1 shows a series of spectra taken for 0.1 mol % CSL in DMPC, as the temperature is raised from −30 to 60 °C. This progression is typical of the increasing mobility of this spin-label in the melting of a lipid bilayer. The −30 °C spectrum is that of immobilized CSL, anisotropically positioned in unoriented liposomes, while at 60 °C it is undergoing rapid rotation about its long axis and wobbling within the bilayer.

Low Spin-Label Concentrations. Plots of $2A_\perp$ vs. temperature for CSL-containing multibilayers yielded S-shaped transition curves centered around 20 °C for nearly all lipids studied. The main gel–liquid-crystal transition was also present at the appropriate temperature. No pretransitions were detected in any samples. Figure 2 shows the results for the series of phosphatidylcholine lipids. All but DMPC, whose main transition occurs in this temperature region as the sole mobility change, exhibit the anomalous mobilization at 20 °C. This transition provides the primary melting phenomenon for DPPC and DSPC, whose main gel–liquid-crystal transitions induce only a minor additional change in CSL mobility. However, the 20 °C transition for DLPC produces only a small increase in CSL mobility, as it is already highly mobilized above the lower temperature main lipid transition. It is apparent that varying the length of the hydrocarbon chains while keeping the phosphatidylcholine head group has little effect on the position of the 20 °C transition. Also, changing the head group to phosphatidylethanolamine (DPPE) or including an unsat-

[1] Abbreviations: ASL, androstane spin-label, 17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxy; CHOL, cholesterol; CSL, cholestane spin-label, 4′,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidin]-3′-yloxy; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DSPC, distearoylphosphatidylcholine; ESR, electron spin resonance spectroscopy.

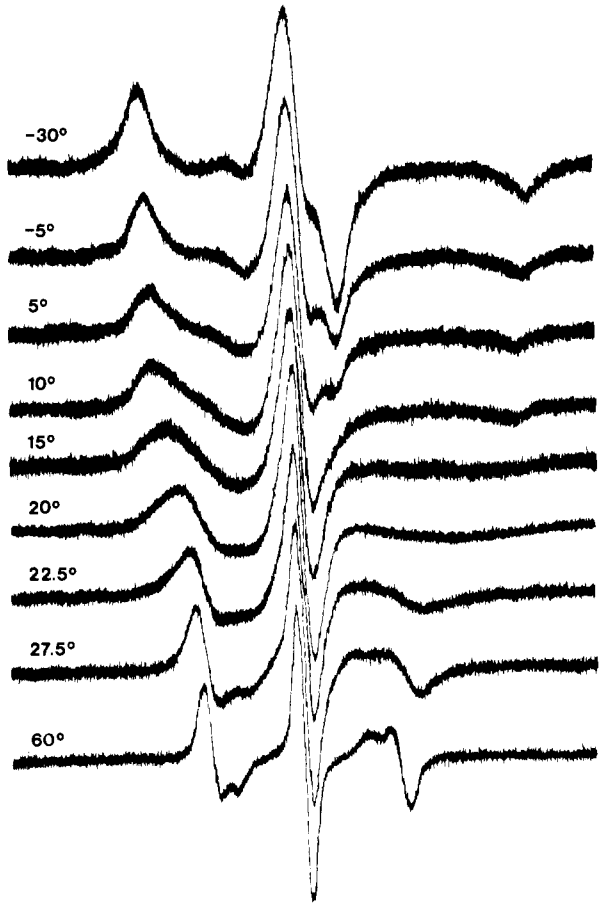

FIGURE 1: Series of ESR spectra taken as temperature is increased for a sample of 0.1 mol % CSL in DMPC liposomes. Note decreasing separation between outermost hyperfine peaks ($2A_\perp$).

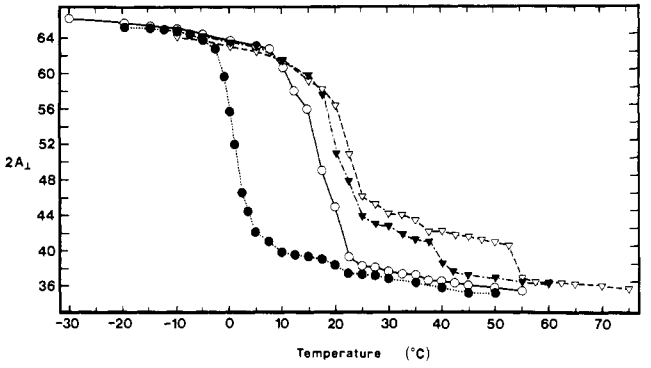

FIGURE 2: Plots of ESR spectral parameters $2A_\perp$, the separation in gauss between the two outer extrema of CSL spectra, as a function of temperature, for aqueous multibilayer samples prepared from phosphatidylcholine lipids of different acyl chain lengths: (●) DLPC, C_{12}; (○) DMPC, C_{14}; (▼) DPPC, C_{16}; (▽) DSPC, C_{18}. All samples contain 0.1 mol % CSL.

urated fatty acid chain (DOPC) has only a slight effect on the temperature of the CSL-induced transition (Presti, 1981).

ASL has its nitroxide group buried in the hydrocarbon region of the bilayer; its head group, like that of cholesterol, is a hydroxyl group. This spin-label also induces an additional transition when incorporated into phospholipid multibilayers, as shown in Figure 3. The mobilization is centered near 14 °C for the longer chained phosphatidylcholines and is also now observed in DMPC. In contrast to the CSL-induced transition, which is apparently coincident with the DMPC main transition, the ASL-induced transition is at low enough temperature to resolve the two. In the presence of ASL, DLPC exhibits

Table I: Thermal Data for Phospholipids[a]

	CSL probe		ASL probe		Chapman,[b]	Van Dijck et al.,[c]	Mabrey & Sturtevant[d]	
lipid	T_m	T_l	T_m	T_l	T_m	T_m	T_m	T_p
DSPC	54	22	51	15	58		54.9	51.5
DPPC	39	19.5	38	14	41	41.5	41.4	35.3
DMPC	17		24	11	23	23	23.9	14.2
DLPC	1	20.5	−1.5	−12.5	0	0	−1.8	4
DOPC	−12	13.5			−22	−14		
DPPE	60	17			63	60	63.8	

[a] T_m = main gel-liquid-crystal phase transition temperature (degree Celsius); T_l = spin-label-induced phase transition temperature (degree Celsius); T_p = pretransition temperature (degree Celsius). [b] Chapman (1975). [c] Van Dijck et al. (1976). [d] Mabrey & Sturtevant (1978).

a large mobilization at about −12 °C, in addition to its now small main transition at −1.5 °C. Apparently ASL destabilizes this lipid, producing a lower temperature induced transition, while CSL tends to stabilize it, inducing its additional transition well above 0 °C. DMPC is also destabilized by ASL, whereas CSL has little effect on its melting. DPPC and DSPC show the same behavior with ASL as they did with CSL.

It is evident that CSL, which is about the same length as DMPC (see Figure 4), destabilizes all lipids longer than this, instituting a lower temperature melting transition in the lipids immediately around the probe. It appears to stabilize the one lipid (DLPC) that is shorter than itself. ASL has no side chain and is shorter than all the lipids used here. It also destabilizes all the lipids, including the shortest, DLPC, so its induced transition is always below the main transition temperature. Table I provides a summary of the transition information for these lipid systems, along with calorimetry data from the literature, with which they show good agreement.

Corroboration by Calorimetry. The presence of an induced transition around 20 °C was corroborated by means of differential scanning calorimetry of CSL-containing DPPC liposomes. Some of these results are shown in Figures 5 and 6, for samples containing 10, 20, and 50 mol % CSL. The endotherm near 25 °C is small and broad, and therefore highly uncooperative, suggesting that it arises from local fluidization of phospholipid molecules in the immediate vicinity of the sterol label. For samples with total lipid concentrations much less than 50 mg/mL, or with less than 10 mol % CSL, the CSL-induced transition is not discernible, presumably because of the breadth of the transition.

Less than 5 mol % cholesterol is sufficient to abolish the pretransition of DPPC aqueous multilayers (Estep et al., 1978). However, the pretransition at 31–34 °C is still evident even at 50 mol % CSL. This is quite striking evidence for behavioral differences between the two steroids. Concentrated samples (50 mg/mL) containing 50 mol % cholesterol with no added spin-label exhibited no endotherm near 20–25 °C and no pretransition, as found by other researchers at lower sample concentrations. Further characterization of CSL by calorimetry will be described in a forthcoming publication.

Cholesterol Effects. The effects of cholesterol on the transition curve for DPPC are presented in Figure 7. There is a shift to increasing temperature in the midpoint of the larger transition from 19.5 °C to a maximum of 28 °C at 30 mol % cholesterol. This trend is reversed upon the addition of higher cholesterol concentrations, the midpoint decreasing to 21 °C for 60 mol % sterol. This sensitivity of the CSL-induced transition to even small additions of cholesterol indicates that the two sterols prefer to be in close proximity. The higher temperature main lipid transition remains at about 39 °C, until

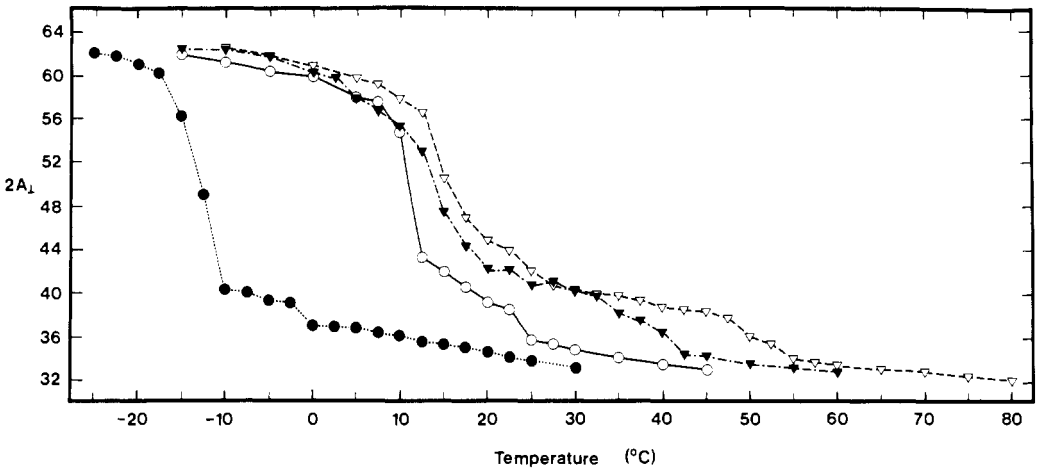

FIGURE 3: Plots of $2A_\perp$ vs. temperature for 0.1 mol % ASL incorporated into a series of phosphatidylcholines of different chain length: (●) DLPC; (○) DMPC; (▼) DPPC; (▽) DSPC.

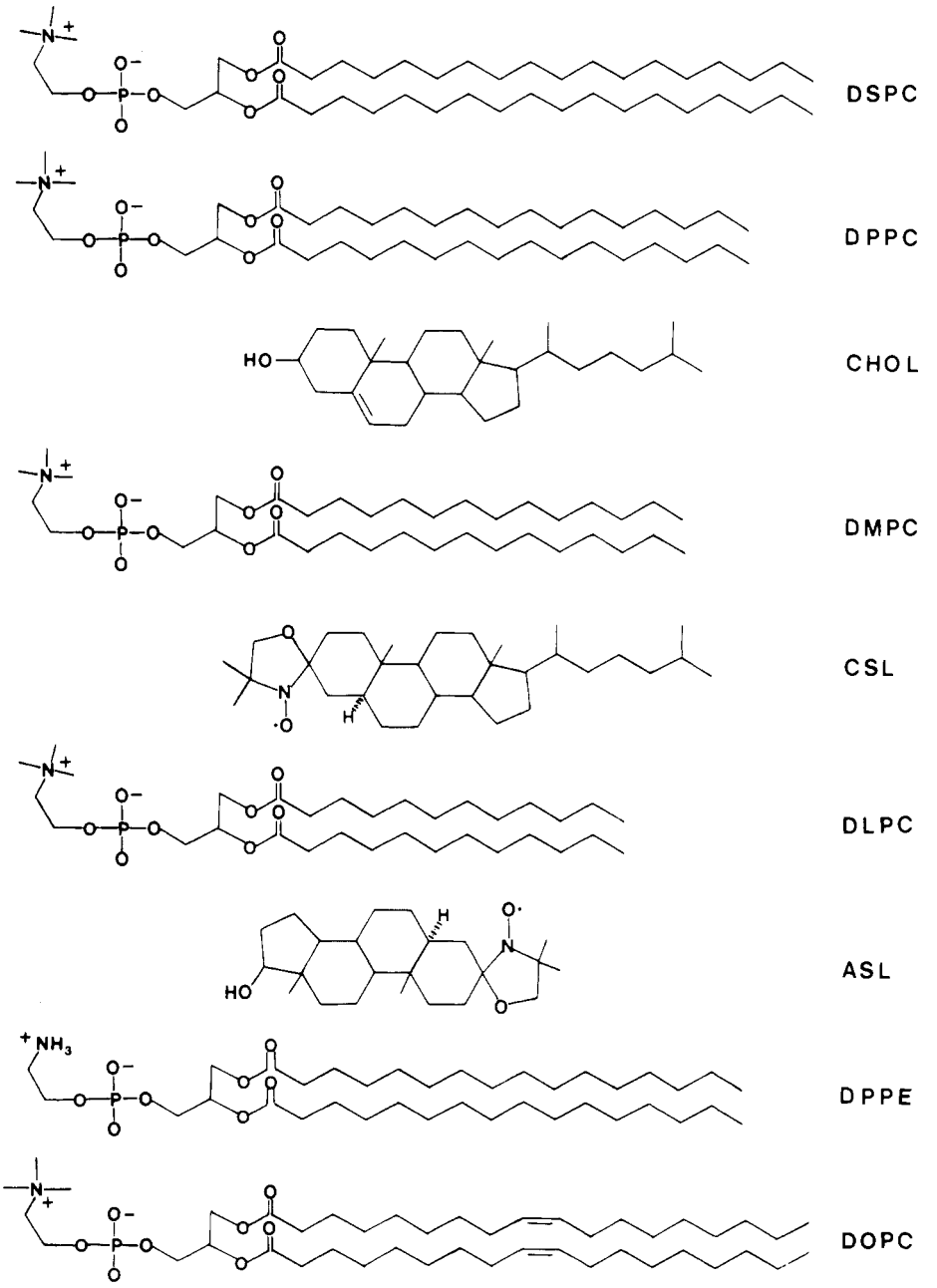

FIGURE 4: Schematic representation of lipids and spin-labels used in this study, illustrating their relative lengths and positions in the bilayer. Relative depth of molecules is from Schreier-Mucillo et al. (1976).

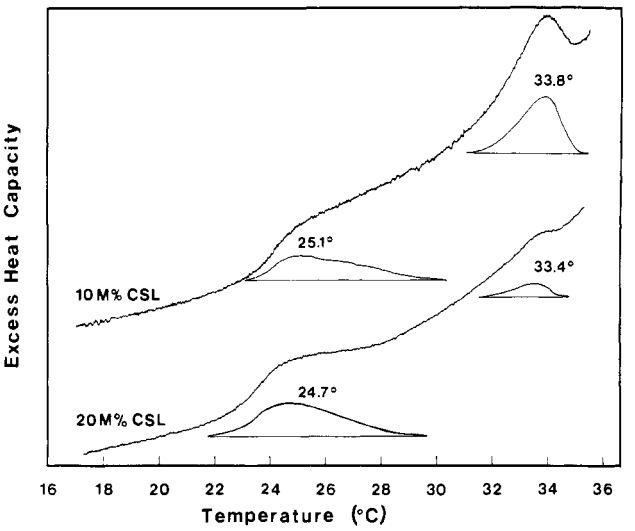

FIGURE 5: Experimental DSC heating curves for samples of DPPC multibilayers containing varying large amounts of CSL. Sample concentration was 50–60 mg/mL. The scans were taken at 0.25 °C/min.

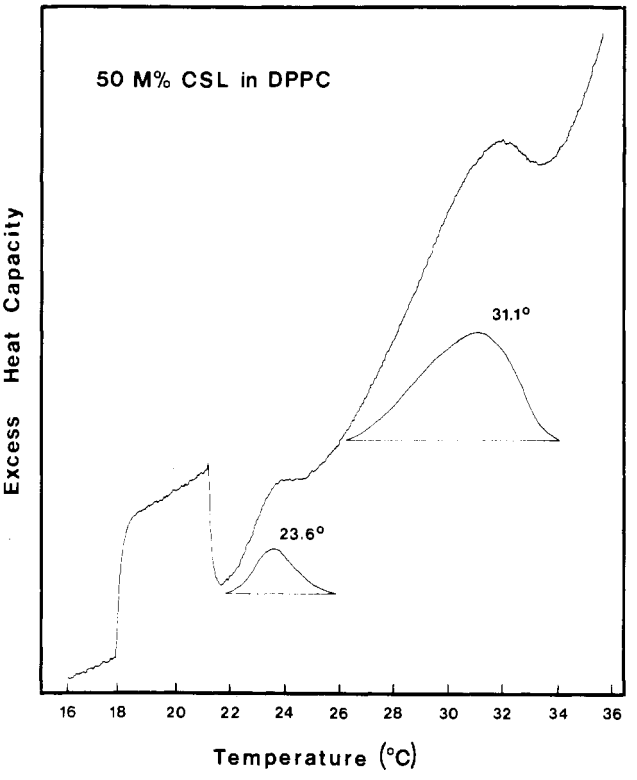

FIGURE 6: Experimental DSC heating curves for samples of DPPC multibilayers containing 50 mol % CSL. Sample concentration was 49 mg/mL. The scans were taken at 0.5 °C/min. A calibration curve is also shown.

the addition of 25 mol % sterol, at which point it disappears.

Similar results were observed for cholesterol in DLPC (Figure 8). There is a small gradual increase in the temperature of the large transition (from 1 to about 6 °C) upon addition of small amounts of cholesterol. However, a large jump occurs at 25 mol % cholesterol, at which point this curve "merges" with the smaller CSL-induced transition. In other words, above 20 mol % cholesterol, there is no longer any melting around 0 °C, the temperature of the main lipid gel–liquid-crystal transition; melting occurs only at the CSL-induced transition temperature. A summary of the observed transitions for these systems is presented in Table II.

Table II: Transition Temperatures Observed from CSL Spectral Data[a]

mol % CHOL	DPPC		DLPC	
	T_l	T_m	T_m	T_l
0	19.5	39	1	20.5
0.1	23.2	39	0	19
0.4	23.7	41		
0.6	25.5	41		
0.9	25.5	38.5	3	21
1.9	27	40		
2.9	26	39.3		
4.9	28	41.3	5	19
9.9	25.7	36.2	5	20
14.9	26.4	38.3	5	22.5
19.9	26.3	39.7	5.5	19.3
24.9	28	*	13	
29.9	28	*	13.5	
33.2	27	*	14	
39.9	24	*	14	
49.9	22	*	15	
59.9	21.3	*	16	

[a] All samples contained 0.1 mol % CSL. T_m = main lipid phase transition temperature (degree Celsius); T_l = spin-label-induced phase transition temperature (degree Celsius); average T_m for DPPC = 39.4 °C. Asterisk means no main lipid transition is observed.

High CSL Concentrations. For further characterization of the behavior of CSL in lipid bilayers, a series of aqueous samples with various spin-label concentrations was investigated by ESR. Significant broadening of the low-temperature spectra becomes evident above 1 mol % CSL. With further addition of spin-label, these spectra consist of a single broad line, which reversibly splits into a triplet at higher temperatures. All lines become progressively broader with increasing spin-label concentration, until, beyond about 33 mol % CSL, the high-temperature triplet is no longer clearly resolved. (These spectra are included in the supplementary material; see paragraph at end of paper regarding supplementary material.)

These results are consistent with those obtained by Träuble (1972) for ASL. Line-shape analyses of the CSL spectra show that the dipolar broadening effect observed with increasing label concentration is accompanied by an increase in exchange interaction between the spin-label molecules (Presti, 1981). In the absence of exchange, dipolar broadening produces a Gaussian-shaped absorption curve, while the exchange interaction renders the line shape closer to Lorentzian (Poole, 1967). The smooth progression from Gaussian line shape at low label concentration to Lorentzian line shape near the critical concentration (about 2 mol %) belies the formation of separate phases in this composition region of the CSL–DPPC system. However, exchange interaction is prevented in multibilayers containing cholesterol, even if fairly large amounts of spin-label are included. Figure 9 includes spectra from such samples (at −5 °C), arranged with increasing CSL content. The hyperfine structure clearly present in spectra from samples containing both sterols is all but totally obscured in spectra obtained from mixtures lacking cholesterol but containing comparable amounts of CSL. In fact, the central peak in the spectrum from the 6.67 mol % CSL sample is a pure Gaussian shape, indicating that cholesterol is effectively preventing the close approach of spin probes that is necessary to observe the Lorentzian shape associated with exchange interactions. Increase in spin-label content is accompanied by growth of a very broad background peak, which is clearly observed from the rising base line upon which the structured spectrum sits. This provides evidence that phase separation

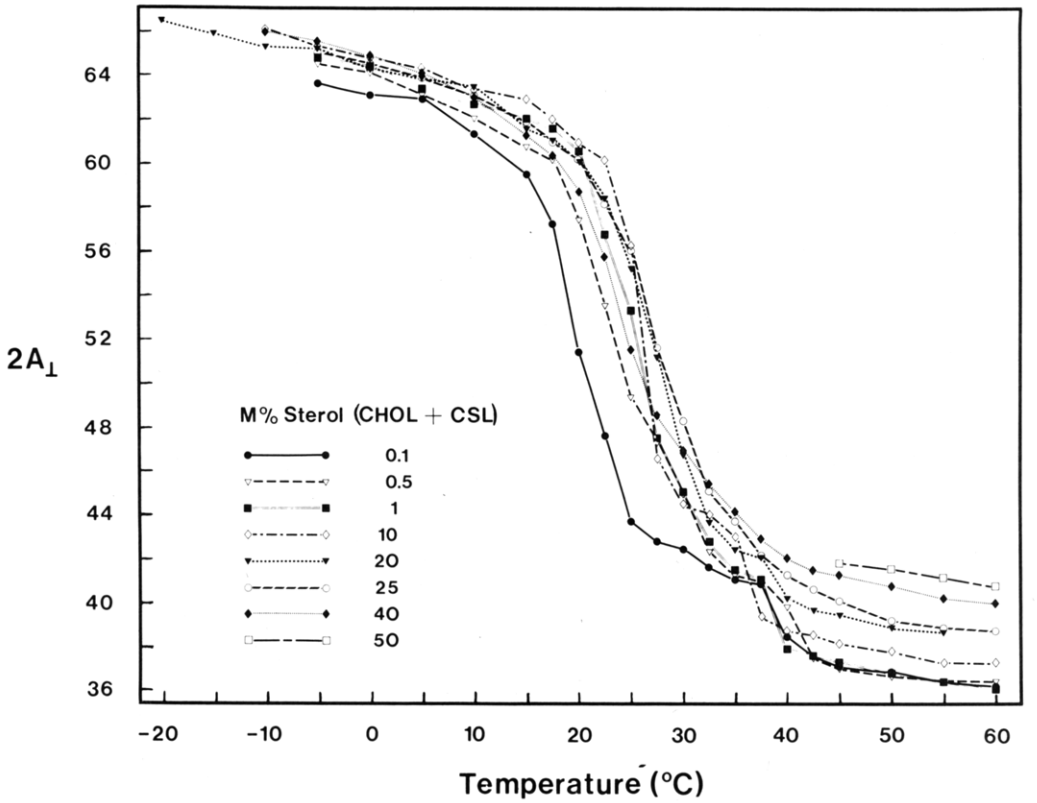

FIGURE 7: Plots of ESR spectral parameter vs. temperature for a series of samples containing various concentrations of cholesterol in DPPC multilayers. All samples contain 0.1 mol % CSL.

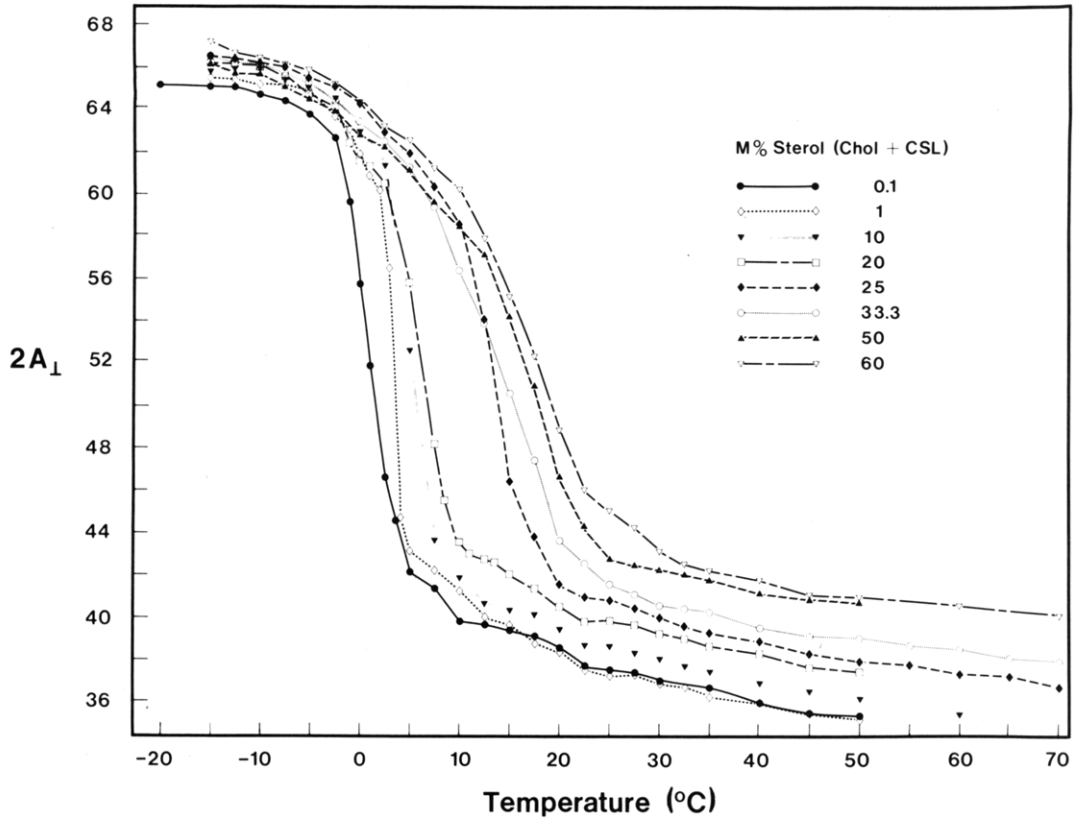

FIGURE 8: Plots of $2A_\perp$ vs. temperature for a series of multibilayer samples containing various concentrations of cholesterol in DLPC. All samples contain 0.1 mol % CSL.

does occur in multibilayers containing cholesterol and that CSL preferentially joins the cholesterol-rich phase, in which exchange broadening is absent. The relatively few spin-probe molecules that remain widely separated within the pure lipid phase are not close enough together, on average, to elicit exchange interactions. The 22.2 mol % CSL sample evidently does not contain enough cholesterol to accommodate all the CSL in the cholesterol-rich phase. The broadening observed in this spectrum is actually comparable to that observed in a sample containing 10 mol % CSL only.

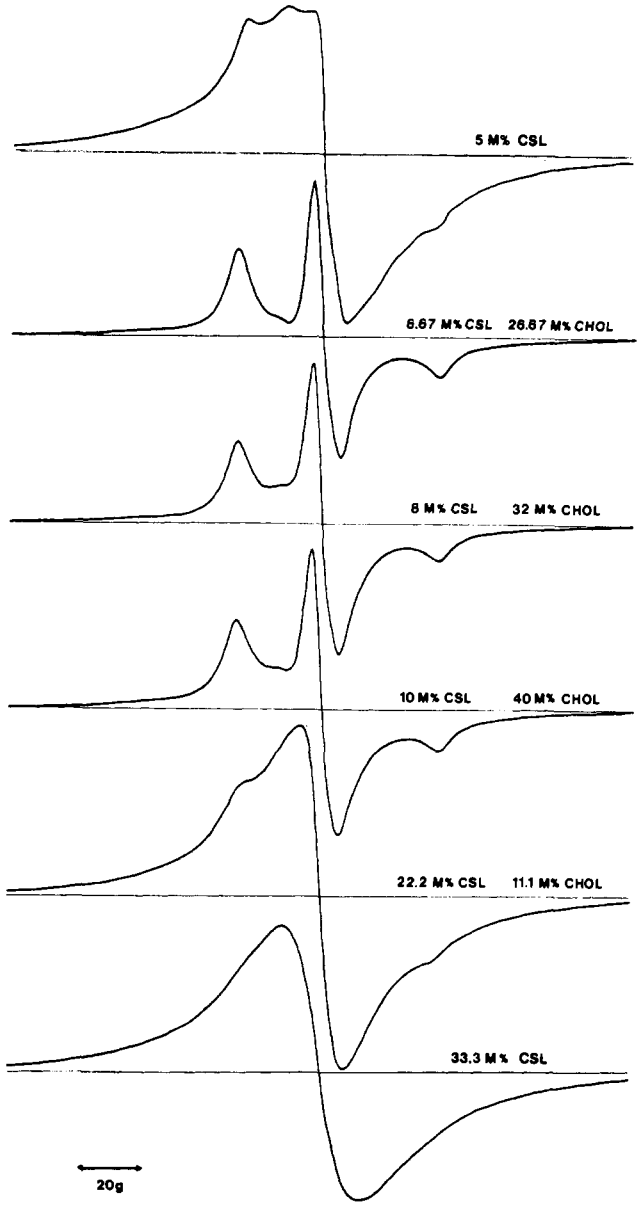

FIGURE 9: Examples of ESR spectra for samples containing increasing amounts of CSL. Fine structure is apparent in the presence of cholesterol, which is not observed if cholesterol is absent. Cholesterol prevents the close contact of spin-label molecules, which leads to exchange broadening. All spectra were recorded at −5 °C.

Discussion

In any concentration, CSL induces a fluidization, probably melting, in neighboring phospholipids, as indicated in these ESR and calorimetric studies of CSL–DPPC mixtures. The cooperativity of this induced melting phenomenon may be estimated from the breadth of the transition by (Mabrey & Sturtevant, 1978)

$$\text{cooperativity} \approx (6.9T_m^2/\Delta T_m)/\Delta H_{cal} \qquad (1)$$

where ΔT_m is the width at half-height of the transition centered at T_m and ΔH_{cal} is the transition enthalpy. A lipid transition with a width of about 15 °C, which is observed for the CSL transition by ESR, would have a cooperativity of 5, assuming the enthalpy of lipid melting has the value observed for pure lipids [ΔH_{cal} = 8740 cal/mol (Mabrey & Sturtevant, 1978)]. Such a highly uncooperative melting would produce a transition that is too broad to observe as a true peak by calorimetry. This might explain why the CSL transition is not observed by calorimetry with less than 10 mol % spin-label present. The small enthalpic change that can be measured at higher CSL concentrations may result from a small fraction of spin-label-associated lipids that melts more cooperatively. The highly sloped backgrounds of these curves preclude accurate determination of the line shapes and areas under the transition regions, particularly at low CSL concentrations. Thus, the cooperativity estimate of 15–20 obtained for the calorimetric peaks with $\Delta T_m \sim 4$ °C provides only an upper limit. This low cooperativity suggests that the induced transition reflects a local melting phenomenon of those lipids immediately surrounding the spin-label.

This transition cannot reflect the phosphatidylcholine pretransition since DPPE, which does not exhibit a pretransition by calorimetry (Chapman et al., 1974), does undergo this low-temperature mobilization. Low-temperature melting of longer chain phospholipids causes shortening of the chains to a length more compatible with the neighboring CSL molecules; less void space is present if the molecules are of comparable length. Lipids shorter than the perturbing spin probe are prevented from melting at their normal gel–liquid-crystal transition temperature because the accompanying shortening of the lipid chains increases the discrepancy in length.

Whichever transition occurs at lower temperature produces the greater change in $2A_\perp$ and is attributed to the onset of long-axis rotation of the spin probe. Melting of the longer chain bulk lipid at the higher gel–liquid-crystal transition temperature provides more freedom for the spin-label to undergo "wobble", and this is responsible for the small additional drop in $2A_\perp$. In the case of DLPC and DOPC, whose main transitions occur below the CSL-induced transition, one may picture the melting of the surrounding bulk lipid as releasing the CSL–lipid domain "icebergs" to move freely in a sea of mobile lipid. When the nearest-neighbor lipids finally melt, the spin-label becomes free to wobble.

The spin-label-induced transition is sensitive to even small additions of cholesterol (see Figures 7 and 8), indicating that CSL is in the vicinity of cholesterol-rich regions of the bilayer. Thus CSL mobility apparently reflects the influence of cholesterol on lipid fluidity, as well as its own perturbation. It is also sensitive to the pure bulk lipid phase, as the phospholipid gel–liquid-crystal transition is observed as an increase in CSL motional freedom. The disappearance of the main lipid transition with more than 20 mol % added cholesterol is consistent with calorimetry data (Mabrey et al., 1978; Estep et al., 1978, 1979) in which cholesterol–phospholipid mixtures exhibit two peaks at the gel–liquid-crystal transition temperature. The sharp peak is abolished by 20–25 mol % cholesterol, while the broad component persists to 50 mol %. The observation that the pure phospholipid phase is abolished by 20 mol % cholesterol is used to elucidate the model for cholesterol–phospholipid interaction that is presented in the following paper (Presti et al., 1982).

From Figures 7 and 8 it is apparent that at high cholesterol concentrations the extent of probe mobilization at high temperatures never reaches that observed at lower concentrations. The value for $2A_\perp$ only reduces to the level that is maintained between the two transitions of the low-cholesterol curves. Since wobble in CSL does not commence until after the lipid melts (Shimoyama et al., 1978), it is likely that this motion is no longer contributing to label mobilization in the presence of high cholesterol. Indeed, Hemminga (1975) notes that cholesterol only influences the wobbling motion of the spin-label and hardly affects the motion about the long molecular axis.

The influence of cholesterol on membrane fluidity is clearly illustrated by the plots in Figures 10 and 11 of the spectral

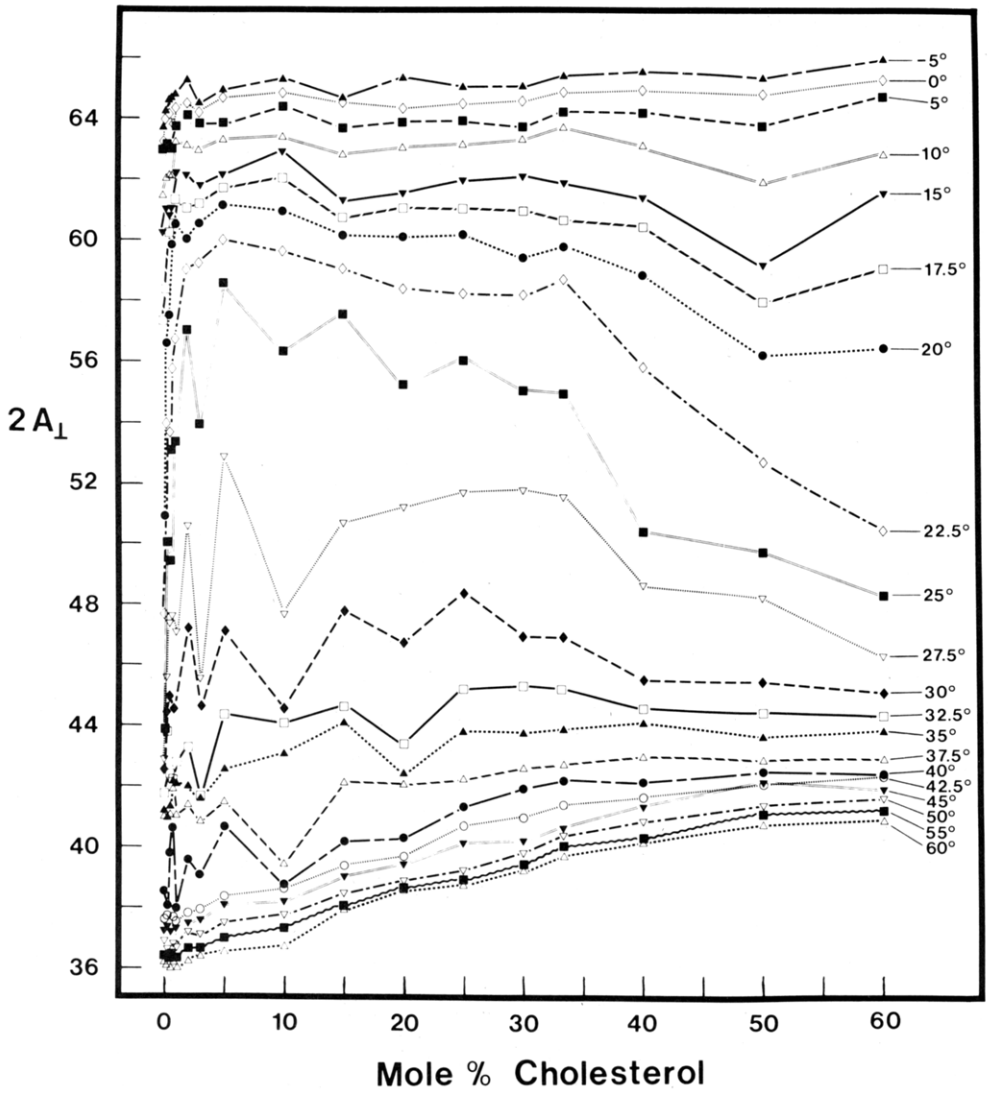

FIGURE 10: Plots of $2A_{\perp}$ vs. mol % cholesterol at various temperatures for 0.1 mol % CSL incorporated into DPPC multibilayers. Cholesterol causes a monotonic decrease in fluidity (increase in $2A_{\perp}$) at temperatures above the phospholipid phase transition. At intermediate temperatures, small additions of sterol produce an initial decrease in fluidity, but more than about 33 mol % cholesterol increases lipid mobility. Very little change with cholesterol is observed at low temperature.

parameter vs. mol % cholesterol at various temperatures. At very low temperatures, when lipid molecules cease all rotation about their long axes (Davis, 1979), cholesterol does not seem to affect their rigid state. At high temperatures, above the lipid phase transitions, the rigidifying effect of cholesterol is apparent in both lipids, manifested by the gradual monotonic increase in $2A_{\perp}$ with added cholesterol. The interpretation of these curves is not so simple for the intermediate temperatures. Very small additions of cholesterol actually rigidify gel-state phospholipids, at least in the vicinity of the spin-label. However, upon addition of more than about $33^{1}/_{3}$ mol % cholesterol, DPPC mobility again increases. From these plots it is quite apparent that the major lipid fluidization occurs in the region of the CSL-induced transition (20–30 °C), which is also the temperature range over which cholesterol exerts its greatest influence. The comparatively small fluidity change that occurs at the main transition (between 37.5 and 42.5 °C) is gone at 25 mol % cholesterol. This is also observed for DLPC near its phase transition; virtually all melting occurs below 7.5 °C for samples with small amounts of cholesterol, whereas at 25 mol % cholesterol, there is virtually no fluidization initiated in this region. At the higher cholesterol concentrations, melting occurs primarily in the CSL transition region (15–20 °C).

Many workers have concluded that the effect of cholesterol is to increase lipid mobility at temperatures below its phase transition (Delmelle et al., 1980). This is shown in this study to be true, but only for additions of 33 mol % or more cholesterol, which happens to be the only concentration many experimenters study. Oriented multibilayers are most easily prepared with a 1:2 cholesterol:lipid ratio, and this is often used even if cholesterol effects are not of interest.

Transition temperatures (Ladbrooke & Chapman, 1969) and hyperfine splittings (Hemmingra & Berendsen, 1972) are known to depend on water content of multibilayers that are not fully hydrated. This is undoubtedly the reason that the results presented here do not agree with similar ESR studies with oriented phospholipid multibilayers. Shimoyama et al. (1978), whose samples contained only 17–22% water, observed a single broad transition centered at 36 °C, which they interpret as representing the main lipid transition. However, this fluidity change may be a temperature-shifted CSL transition. Also, differences in sample preparation may be responsible for conflicting results. Indeed, cursory attempts to repeat Shimoyama's experiments yielded melting only around 65 °C, for oriented multibilayers of DPPC containing 0.75 mol % CSL and 29.25 mol % cholesterol. These samples were hydrated at room temperature by soaking in water for several

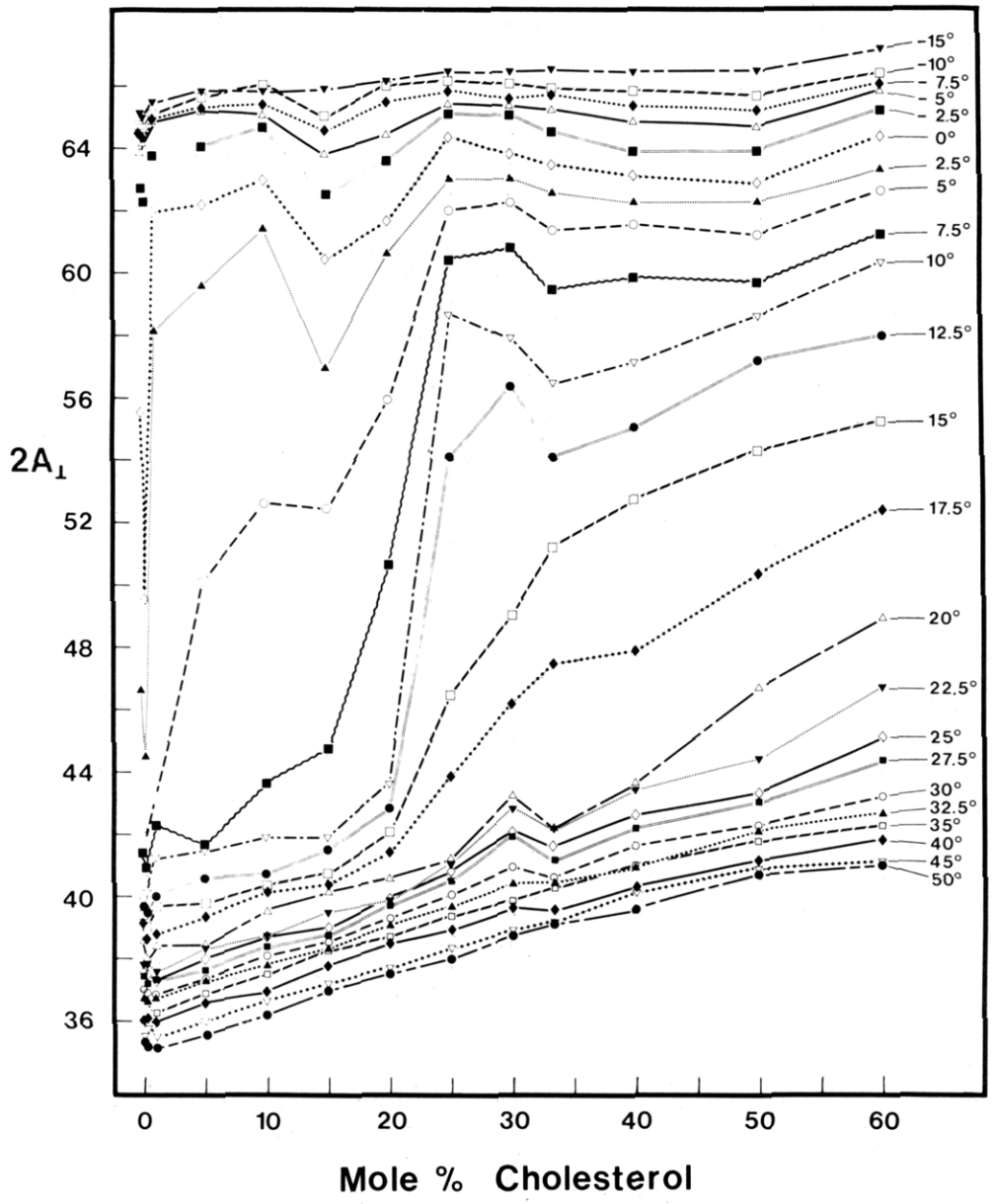

FIGURE 11: Plots of $2A_{\perp}$ vs. mol % cholesterol at various temperatures for 0.1 mol % CSL in DLPC multibilayers. The curves at high and low temperature exhibit the same behavior as those presented for DPPC in Figure 9. Only a small amount of cholesterol is necessary to rigidify DLPC near its main gel–liquid-crystal phase transition temperature. At higher temperatures, 25 mol % cholesterol is required to reduce their mobility.

hours, while Shimoyama's samples were annealed for 30 min under controlled relative humidity at 60 °C. When these conditions were duplicated, resulting in better hydration, the transition curve shown in Figure 12 was obtained. Here, the primary fluidity change occurs at 41.2 °C, with a smaller transition at about 47.7 °C. The appearance of this curve is reminiscent of those obtained with DPPC liposomes containing less than 25 mol % cholesterol. The transition temperatures are higher because the oriented sample contains only about 20% water, whereas 25–40% water (depending on temperature) is required to fully hydrate DPPC (Janiak et al., 1976). The discrepancy in the results obtained by the two laboratories demonstrates the difficulty in reproducing oriented samples with identical properties.

Recently Marsh (1980) obtained temperature-dependent ESR spectra of CSL spin-label in oriented bilayers of DPPC, hydrated by soaking in aqueous buffer for a few hours at room temperature. He observed cooperative onset of long-axis rotation at about 27.5 °C, which he attributed to the pretransition, as well as a smaller transition at 40 °C. This observation is consistent with the results presented in this study. The large mobility change observed at 27.5 °C with CSL as the probe is comparable to the change seen at the DPPC main gel–liquid-crystal transition temperature when Marsh used a stearic acid spin-label. Relatively little change occurs in the long-axis rotation of these lipid probe molecules at temperatures lower than 40 °C. This suggests that true lipid melting is occurring locally around the CSL spin-label molecules at 20–30 °C.

It thus appears that the packing of CSL in the lipid matrix is less thermodynamically stable than that of cholesterol, a factor that permits mobilization of the lipid chains in the immediate vicinity of CSL molecules. The major structural difference between the two sterols is in their head groups, and it is this difference that is undoubtedly reflected in their behavioral differences in aqueous multibilayers. However, disruption of head group interactions between phospholipid and sterol may also be caused by perturbations in other regions of the molecule. For example, the bulky nitroxide group affixed to the interior end of the sterol ring system of ASL

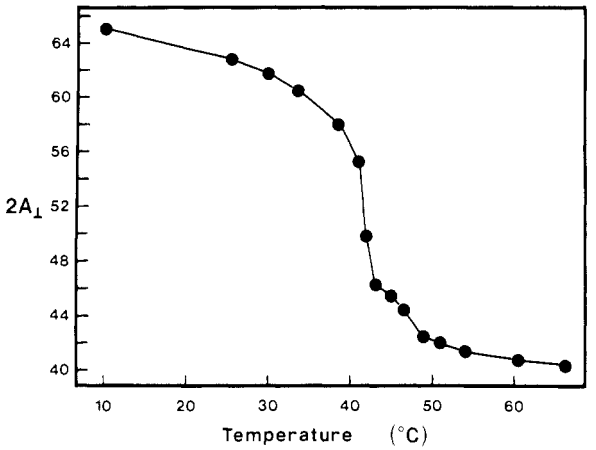

FIGURE 12: ESR transition curve for sample of oriented DPPC multibilayers containing 1 mol % CSL and 19 mol % cholesterol. Sample was hydrated over a saturated KCl solution at 60 °C for 30 min, as in Shimoyama et al. (1978).

probably results in steric hindrance to proper orientation of its hydroxyl head group. This will be discussed in more detail in the following paper (Presti et al., 1982), in conjunction with a proposal for cholesterol–phospholipid molecular interactions.

In conclusion, cholestane spin-label has been shown to induce a characteristic melting of the lipids surrounding it, in contrast to cholesterol, which inhibits the gel–liquid-crystal transition in its neighboring phospholipid. This striking behavioral difference between the two sterols should serve as a caution to those experimenters who would use CSL as an indicator for cholesterol's action in bilayers. However, CSL's similarities to cholesterol make it a relatively nonperturbing probe into cholesterol-rich regions of the bilayer.

We have shown that cholesterol–phospholipid mixed bilayers are composed of two separate phases, a cholesterol-rich phase and a pure phospholipid phase. CSL preferentially joins the cholesterol-rich phase, as indicated by the acute sensitivity of its induced local melting to even small additions of cholesterol. However, it is still sensitive to the state of the pure phospholipid phase, since the lipid's phase transition is observed to affect the probe's mobility. Thus, it has been a useful tool in monitoring fluidity changes in the phospholipid bilayer, including the disappearance of the pure lipid phase at 20 mol % cholesterol content.

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Professor Julian M. Sturtevant provided invaluable assistance in obtaining calorimetry data with his high-sensitivity Privalov instrument.

Supplementary Material Available

Temperature-dependent ESR spectra of DPPC liposomes containing various concentrations of CSL (0.2, 1, 3, 5, 10, 20, $33^{1}/_{3}$, 40, and 50 mol %) (9 pages). Ordering information is given on any current masthead page.

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