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THERMAL EFFECTS ON MOTION AND ORIENTATION OF THE CHOLESTANE SPIN LABEL IN PLANAR MULTIBILAYERS OF DIMYRISTOYLPHOSPHATIDYLCHOLINE AND DIPALMITOYLPHOSPHATIDYLCHOLINE

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A detailed picture of the orientation and restricted motion of the cholestane spin label (3-spiro-doxyl-5α-cholestane) in planar multibilayers of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine has been recorded by simultaneous simulation of ESR spectra obtained with the magnetic field parallel and perpendicular to the bilayers (Shimoyama, Y., Eriksson, L.E.G. and Ehrenberg, A. (1978) Biochim. Biophys. Acta 508, 213–235). The analysis has been made over the temperature range −30°C to 60°C on samples containing 20 to 22% water. At low temperatures the cholestane spin label is tilted with respect to the lipid bilayer normal by an angle of approx. 30° which disappears at the pretransition. In this low temperature range the restricted twisting motion has an activation energy of 5.5 kJ · mol^{-1}. Above the main transition the twisting motion is unrestricted and has the activation energy 20 kJ · mol^{-1}. From below the pretransition to above the main transition the velocity of the twisting motion increases by an order of magnitude. The amplitude of the wobbling motion increases abruptly from 0° to 35° at the main transition.

Introduction

The phase behaviour of synthetic phospholipid-water multibilayer systems has been the subject of many investigations. Techniques such as X-ray diffraction [1–5], infrared and Raman spectroscopy [6,7], ^{2}H-NMR [8,9] and electron spin resonance [10–12] have been used to provide information concerning molecular arrangement and dynamics under various conditions of hydration and temperature. Thermotropic phase transitions have been characterized by differential scanning calorimetry [2,13].

The saturated lipids dimyristoyl- (DMPC) and dipalmitoylphosphatidylcholine (DPPC) exhibit an order-disorder transition associated with the melting of the hydrocarbon chains. At high water content (20% w/w or more) an additional broader pretransition occurs at temperatures below the chain-melting transition. Below the pretransition the hydrocarbon chains are highly ordered and tilted with respect to the bilayer normal. The tilt angle has been measured by X-ray diffraction [1,14–16], neutron scattering [17] and electron spin resonance using spin labels [18,19]. The ESR work has been made on oriented phospholipid multibilayers. Birrell and Griffith [18] used spin-labeled phosphatidylcholine to show that a significant angle of tilt, varying between 33° at the polar interface and 21° towards the center of the bilayer, exists along the entire length of the lipid chains in

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Abbreviations: CSL, cholestane spin label; 5-SASL, stearic acid spin label; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

DPPC. Hemminga [19] provided evidence that the cholestane spin label is tilted by about 25° in a similar system with a lower water content. The X-ray diffraction investigations agree on a tilt angle of the order of 30° at room temperature for fully hydrated DPPC. Neutron scattering data are conflicting. Zaccai et al. [17] have reported an absence of tilting in DPPC multibilayers containing 10% water.

The steroid analogue cholestane spin label (CSL, 3-spirodoxyl-5α-cholestane) is a good qualitative reporter of bulk effects in membranes [20] and in some respects resembles cholesterol, a common constituent of biological membranes, in its chemical and physical properties [21]. The CSL is incorporated into lipid bilayers with the steroid nucleus embedded in the hydrophobic region and the nitroxide located near the polar headgroups of the phospholipids [22]. Due to its rigid nature, the molecule is expected to be sensitive to and reflect orientation and mobility of the fatty acyl chains. The major axis of the hyperfine tensor is closely perpendicular to the long molecular axis which gives a possibility to resolve both rotation around the long molecular axis and perpendicular to this axis [10,23].

CSL incorporated into lipid bilayers exhibits a restricted anisotropic motion. The motional parameters and associated correlation times can be determined by computer simulation of the ESR spectra. We have previously [24,25] developed a model for the rapid restricted motion of a spin labeled molecule. The motion is composed of a rotation around the long molecular axis and a simultaneous tumbling of this axis within the confines of a cone. The model was applied to the evaluation of ESR spectra from spin labels incorporated into phospholipid dispersions [25] and was extended to oriented multibilayers [23]. The order and motion of CSL incorporated into egg yolk phosphatidylcholine, DMPC and DPPC, pure and in mixture with cholesterol, were studied at various temperatures. For pure DMPC and DPPC it was only possible to simulate spectra obtained above the main transition of the lipids. The lack of success at lower temperatures was due to an imperfection in the computer program with regard to handling the spatial distribution of the tilt angle. The program has now been revised (Shimoyama, Y., personal communication).

Related studies concerning the motion of CSL in gel phase phospholipids have been performed in oriented multibilayers by Hemminga [10] and Marsh [11] and in dispersions by Polnaszek et al. [12]. Hemminga uses a system with very low level of hydration and gives a static description. Marsh and Polnaszek only consider rotation about the main molecular axis of the CSL and the correlation time is deduced from line splittings and line heights in the experimental spectra using empirical calibrations.

From calorimetry [13] and density measurements [26] the transitions between the crystalline (gel) state and the liquid-crystalline state are known to be centered at approx. 41.5 and 24.0°C for DPPC and DPMC, respectively. In addition, by the same methods, the minor, so called pretransitions, have been determined for the two lipids to take place at approx. 34 and 14°C, respectively. In the present work the ESR spectra of CSL incorporated into oriented multibilayers of DPPC and DMPC are recorded and analyzed over a wide range of temperatures, −30°C to +60°C. Particular attention is paid to the behaviour of the various parameters of the spin label, measured directly on the ESR spectra, and determined by computer simulations, in relation to the known transitions of the pure lipids. As a result a detailed picture is presented of the temperature dependence of the motional parameters of the CSL probe. The extent to which they reflect changes in order and motion of the lipids in the host multibilayers of DPPC and DMPC is analyzed and discussed.

Materials and Methods

L-α-Dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma with a purity of 98% and 99%, respectively. The purity was checked by thin-layer chromatography and the lipids were used without further purification. The cholestane spin label (3-spiro-doxyl-5α-cholestane) was synthesized by the method of Keana et al. [27]. The stearic acid spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-SASL) was purchased from SYVA Research Chemicals and used without further purification.

Preparation of oriented multibilayers

The oriented multibilayers were prepared according to a modification of the method described previously [23].

The phospholipid and the spin label were dissolved in chloroform/ethanol (7 : 3, v/v). The solution was thoroughly mixed and then distributed in 20-μl aliquots on a glass plate 3 mm × 30 mm mounted on a plexiglass rod. The solvent was evaporated at a temperature above the transition temperature of the phosphatidylcholine under a stream of wet nitrogen. Residual solvent was removed by exposure to high vacuum.

Hydration was achieved by equilibration against a constant relative humidity of 98% unless otherwise stated. The water content was 20–22% w/w, determined by gravimetric analysis as described elsewhere [23].

The samples contained 3000 to 5000 bilayers and the molar ratio of spin label to phospholipid was 0.5% in all cases.

Electron spin resonance measurements

ESR measurements were made on a JEOL ME-1X, or occasionally on a Varian E9, X-band spectrometer. The sample temperature was regulated with a heater-sensor system which is a modification of the Varian V-4557 variable temperature accessory. The system consists of a transfer dewar (Varian S-837) equipped with a heater. Compressed air or nitrogen, precooled by passage through a copper loop submerged in a dry ice-ethanol bath, is flushed through the system. The temperature is measured by a platinum resistor located below the sample in the spectrometer cavity. This sensor is part of a bridge circuit which via the heater controls the temperature.

The temperature was calibrated to ± 0.2°C in a series of separate measurements with an ESR tube containing silicon oil and a second platinum resistor in the sample position. The gas flow was measured with a rotameter and kept at 4 l/min. The use of dry ice-ethanol as a cooling medium instead of liquid nitrogen has the advantage of increasing the precision of the regulation since the gas when leaving the heat exchanger has a temperature just below the range of temperatures used in the experiments.

The hydration of the sample during ESR measurements was kept at the preequilibrated level by having a saturated solution of potassium sulfate [28] at the bottom of the ESR tube in which the sample was mounted [23].

The sample could be oriented in the cavity of the spectrometer by means of a goniometer. The angular positions for maximum and minimum hyperfine splittings were determined with the precision of 1 degree. The goniometer settings for these orientations were best determined at a temperature above 25°C. ESR spectra were recorded at various temperatures for these two orientations, i.e. with the plane of the multibilayers parallel and perpendicular, respectively, to the static magnetic field. For each pair of spectra, the hyperfine splittings $T'_{\parallel}$ and $T'_{\perp}$ were measured as indicated in Fig. 3. In some cases spectra were recorded with the sample oriented at various angles θ' away from the perpendicular orientation. In these spectra the splitting T' between the low and middle field baseline crossings was measured as indicated in Fig. 3 for $T'_{\perp}$.

Simulation procedure

Each pair of spectra recorded in the parallel and perpendicular directions was simulated with a single set of motional parameters as described in Ref. 23. An Amdahl 470/V7 computer was used.

The following values for the hyperfine T and g tensor components were applied [23,25]:

$T_{xx} = 0.56$ mT $\quad T_{yy} = 3.42$ mT $\quad T_{zz} = 0.56$ mT

$g_{xx} = 2.0083 \quad g_{yy} = 2.0021 \quad g_{zz} = 2.0064$

T_{yy} and g_{yy} were determined from powder spectra of lipid dispersions at -196°C [25]. T_{zz} and g_{zz} were obtained in the perpendicular direction for samples with $\beta_0 = 0$, vide infra [23]. T_{xx} and g_{xx} were selected to fit spectra in both directions of different samples at various temperatures. On several occasions other published sets of parameters [10,29] were used for comparison but the overall fit was not improved and deteriorated particularly at lower temperatures with $T_{yy} < 3.42$ mT. From our experience other minor changes in the T and g tensor components did not affect significantly the pattern of changes in estimated motional parameters.

The g and T tensor components are defined in a

right-handed molecular reference frame where the z axis is in the direction of the principal axis of the cholestane spin label molecule. This nomenclature [25] is different from that commonly used in the literature, where the z-axis is defined by the largest hyperfine splitting [29].

Two modes of motion are considered [24,25]:

(1) Restricted twisting motion around the main molecular axis with angular amplitude $\pm\phi_0$ and correlation time τ_a.

This is valid when (a) $T'_{\parallel} > \frac{1}{2}(T_{xx} + T_{yy})$ and (b) $T'_{\perp} \sim T_{zz}$. There is no tumbling of the long molecular axis, i.e. $\beta_0 = 0$.

A starting value, ϕ'_0, of the twisting amplitude was obtained from the relationship (Ref. 23, Eqn. 4):

$$\frac{\sin 2\phi'_0}{2\phi'_0} = \frac{2T'_{\parallel} - (T_{xx} + T_{yy})}{T_{yy} - T_{xx}} \tag{1}$$

(2) Rapid fully liberated twisting motion ($\phi_0 = 90°$) and restricted wobbling within a cone of semi-cone angle β_0. The correlation time is τ_t for the tumbling motion and τ_{at} for the axial motion.

This is valid when (a) $T'_{\parallel} < \frac{1}{2}(T_{xx} + T_{yy})$ and (b) $T'_{\perp} > T_{zz}$. A preliminary value, β'_0, of the semi-cone angle was obtained from the equation (Ref. 23, Eqn. 5):

$$\tfrac{1}{2}\cos\beta'_0(1+\cos\beta'_0) = \frac{T'_{\parallel} - T'_{\perp}}{\frac{1}{2}(T_{xx} + T_{yy}) - T_{zz}} \tag{2}$$

From the final best fit value of β_0 the order parameter was calculated according to

$$S_{zz} = \tfrac{1}{2}\cos\beta_0(1+\cos\beta_0) \tag{3}$$

The distribution of the cone axes is supposed to be symmetric with respect to the normal of the bilayer plane and is accounted for by a Gaussian distribution function [29]

$$P(\vartheta) = \sin\vartheta \exp\left\{-\frac{(\vartheta - \bar{\vartheta})^2}{2\vartheta_0^2}\right\} \tag{4}$$

Here the tilt angle $\bar{\vartheta}$ is the angle between the director and the normal, and the spread angle ϑ_0 defines the width of the distribution around the director. The weighting function $\sin\vartheta$ accounts for the solid angle over the whole sphere. In this model each local microregion of the sample has its own tilt direction, but taken over all microregions all tilt directions are equally probable.

A tilt angle $\bar{\vartheta} > 0$ is needed only for lipids in the gel state, i.e. case 1 with $\beta_0 = 0$. In this case condition (b) has to be relaxed since $T'_{\perp} > T_{zz}$ when there is a tilt.

The residual linewidth is mainly attributed to unresolved proton hyperfine splittings and is orientation dependent [23]. The linewidths $w_{\parallel}$ and $w_{\perp}$ used in the simulations were determined empirically for both directions. The lineshape function is a mixture of Lorentzian and Gaussian shapes in a ratio of 4 : 1.

Results

Directly measured spectral parameters $\parallel$ and $\perp$ to the bilayers

ESR spectra of the cholestane spin label incorporated into planar multibilayers of DMPC and DPPC were recorded over the temperature range -30 to 60°C and -35 to 70°C, respectively, at intervals of 2.5 or 5 C deg, depending on the temperature sensitivity of the spectral shape. At each temperature the sample was equilibrated for 10 min before spectra were recorded with the sample oriented parallel and perpendicular to the magnetic field. No hysteresis effects, depending on direction of temperature variation, could be detected. In general, measured values of $T'_{\parallel}$ and $T'_{\perp}$ were reproducible within ± 0.02 mT at a given temperature. However, in the steepest region of change variations of up to ± 0.05 mT were observed between individual samples, and in the region 25 to 35°C for DPPC and 10 to 20°C for DMPC the uncertainties were also larger because of ill defined extrema. The shapes of the curves for the variation of $T'_{\parallel}$ and $T'_{\perp}$ with temperature shown in Figs. 1 and 2 were reproducible between different samples.

In the case of DPPC it was tested whether the magnetic field had any influence on the orientation of the lipids as was previously observed in the field of a Q-band spectrometer [23]. In particular it was also examined if the orientation of the sample in the magnetic field while lowering the temperature from above the transition temperature

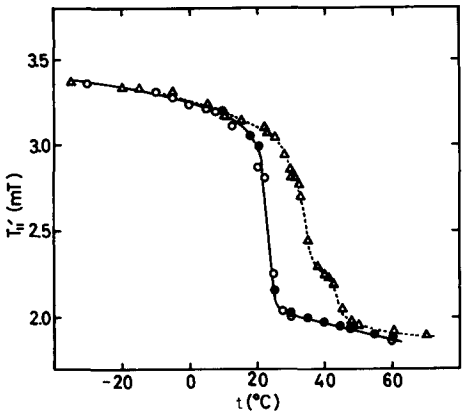

Fig. 1. Temperature variation of the apparent hyperfine splitting $T'_{\parallel}$ measured from the ESR spectra of cholestane spin label in oriented multibilayers of DMPC (○, ●), two samples, and DPPC (△). $T'_{\parallel}$ is half of the distance between the derivative peaks at high- and low-field extrema of spectra taken with the magnetic field parallel to the bilayer surface.

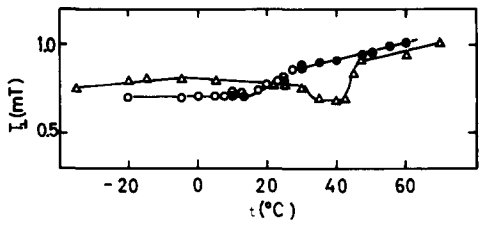

Fig. 2. Temperature variation of the apparent hyperfine splitting $T'_{\perp}$ measured from the ESR spectra of cholestane spin label in oriented multibilayers of DMPC (○, ●), two samples, and DPPC (△). $T'_{\perp}$ is the distance between the derivative peaks at high- and middle-field zero crossings of spectra taken with the magnetic field perpendicular to the bilayer surface.

to below −20°C had any influence on the ESR spectra. No effect of the magnetic field was detected.

The variation with temperature of $T'_{\parallel}$ for CSL in planar multibilayers of DMPC and DPPC is shown in Fig. 1. For DPPC there is a slow decrease of $T'_{\parallel}$ going from −30 to 25°C with an indication of a change of slope at about −5°C. Between 25 and 50°C the decrease is steep, but with a shelf at 40°C, and above 50°C it is again small and nearly linear. For DMPC the level and slope between −35 and 20°C are nearly the same as for DPPC in the same range, there is a steep transition between 20 and 26°C, and above the latter temperature the decrease of $T'_{\parallel}$ with increasing temperature is small but somewhat larger than in DPPC above 50°C.

The temperature dependence of $T'_{\perp}$ is shown in Fig. 2. For DPPC $T'_{\perp}$ decreases only slightly from −22 to 30°C but between 30 and 35°C there is a marked drop. Between 35 and 42.5°C $T'_{\perp}$ is nearly constant, between 42.5 and 47°C there is a small but sharp increase, and above 47°C there is a small linear increase. With DMPC $T'_{\perp}$ is slightly increasing from 10 to 20°C, increases comparatively steeply between 20 and 27.5°C, and increases above 27.5°C less steeply and with about the same slope as for DPPC above 47.5°C.

The midpoint of the steep transition of $T'_{\parallel}$ for DMPC (Fig. 1) is centered at 23°C and the half-width, i.e. the width between the points corresponding to 1/4 of the transition at each side of the center, is 3.5 C deg. The same transition is revealed by $T'_{\perp}$ (Fig. 2). It most likely reflects the main transition between the gel and liquid-crystalline phases which for DMPC has been determined to be at 24°C [13].

For DPPC the steep change of $T'_{\parallel}$ goes in two steps. The largest and steepest change is centered at 34°C and apparently coincides with the pretransition [13]. The smaller transition centered at 44°C is 2.5 C deg. above the main transition [13]. The widths of the two transitions are about 3.5 C deg as for the transition of $T'_{\parallel}$ in DMPC. The two transitions are also seen in the curve for $T'_{\perp}$ (Fig. 2) as a decreasing and increasing step, respectively.

Parameters deduced by simulation of spectra ∥ and ⊥ to the bilayers

Spectra were simulated to best fit by the procedure described in the section of Materials and Methods. A comparison of experimental and simulated spectra in the case of DPPC is given in Fig. 3 for some representative temperatures. The parameter values of the best fit simulations are given in Tables I and II for DMPC and DPPC, respectively, also at selected temperatures.

In particular, it should be noted that for both lipids it was not possible to obtain reasonable simulations of the spectra below 27°C for DPPC and 18°C for DMPC without using a tilt angle (Table II). At −20°C the tilt angle needed is 25° and 28°, respectively. With increasing temperature

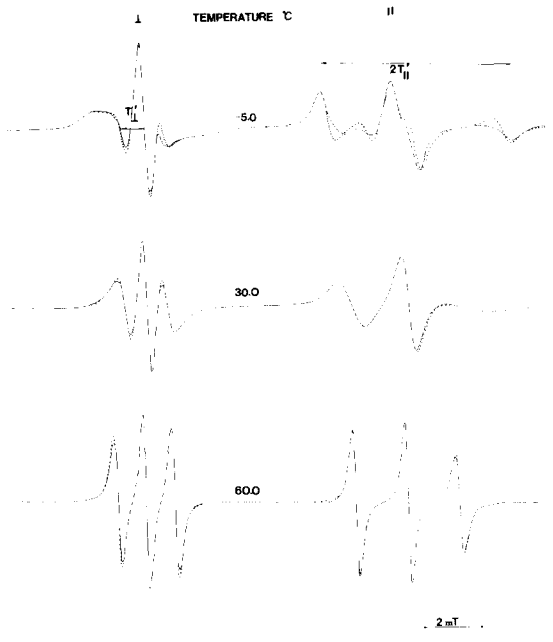

Fig. 3. Experimental (broken line) and simulated (solid line) ESR X-band spectra of the cholestane spin label (0.5 mol%) in oriented multibilayers of DPPC. The sample was equilibrated under the controlled relative humidity of 98% and was oriented with the film plane parallel (||) and perpendicular (⊥) to the static magnetic field directions. The parameters used for the simulated spectra are summarized in Table II.

the tilt angle decreases gradually and above the mentioned temperatures 27°C and 18°C, respectively, it had vanished. To improve the fit a spread angle (cf. Eqn. 4) was also needed: For DPPC a spread angle of 15° at 32.5°C and below, and 8° and 42.5°C and above; for DMPC a spread angle of 17° at 5°C and below, and 6° at 40°C and above. Intermediate values were used in the intermediate regions of temperature.

The variation with temperature of the amplitudes of the twisting motion, ϕ_0, and of the wobbling motion, β_0, is shown in Fig. 4. For temperatures below 25°C for DMPC and 42.5°C for DPPC it was possible to simulate spectra with $\phi_0 < 90°$ and $\beta_0 = 0$. At the mentioned temperatures and above, simulations required $\phi_0 = 90°$ and $\beta_0 > 35°$. The jump of β_0 from 0 to 35° is in both cases apparently coinciding with the temperatures of the main transitions, 24°C and 41.5°C, respectively. For DPPC, at 37.5°C and 40°C, simulations were attempted with $\phi_0 = 90°$ and $\beta_0 > 0$. In the parallel direction, reasonable fits were obtained with $\beta_0 = 25°$, but the fits in the perpendicular direction were quite unsatisfactory. The model does not include the possibility to simulate the spectra with $\phi_0 < 90°$ and $\beta_0 > 0°$.

TABLE I

PARAMETERS USED FOR THE SIMULATION OF X-BAND SPECTRA FROM CHOLESTANE SPIN LABEL IN ORIENTED MULTIBILAYERS OF DIMYRISTOYLPHOSPHATIDYLCHOLINE AT VARIOUS TEMPERATURES

The lineshape function used for simulation is a mixture of Lorentzian and Gaussian shapes in a ratio of 4:1. The effective velocity τ_{eff}^{-1} is related to the various correlation times by $\tau_{eff}^{-1} = \Sigma\tau^{-1}$.

Temperature (°C)	Correlation times (ns)				Motional angles (degrees)		Tilt spread (degrees)		Residual linewidth (mT)	
	τ_a	τ_t	τ_{at}	τ_{eff}	ϕ_0	β_0	$\bar{\vartheta}$	ϑ_0	$w_{\|}$	$w_{\perp}$
−20	3.0	–	–	3.0	30	0	28	17	0.45	0.30
+5	3.0	–	–	3.0	42	0	15	17	0.45	0.30
10	3.0	–	–	3.0	45	0	5	17	0.45	0.30
17.5	3.0	–	–	3.0	55	0	0	17	0.45	0.29
21	3.0	–	–	3.0	65	0	0	12	0.45	0.28
25	–	8.0	1.75	1.44	90	35	0	10	0.35	0.26
35	–	5.0	0.5	0.44	90	43	0	7	0.30	0.26
45	–	2.0	0.35	0.30	90	47	0	6	0.28	0.25
55	–	1.0	0.25	0.20	90	49	0	6	0.25	0.25

Estimated accuracies (± half the difference between values that can be distinguished): τ_a: ±0.25 ns, τ_t: ±0.25 ns for 8 ns > τ_t > 2 ns; ±0.1 ns for τ_t < 2 ns, τ_{at}: ±0.05 ns, ϕ_0: ±1° for $\phi_0 < 50°$; ±2.5° for $\phi_0 > 50°$, β_0: ±1°, $\bar{\vartheta}$: ±1°, ϑ_0: ±1° for $\bar{\vartheta} > 0$; ±0.5° for $\bar{\vartheta} = 0$, $w_{\|}$: ±0.025 mT for $\phi_0 < 50°$; ±0.01 mT for $\phi_0 = 90°$, $\beta_0 > 0$, $w_{\perp}$: ±0.01 mT for $\phi_0 < 50°$; ±0.005 mT for $\phi_0 = 90°$, $\beta_0 > 0$.

TABLE II

PARAMETERS USED FOR THE SIMULATION OF X-BAND SPECTRA FROM CHOLESTANE SPIN LABEL IN ORIENTED MULTIBILAYERS OF DIPALMITOYLPHOSPHATIDYLCHOLINE AT VARIOUS TEMPERATURES

The lineshape function used for simulation is a mixture of Lorentzian and Gaussian shapes in a ratio of 4:0. The effective velocity τ_{eff}^{-1} is related to the various correlation times by $\tau_{eff}^{-1} = \Sigma\tau^{-1}$. For estimated accuracies see footnote to Table I.

Temperature (°C)	Correlation times (ns)				Motional angles (degrees)		Tilt spread (degrees)		Residual linewidth (mT)	
	τ_a	τ_t	τ_{at}	τ_{eff}	ϕ_0	β_0	ϑ	$\bar{\vartheta}_0$	$w_{\|}$	$w_{\perp}$
−20	3.0	–	–	3.0	33	0	25	15	0.45	0.45
−5	3.0	–	–	3.0	35	0	20	15	0.45	0.38
+15	3.0	–	–	3.0	45	0	15	15	0.45	0.35
+20	3.0	–	–	3.0	50	0	10	15	0.45	0.34
+25	3.0	–	–	3.0	55	0	0	15	0.45	0.30
+30	2.75	–	–	2.75	70	0	0	15	0.45	0.30
+35	2.0	–	–	2.0	85	0	0	12	0.42	0.30
+40	1.5	–	–	1.5	89	0	0	10	0.40	0.30
+42.5	–	4.0	0.8	0.67	90	35	0	8	0.40	0.30
+45	–	2.0	0.4	0.33	90	37	0	8	0.34	0.28
+60	–	0.7	0.2	0.18	90	43	0	8	0.28	0.28

From Fig. 4 it is further noted that for both lipids, and at temperatures below 0°C, ϕ_0 approaches a value of about 30°, and at high temperatures, β_0 goes towards an asymptotic value of about 50°. For DMPC the steepest change of ϕ_0 is close to the main transition, 24°C, whereas for DPPC ϕ_0 has its steepest change at 30°C, which is well below the pretransition, and only gradually approaches 90° when temperature increases towards the main transition.

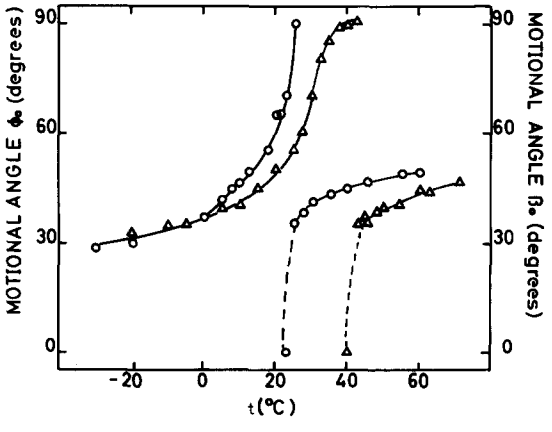

Fig. 4. Temperature dependence of the motional angles ϕ_0 and β_0 for cholestane spin label in oriented multibilayers of DMPC (○) and DPPC (△), determined by spectral simulation.

We earlier [23] studied the temperature dependence of the rotational motion of CSL in lipid bilayers by making Arrhenius graphs with log τ_{eff}^{-1} plotted versus T^{-1}, with

$$\tau_{eff}^{-1} = \tau_a^{-1} \quad \text{for } \phi_0 < 90°,\ \beta_0 = 0, \text{ and} \tag{5a}$$

$$\tau_{eff}^{-1} = \tau_{at}^{-1} + \tau_t^{-1} \quad \text{for } \phi_0 = 90°,\ \beta_0 > 0. \tag{5b}$$

The corresponding Arrhenius plots for the correlation times obtained in the present experiments with DMPC and DPPC are shown in Fig. 5.

Above 45°C the slopes of the two curves are nearly the same and an average apparent activation energy of 20 kJ/mol is obtained. Below 21°C the two curves coincide, the slope and hence the apparent activation energy are both 0. In the intermediate temperature range there is for both lipids a transition with the steepest change somewhat above the temperature of the main transition of the lipid. For DPPC the shape of the curve suggests a second transition in the range 30 to 35°C.

The vanishing activation energy below 20°C is of course only apparent. It should be noted that the twisting motion is restricted, with the mean amplitude ϕ_0 also changing with temperature (Fig. 4). Hence, we have found it worthwhile to explore the temperature dependence of the quantity $\phi_0 \cdot \tau_{eff}^{-1}$, which has the dimension of angular velocity.

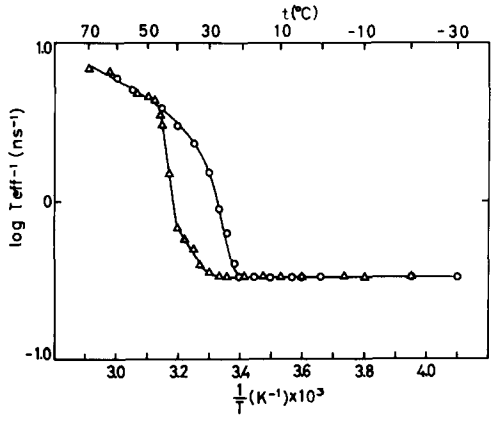

Fig. 5. Arrhenius type plot. Temperature dependence of the reciprocal correlation time τ_{eff}^{-1} for cholestane spin label in oriented multibilayers of DMPC (○) and DPPC (△), determined by spectral simulation.

Since τ_t is not directly connected with ϕ_0, we have discarded the second term on the right side of Eqn. 5b, which has a quite small influence anyhow, and constructed the Arrhenius plots of Fig. 6. At temperatures above the main transitions, the curves of Figs. 6 and 5 are coinciding, except for the small change caused by neglect of the term with τ_t. However, at low temperatures there is a measurable activation energy and the second transition of DPPC is better expressed.

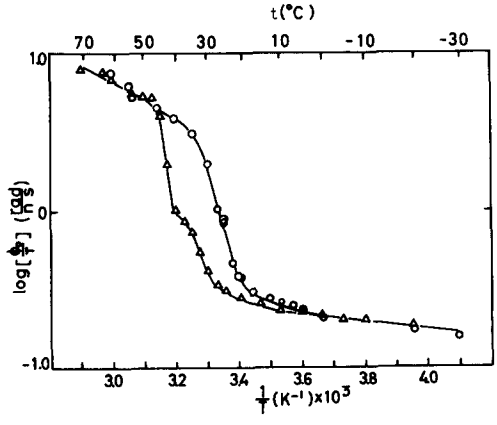

Fig. 6. Arrhenius type plot. Temperature dependence of the angular velocity ϕ_0/τ for the twisting motion around the main molecular axis of cholestane spin label in oriented multibilayers of DMPC (○) and DPPC (△). Below the main transition, $\tau = \tau_a$ and above the transition, $\tau = \tau_{at}$. The motional angle ϕ_0 is expressed in radians, and τ in ns.

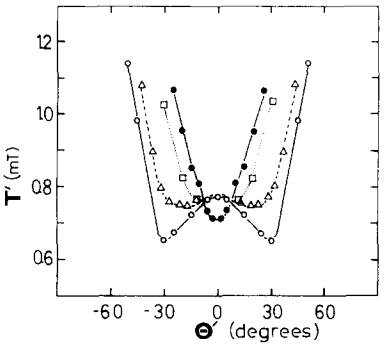

Fig. 7. Angular dependence of the apparent hyperfine splitting T' between the low- and middle-field baseline crossings, measured at −20 (○), +5 (△), +22 (□) and +35°C (●).

Variation of the splitting T' with bilayer orientation and temperature

The apparent hyperfine splitting T' between the low and middle field baseline crossings (cf. measurement of $T'_\perp$, Fig. 3) was measured for DPPC at various temperatures and various settings of the goniometer angle θ' away from the direction with

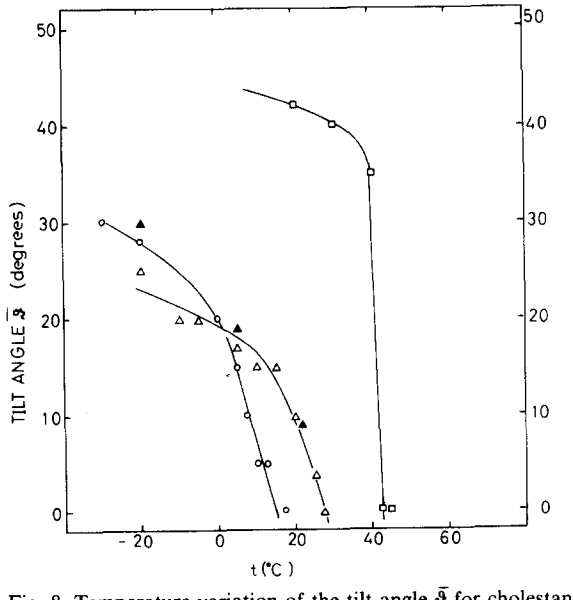

Fig. 8. Temperature variation of the tilt angle $\bar{\vartheta}$ for cholestane spin label in oriented multibilayers of DMPC (○) and DPPC (△), obtained by spectral simulation. Experimental values (▲) of $\bar{\vartheta}$ for CSL in DPPC, estimated from the angular variation of the apparent hyperfine splitting T' at various temperatures (Fig. 7), and the experimental values (□) for 5-stearic acid spin label are included for comparison.

the field perpendicular to the bilayers. The results are shown in Fig. 7. At the lowest temperatures T' has a distinct maximum for $\theta' = 0$ and passes through minima some angle away on either side. Half the distance between these minima is in degrees: 30 (−20°C), 19 (5°C), and 10 (22°C) at the temperatures given within brackets. The tilt angles determined at the same temperatures by simulation of the spectra in the parallel and perpendicular directions are 25, 17 and 8 degrees, respectively. Above 25°C there is only a central minimum for $\theta' = 0$ in the plots of Fig. 7 and no tilt angle is needed in the simulation. Apparently the half distance between the minima is a good measure of the tilt angle. The angles measured this way are also plotted in Fig. 8.

Discussion

The presented results reveal several interesting features about the ordering and motion of CSL in lipid bilayers of DPPC and DMPC hydrated at 98% relative humidity. These results will now be discussed in relation to other data available for this type of system.

The structure and dynamics of saturated diacylphosphatidylcholines have been studied under different experimental conditions by a variety of spectroscopic techniques [1–12]. Several temperature-water composition phase diagrams have been constructed for the lipids [1,7,30]. A model for the generalized behaviour of hydrated synthetic phosphatidylcholines has been proposed by Janiak et al. [3].

At low temperatures and at low water content the lipids form a one-dimensional lamellar structure, called $L_{\beta'}$ in the nomenclature of Luzzati [31]. The phospholipid acyl chains are fully extended and packed in a distorted hexagonal lattice [2]. The chains are tilted with respect to the normal of the bilayer in order to accommodate the choline headgroup in its conformation parallel to the bilayer surface [4]. At very low temperatures the lattice becomes more distorted, assuming an orthorhombic or monoclinic form [8] and the tilt angle increases. The tilt angle has been measured directly by X-ray [1,14–16] and by ESR using spin labels [18,19].

In samples containing 20% water by weight or more a broad, low enthalpy, transition occurs at about 14°C for DMPC and 34°C for DPPC [13]. This pretransition is associated with a structural transformation from a one-dimensional lamellar to a two-dimensional monoclinic lattice consisting of lipid lamellae distorted by a periodic ripple [2,32]. Rand et al. [33] and Brady and Fein [34] concluded that at the pretransition the tilted hydrocarbon chains become perpendicular to the bilayer forming a new phase (P_β) whereas according to Janiak [2] the chains are still tilted in this phase. Recently Stomatoff et al. [5] have measured the rippling period and suggested that rippling produces a large enough out-of-plane displacement to accommodate straight hydrocarbon chains and large headgroups.

The rippled structure persists to the main gel to liquid-crystal phase transition, which occurs at about 24°C for DMPC and 41.5°C for DPPC [13,35] at the level of hydration of our samples. Above the main transition, the lipids are once more in a one-dimensional lamellar phase, L_α, with the hydrocarbon chains highly distorted, melted [7].

Our simulations of the ESR spectra of CSL show that both in DPPC and DMPC a tilt angle is needed below the pretransition temperature (Fig. 8). Below −20°C the tilt angle is larger than 25° and with increasing temperature it decreases, more and more steeply the closer the temperature of the pretransition is approached. Confirming results were obtained by studying the splittings in the spectra as the orientation of the planar sample in the magnetic field was varied (Fig. 7). The samples contained 0.5 mol% of CSL in the pure lipids, and 20 to 22% of water.

These observations are in agreement with several data found in the literature. Most X-ray studies performed on samples containing at least 20% water give a tilt angle of 30–35° below the pretransition [1–5,14,34]. Above the pretransition the evidence is conflicting. According to Janiak et al. [2] the tilt angle reaches a minimum value of 30° at the pretransition whereas most other authors claim that the tilt is zero above this temperature [4,14,15,33,34].

Hemminga [19] has provided some evidence that the cholestane spin label exhibits an angle of tilt of about 25° in DPPC at room temperature,

which is larger than the 10° at 20°C we found by spectral simulation. However, his samples have a low degree of hydration, as shown by the high gel phase to liquid crystalline phase transition temperature (52°C) reported in this study, and are hence not directly comparable to ours. Reducing the relative humidity to 30%, we obtained a tilt angle of 10° at 25°C, about 7° at 40°C and 0° at 55°C.

Birrell and Griffith [18] used phospholipids spin-labeled at different positions along the hydrocarbon chains to measure the tilt angle in oriented multibilayers of DPPC prepared in a fashion similar to ours but with somewhat less hydration. They studied the variation of the splitting with orientation of the sample and showed that at 22°C there exists a significant tilt along the entire chain length, which varies between 37° near the polar head group and 28° deep in the hydrophobic region. The degree of time-average alignment decreases towards the center of the bilayer. We have measured the tilt angle in the same way as described by Birrell and Griffith [18] for the stearic acid spin label 5-SASL in DPPC at various temperatures in the range 20 to 45°C. The results are included in Fig. 8 and show that a tilt angle of 35 to 40° persists until the main transition is reached when it drops to zero. This is very different from the behaviour of the CSL: The tilt of 5-SASL is much larger than for CSL and is not abolished at the pretransition. Both CSL and 5-SASL probe the upper region of the hydrocarbon chains and are sensitive to motions on the same time scale but they do not yield the same information concerning the orientation of the lipid chains. Taylor and Smith [20] have proposed that tilting of the nitroxide moiety is a conformational adaptation of the stearic acid probe to insert the bulky doxyl group into the tightly packed upper region of the bilayer. This argument may be extended to the spin labeled phospholipids used by Birrell and Griffith.

The estimates of the tilt angle obtained through our simulations are generally smaller than those found by other techniques in similar systems. Only below −20°C are the values determined in our experiments approaching those measured from X-ray data in pure lipids at temperatures up to the pretransition. CSL is likely to have a strong tendency to align perpendicular to the bilayer surface in analogy with what has been determined for cholesterol [36]. The pretransition represents only a small enthalpy change [37]. Small amounts of CSL might appreciably decrease the temperature of the pretransition. As mentioned earlier the samples must be composed of a great number of microregions with various tilt directions so that all tilt directions have the same representation, probability. The form of the curves for CSL in Fig. 8 and their deviation from what would be expected for an ideal phase transition might reflect how the different microregions due to variations in the local concentration of CSL have different phase transition temperatures. In fact, the spectra are equally well simulated by properly weighted sums of spectra with full tilt, 30°, and no tilt. Those mixtures should be dynamic in nature at least at higher temperatures. The change from tilt to no tilt and vice versa with a certain microregion is, however, too slow to be detected by regular ESR. Preliminary results with the saturation transfer ESR technique by ourselves (unpublished) and by others [11,38] do not contradict the possibility of a motion of the long axis of CSL with a correlation time that could be of the order of 10^{-5} s. However, these spectra depend on anisotropic motion and a more detailed analysis has to await the development of a satisfactory simulation procedure.

Between −20 and +20°C we find the correlation time of the restricted rotational motion of CSL to be 3 ns in both DMPC and DPPC. For isotropic unrestricted motion, the motional narrowing formalism is applicable for $\tau < 2$ ns [39]. In the case of a restricted motion, as applied in our model of the motion, the formalism is, however, applicable for longer τ-values [25]. Hence, the constancy of τ in the temperature range from −20 to +20°C is considered to be real. In the same range of temperature the amplitude of the twisting motion, ϕ_0, is steadily increasing with temperature (Fig. 4). Hence, the angular velocity of the motion is also increasing. The temperature dependence of the quantity $\phi_0 \cdot \tau_a^{-1}$ may be used as a measure of how the mean angular velocity is changing with temperature. From the Arrhenius plot, Fig. 6, the activation energy of the twisting motion is found to be 5.5 kJ · mol^{-1} in the low temperature range. This low value strongly suggests that the molecular packing in the $L_{\beta'}$ phase can accommodate the

dissolved CSL in holes, lattice defects, which permit a restricted twisting motion, are dynamic in nature, and which change only slightly with temperature. Infrared spectroscopy [6] and ^{2}H-NMR [8] have shown that in the hexagonal lattice of the $L_{\beta'}$ phase the acyl chains undergo rapid motion about their long axes, the motion being torsional rather than rotational due to restrictions imposed by the choline headgroup. The occurrence of monoclinic or orthorhombic packing at low temperatures restricts the angular amplitudes [6]. These results are consistent with our observations concerning the variation of ϕ_0 with temperature in the range from -20 to $+20$°C.

For DPPC it is found that, coinciding with the disappearance of the tilt angle (Fig. 8) the restricted rotational motion is accelerated, the correlation time τ_a decreases (Fig. 5), and the amplitude, ϕ_0, of the twisting motion increases rapidly (Fig. 4). When these two changes are combined in the Arrhenius plot of Fig. 6 the pretransition at 34°C is well expressed. In the case of DMPC there is only an indication of a weak and broad transition stretching from 15 to 40°C (Fig. 6). It is known from other works that the pretransition in DMPC has a lower enthalpy change and often is observed to be broader than in DPPC [37]. The disappearance of the tilt angle at the pretransition (Fig. 8) is, however, about equally sharp in the two lipids.

At the main transition of each of the two lipids, 24°C for DMPC and 41.5°C for DPPC, there is a steep increase of the twisting velocity as seen in Fig. 6. Above the main transition the twisting motion is an order of magnitude faster than below the pretransition. The most conspicuous change is, however, the onset of the wobbling motion, the amplitude of which is sharply jumping from 0 to 35° within less than 2.5°C (Fig. 4). At higher temperatures the activation energy of the twisting motion around the long axis of CSL is 20 kJ · mol^{-1} in both lipids (Fig. 5). The same value is obtained for the tumbling motion within a cone of the long axis itself. This is close to the value of 25 kJ · mol^{-1} determined earlier for DPPC [23].

In summary we conclude that the orientation and motion of the cholestane spin label in oriented lipid bilayers as determined by simulation of the ESR spectra in parallel and perpendicular direction rather faithfully respond to the properties of the host lipids. The spectral simulation results lead to a detailed picture of the temperature dependence of the tilt angle, and of the twisting and wobbling motional velocities and amplitudes. Deviations from the ideal behaviour are attributed to the disturbance by the cholestane spin label itself on the surrounding. Some properties may be read directly from the temperature dependence of the apparent hyperfine splittings in the two major directions. Such information must be more difficult to read directly from the ESR spectra obtained with unoriented samples, such as liposomes. In general the most reliable and informative results are obtained by spectral simulations.

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