Librational Motion of Spin-Labeled Lipids in High-Cholesterol Containing Membranes from Echo-Detected EPR Spectra

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ABSTRACT Two-pulse, echo-detected (ED) electron paramagnetic resonance (EPR) spectroscopy was used to study the librational motions of spin-labeled lipids in membranes of dipalmitoylphosphatidylcholine + 50 mol % cholesterol. The temperature dependence, over the range 77–240 K, and the dependence on position of spin-labeling in the *sn*-2 chain ($n = 5$, 7, 10, 12, and 14) of the phospholipid, were characterized in detail. The experimental ED-spectra were corrected for instantaneous spin diffusion arising from static spin-spin interactions, by using spectra recorded at 77 K, where motional contributions are negligible. Simulations according to a model of rapid, small-amplitude librations about an axis whose direction is randomly distributed are able to describe the experimental spectra. Calibrations, in terms of the amplitude-correlation time product, $\langle\alpha^2\rangle\tau_c$, were constructed for diagnostic spectral line-height ratios at different echo delay times, and for relaxation spectra obtained from the ratio of ED-spectra recorded at two different echo delays. The librational amplitude, $\langle\alpha^2\rangle$, was determined for a spin label at the 14-C position of the lipid chain from the partially motionally averaged hyperfine splitting in the conventional EPR spectra. The librational correlation time, τ_c, which is deduced from combination of the conventional and ED-EPR results, lies in the subnanosecond regime and depends only weakly on temperature. The temperature dependence of the ED-EPR spectra arises mainly from an increase in librational amplitude with increasing temperature, and position down the lipid chain. A gradual transition takes place at higher temperatures, from a situation in which segmental torsional librations are cumulative, i.e., the contributions of the individual segments add up progressively upon going down the chain, to one of concerted motion only weakly dependent on chain position. Such librational motions are important for glass-like states and are generally relevant to high lipid packing densities, e.g., in cholesterol-containing raft domains and condensed complexes.

INTRODUCTION

Spectroscopic studies of biological systems at cryogenic temperatures are important to understand their structure-function relationship. Indeed, in many biophysical instances, low temperatures are used to dissect out specific dynamic, kinetic, and structural features of supramolecular aggregates that inevitably are present also at higher physiological temperatures. High-resolution studies in structural biology are now carried out routinely at cryogenic temperatures. Moreover, studies at low temperatures are currently the subject of intensive research because of the relevance to cryopreservation of biological tissues (Fahy et al., 2004), as well as genetic materials (Anchordoguy et al., 1988). Several experimental methods and theoretical approaches, sampling different timescales, have been used to investigate both natural and model biosystems in different environments and under different conditions. Among these are: neutron scattering (Fitter et al., 1999; Gabel et al., 2002 and references therein), x-ray crystallography (Frauenfelder et al., 1979; Rasmussen et al., 1992), molecular dynamics simulations (Steinbach and Brooks, 1996), and infrared (Demel et al., 1997), optical (Nocek et al., 1991; Di Pace et al., 1992) and pulsed electron paramagnetic resonance (EPR) spectroscopies (Millhauser and Freed, 1984; Crepeau et al., 1984; Kar et al., 1985; Saxena and Freed, 1997) . As regards spin-labeled bilayer membranes, pulsed EPR techniques in the time and frequency domains have proved to be a powerful means for providing direct evidence of water penetration into phospholipid membranes (Bartucci et al., 2003a), and in defining details of the librational dynamics of phospholipid chains in the low-temperature phases of bilayer membranes (Bartucci et al., 2003b).

High-frequency torsional librations are an important feature of the rotational dynamics in the glassy state (Dzuba, 1996; Kirilina et al., 2001). In the present work, we have characterized the temperature dependence and positional dependence of the fast librational motion of spin-labeled lipid chains in membranes of dipalmitoylphosphatidylcholine containing 50 mol % cholesterol in the frozen state. Phase diagrams for mixtures of dipalmitoylphosphatidylcholine with cholesterol (Vist and Davis, 1990), and of dimyristoylphosphatidylcholine with cholesterol (Almeida et al., 1992), indicate that the equimolar mixtures produce homogeneous bilayers. These compositions correspond to the liquid-ordered phase that has been implicated in the formation of raft-like domains in cell membranes (Simons and Ikonen, 1997; Schroeder et al., 1998).

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For this study, it was necessary to correct the two-pulse echo-detected (ED) EPR spectra for instantaneous spin diffusion by using spectra recorded at 77 K, where motion is essentially absent. Calibrations obtained by spectral simulation are used to determine the librational amplitude-correlation time product from the dependence of the ED-spectra on echo delay time. Conventional EPR spectra are used additionally to obtain the librational amplitude from the partial motional averaging of the outer hyperfine splitting. Changes in relaxation rate of the ED-spectra are attributable principally to changes in librational amplitude, rather than in correlation time. A gradual transition takes place from independent torsional motions to concerted motion, largely independent of chain position, as the temperature increases.

Characterization of chain motions in saturated phosphatidylcholine/cholesterol mixtures at the high packing densities achieved by low temperatures is important for several reasons. It is relevant not only to possible glass-like behavior of membranes, but also to the dynamical behavior in membrane raft domains, and particularly in condensed cholesterol complexes (McConnell and Radhakrishnan, 2003; McConnell and Vrljic, 2003).

MATERIALS AND METHODS

Materials

Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol were obtained from Sigma-Aldrich (St. Louis, MO). Phosphatidylcholines spin-labeled in the *sn*-2 chain (*n*-PCSL; 1-acyl-2-(*n*-doxyl)-stearoyl-*sn*-glycero-3-phosphocholine) were synthesized according to Marsh and Watts (1982). Certain positional spin-label isomers were also obtained from Avanti Polar Lipids (Birmingham, AL). Reagent grade salts for the 10 mM phosphate buffer solution at pH = 7.5 were from Merck (Darmstadt, Germany). All materials were used as purchased with no further purification. Distilled water was used throughout.

Sample preparation

DPPC with 50 mol % cholesterol and 1 mol % of *n*-PCSL were codissolved in chloroform. The solvent was evaporated in a nitrogen gas stream, and then residual traces of solvent were removed by drying under vacuum overnight. The lipids were dispersed at a concentration of ~100 mg/ml in pH 7.5 phosphate buffer by vortex mixing with heating to 60°C (i.e., above the chain-melting transition of DPPC). The hydrated lipid bilayers were transferred to a standard 4-mm-diameter, quartz EPR tube, concentrated by pelleting in a bench-top centrifuge, and the excess water was removed. Samples were incubated for 24 h at 10°C before measuring.

EPR spectroscopy

Two-pulse ($\pi/2$-τ-π-τ-echo) echo-detected EPR spectra were collected on an ELEXSYS E580 9-GHz Fourier Transform FT-EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with a MD5 dielectric resonator and a CF 935P cryostat (Oxford Instruments, Eynsham, Witney, Oxfordshire, UK). ED-EPR spectra were obtained by recording the integrated spin-echo signal when sweeping the magnetic field. The integration window was 160 ns. The microwave pulse widths were 32 ns and 64 ns, with the microwave power adjusted to provide $\pi/2$ and π-pulses, respectively. Additional experiments were performed on the microwave power dependence at 77 K, with the amplitude of the pulses reduced by two, four and eight times. Conventional, continuous-wave EPR spectra were recorded on an ESP-300 9-GHz spectrometer with 100-kHz field modulation (Bruker).

Simulation of echo-detected spectra

Theoretical background

The amplitude of a two-pulse echo, in the limit of fast small-amplitude motion ($\Delta\omega^2\tau_c^2 \ll 1$) that is an appropriate approximation here, depends on the rotational correlation time, τ_c, and the shift in resonance frequency, $\Delta\omega$, that is induced by the motion. For a polar orientation θ, φ of the magnetic field, B, relative to the nitroxide x, y, and z axes, the echo decay curve after correction for intrinsic T_2-relaxation is given approximately (see Dzuba et al., 1992; Dzuba, 1996) by

$$E(2\tau,\theta,\varphi) \approx \exp(-2\Delta\omega^2(\theta,\varphi)\tau_c\tau), \tag{1}$$

where τ is the interpulse spacing. The shift in resonance frequency caused by a libration of small amplitude, α, about the nitroxide x axis is given by

$$\Delta\omega(\theta,\varphi) = \alpha\left\{\frac{\beta}{\hbar}B(g_{YY}-g_{ZZ}) + \frac{m_I(A_{YY}^2-A_{ZZ}^2)}{a_{zz}(\theta,\varphi)}\right\} \times \sin\theta\,\cos\theta\,\sin\varphi, \tag{2}$$

where g_{XX}, A_{XX}, etc. are the principal values of the g-tensor and of the hyperfine interaction tensor, respectively; m_I is the ^{14}N nuclear magnetic quantum number; and $a_{zz}(\theta,\varphi)$ is the angular-dependent hyperfine splitting constant (in angular frequency units). The latter is given (see Libertini and Griffith, 1970) by

$$a_{zz}(\theta,\varphi) = (A_{XX}^2\sin^2\theta\cos^2\varphi + A_{YY}^2\sin^2\theta\sin^2\varphi + A_{ZZ}^2\cos^2\theta)^{1/2}. \tag{3}$$

For motion around the nitroxide y or z axes, the shift in resonance frequency is given similarly to Eq. 2 (see Dzuba et al., 1995) by

$$\Delta\omega(\theta,\varphi) = \alpha\left\{\frac{\beta}{\hbar}B(g_{ZZ}-g_{XX}) + \frac{m_I(A_{ZZ}^2-A_{XX}^2)}{a_{zz}(\theta,\varphi)}\right\} \times \sin\theta\cos\theta\cos\varphi \tag{4}$$

and

$$\Delta\omega(\theta,\varphi) = \alpha\left\{\frac{\beta}{\hbar}B(g_{XX}-g_{YY}) + \frac{m_I(A_{XX}^2-A_{YY}^2)}{a_{zz}(\theta,\varphi)}\right\} \times \sin^2\theta\cos\varphi\sin\varphi, \tag{5}$$

respectively.

The so-called ''isotropic'' librational model assumes stochastic changes in orientation of the librational axis in all possible directions at different moments in time. This situation can be modeled by assuming simultaneous, independent librational motions, each of amplitude α and with correlation time τ_c, around each of the three perpendicular x, y, and z axes of the nitroxide (i.e., the motion can be decomposed into three independent librational motions around any set of three mutually perpendicular axes). The net relaxation is then given by the product of the three independent

relaxations induced by each librational mode. As a result, the angular dependence of the term $\Delta\omega^2(\theta,\varphi)$ in Eq. 1 is much reduced, relative to that for libration about a single, fixed axis. For this reason, the model is referred to as *isotropic*. It is found that the isotropic model fits the experimental ED-spectra from spin-labeled lipid chains in bilayer membranes much better than does any model for libration about a single axis (Erilov et al., 2004). This is because the reduced angular dependence of $\Delta\omega^2(\theta,\varphi)$ for the isotropic model results in near-exponential relaxation, as is observed experimentally (see also later below).

The echo-detected EPR lineshape, *ED*, is finally given by summation of the echo amplitude over all orientations θ, φ of the magnetic field, and over all three $m_I = 0, \pm 1$ hyperfine manifolds, as

$$ED(2\tau,B) = \sum_{m_I}\frac{1}{4\pi}\iint \sin\theta\, d\theta\, d\varphi f(B - \omega_{m_I}(\theta,\varphi)/\gamma) \times E(2\tau,\theta,\varphi), \quad (6)$$

where $f(\Delta B)$ is the lineshape of an individual spin packet. This is represented by a convolution of Gaussian and Lorentzian lineshapes, where the former takes into account inhomogeneous broadening from unresolved proton hyperfine interactions. The resonance frequency in Eq. 6 is given by the intermediate field approximation (Libertini and Griffith, 1970),

$$\omega_{m_I}(\theta,\varphi) = g_{zz}(\theta,\varphi)\frac{\beta}{\hbar}B + m_I a_{zz}(\theta,\varphi), \quad (7)$$

where the angular-dependent g-value for the magnetic field directed along the laboratory z axis is

$$g_{zz}(\theta,\varphi) = g_{XX}\sin^2\theta\cos^2\varphi + g_{YY}\sin^2\theta\sin^2\varphi + g_{ZZ}\cos^2\theta. \quad (8)$$

Here and throughout, β is the Bohr magneton, and $\hbar$ is Planck's constant divided by 2π.

Calibrations from simulated ED-spectra

Fig. 1 *a* gives simulated echo-detected EPR spectra of a nitroxide spin label that is undergoing librational motion according to the isotropic model. The spectra are normalized to the same maximum line height, so as to display only the field-dependent part of the relaxation. It is seen that the amplitudes in the intermediate spectral regions at low and high field decrease systematically, relative to the corresponding outer peaks (or shoulders) in the spectrum, with increasing pulse spacing, τ. This is a characteristic effect of small-amplitude librational motion in ED-EPR spectra (Dzuba et al., 1992; Dzuba, 1996). The rate of decrease in spectral intensity in the intermediate regions depends on the amplitude-correlation time product, $\langle\alpha^2\rangle\tau_c$. The ratio, P''/P, of line heights in the intermediate regions (P'') to those at the outer turning points (P) has been used previously to quantitate this rate of change (Bartucci et al., 2003b). Fig. 2 gives the dependence of the low-field diagnostic line-height ratios, L''/L, on τ for different values of the amplitude-correlation time product, $\langle\alpha^2\rangle\tau_c$. As can be seen from the linearity of the log-linear plot (Fig. 2 *b*), the dependence of L''/L on τ is closely exponential, as is also found experimentally (Bartucci et al., 2003b). The slope of these plots bears a constant ratio to the amplitude-correlation time product: $d\ln(L''/L)/d\tau = (1.13 \times 10^{17}\ \text{rad}^{-2}\ \text{s}^{-2}) \times \langle\alpha^2\rangle\tau_c$. This simulation result provides a practical calibration between the experimental ED-EPR line-height ratios and the amplitude-correlation time product for the librational motion. Corresponding calibration constants for the high-field diagnostic line-height ratio, H''/H, and from simulations using spin Hamiltonian parameters and line widths appropriate to both 5-PCSL and 14-PCSL, are given in Table 1.

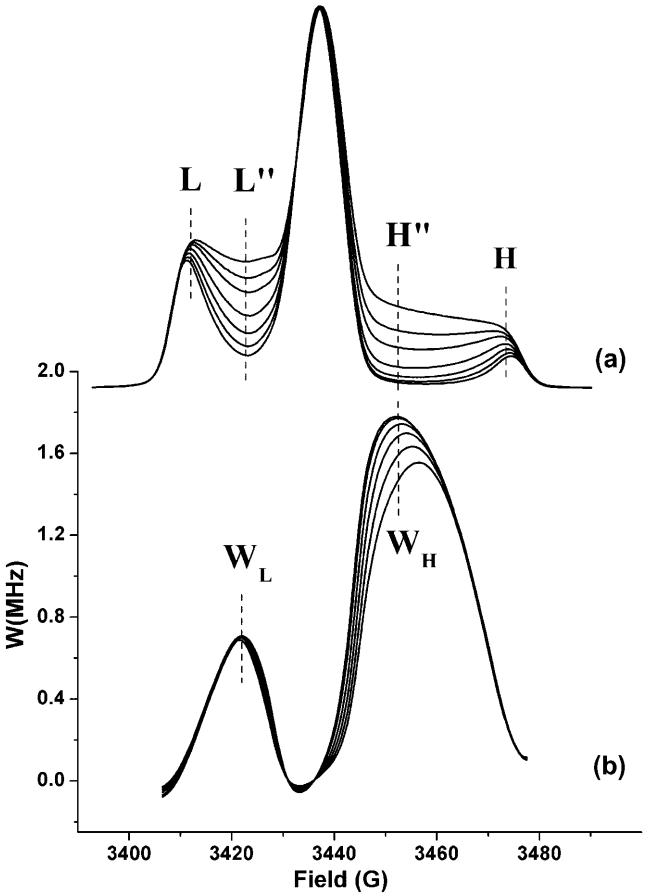

FIGURE 1 (*a*) Echo-detected EPR spectra simulated with the isotropic librational model for interpulse spacings (*top* to *bottom*) of $\tau = 0$, 100, 200, 400, 600, 800, and 1000 ns, and a fixed amplitude-correlation time product of $\langle\alpha^2\rangle\tau_c = 5 \times 10^{-12}$ rad^2 s. Spin Hamiltonian parameters: $g_{XX} = 2.0089$, $g_{YY} = 2.0059$, $g_{ZZ} = 2.0024$, $A_{XX} = 4.2$ G, $A_{YY} = 3.6$ G, and $A_{ZZ} = 34.6$ G are those appropriate to 5-PCSL in DPPC + 50 mol % cholesterol. (*b*) Anisotropic part of the relaxation rate, W, obtained according to Eq. 9 from pairs of ED-spectra in *a* with interpulse separations of $\tau_1 = 0$ ns, $\tau_2 = 100$ ns; $\tau_1 = 0$ ns, $\tau_2 = 200$ ns; $\tau_1 = 0$ ns, $\tau_2 = 400$ ns; $\tau_1 = 0$ ns, $\tau_2 = 600$ ns; $\tau_1 = 0$ ns, $\tau_2 = 800$ ns; and $\tau_1 = 0$ ns, $\tau_2 = 1000$ ns.

An alternative method of analyzing the dependence of the ED-EPR lineshapes on librational dynamics is illustrated in Fig. 1 *b*. Here, the anisotropic lineshapes of Fig. 1 *a* are replotted as the ratio of ED-spectra recorded at two different values, τ_1 and τ_2, of the interpulse delay by using the relation

$$W(B,\tau_1,\tau_2) = \ln\left[\frac{ED(2\tau_1,B)}{ED(2\tau_2,B)}\right]\times\frac{1}{2(\tau_2-\tau_1)}, \quad (9)$$

where $ED(2\tau,B)$ is the ED-spectral line height at field position B. Thus $W(B,\tau_1,\tau_2)$ represents the relaxation rate averaged over the time interval from τ_1 to τ_2. Because the spectra were already normalized to exclude all field-independent relaxation processes, W is the anisotropic part of the relaxation rate only. The near coincidence, in Fig. 1 *b*, of the curves derived from the different pairs of τ-values implies that the anisotropic relaxation is almost exponential, especially on the low-field side, just as was found from the L''/L line-height ratios in Fig. 2. According to Eq. 1, exponential relaxation is expected for a single spin packet, corresponding to a fixed

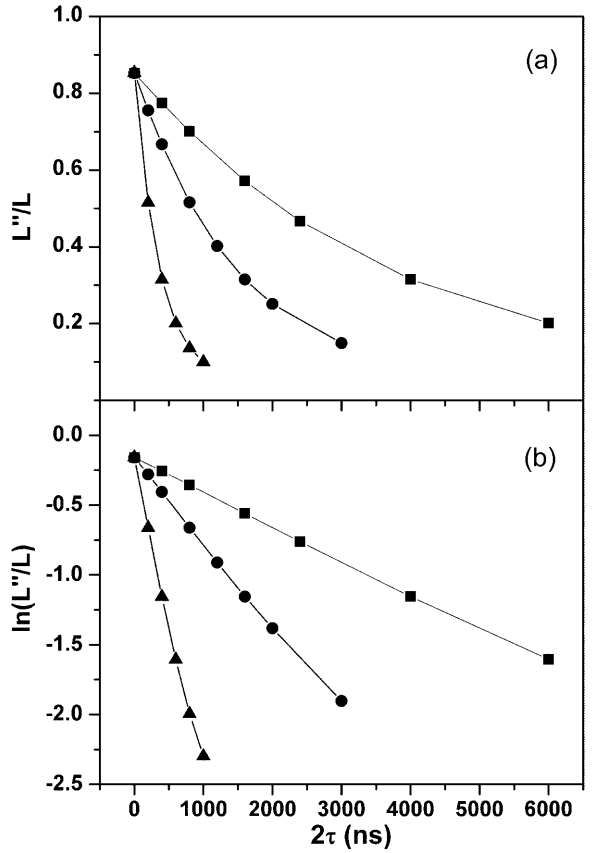

FIGURE 2 Dependence of the low-field diagnostic line-height ratio, L''/L, on interpulse spacing, τ, with a linear (*a*) and a semilogarithmic (*b*) scale, for ED-spectra simulated with the isotropic librational model and values of amplitude-correlation time product: $\langle\alpha^2\rangle\tau_c = 2 \times 10^{-12}$ rad^2 s (*squares*), 5×10^{-12} rad^2 s (*circles*), and 2×10^{-11} rad^2 s (*triangles*).

orientation θ, φ. However, the net intensity of the resonance line at a fixed field position, i.e., $ED(2\tau,B)$, arises from contributions from molecules with different polar orientations, θ and φ. In general, therefore, near-exponential relaxation is not expected for the rate parameter W (see Eq. 9). Nor is it found in simulations for librations about just one of the nitroxide x, y, or z axes (data not shown). Only for the isotropic model, in which the angular dependence of $\Delta\omega^2(\theta,\varphi)$ is greatly reduced, is the W-relaxation expected to be near to exponential. The approximately invariant values, W_L and W_H, of the low-field and high-field peaks in Fig. 1 *b* can also be used to characterize the librational dynamics. For example, $W_L = (1.41 \times 10^{17}$ rad^{-2} s$^{-2}) \times \langle\alpha^2\rangle\tau_c$ for the low-field peak. As expected, this calibration constant is rather similar to that for the low-field line-height ratio, L''/L. Systematic differences arise because the residual angular dependence is reflected slightly differently by the two calibration constants. A complete set of calibration constants for the W parameters is given in Table 1.

TABLE 1 Multiplicative calibration constants (rad^{-2} s^{-2}) relating the τ-dependence of the diagnostic line-height ratios, ln(L''/L) and ln(H''/H), and the averaged relaxation rates, W_L and W_H, to the amplitude-correlation time product, $\langle\alpha^2\rangle\tau_c$

	5-PCSL	14-PCSL
L''/L	$1.13 \pm 0.01 \times 10^{17}$	$9.30 \pm 0.15 \times 10^{16}$
H''/H	$2.3 \pm 0.1 \times 10^{17}$	$2.2 \pm 0.1 \times 10^{17}$
W_L	$1.41 \pm 0.02 \times 10^{17}$	$1.05 \pm 0.02 \times 10^{17}$
W_H	$3.3 \pm 0.1 \times 10^{17}$	$2.9 \pm 0.1 \times 10^{17}$

Values are given from spectral simulations with spin-Hamiltonian parameters and line widths appropriate to the 5-PCSL and 14-PCSL spin labels.

RESULTS AND DISCUSSION

ED-EPR spectra

Fig. 3 *a* shows the experimental ED-EPR spectra of the 14-PCSL spin label in bilayer membranes of DPPC + 50 mol % cholesterol at 200 K. These spectra have been corrected for instantaneous spin diffusion by normalizing with respect to those recorded at 77 K, where little molecular motion is expected. The corrected spectra are plotted according to (see Erilov et al., 2004)

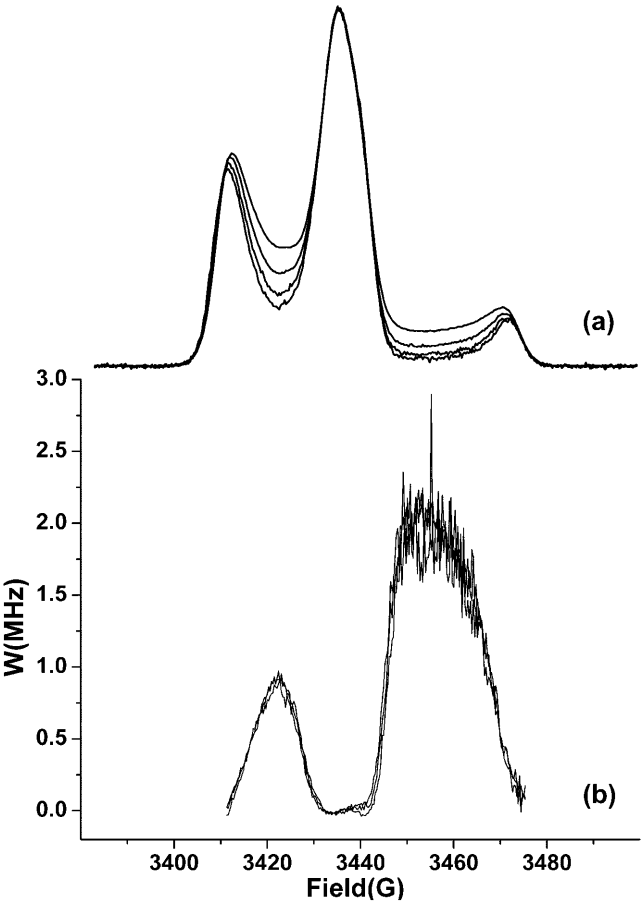

FIGURE 3 (*a*) Echo-detected EPR spectra of 14-PCSL in bilayer membranes of DPPC + 50 mol % cholesterol at 200 K, for interpulse spacings (*top* to *bottom*) of τ = 216, 352, 488, and 624 ns. Spectra are corrected for instantaneous diffusion according to Eq. 10 and are normalized to the maximum line height. (*b*) Anisotropic part of the relaxation rate, W, obtained according to Eq. 9 from pairs of spectra in *a* with interpulse separations of (*top* to *bottom*): 216 ns and 352 ns; 216 ns and 488 ns; and 216 ns and 624 ns, respectively. Note that all three curves coincide within the noise.

$$ED_{\mathrm{T}}^{\mathrm{corr}}(2\tau,B) = ED_{\mathrm{T}}(2\tau,B)\frac{ED_{77K}(2\tau_0,B)}{ED_{77K}(2\tau,B)}, \qquad (10)$$

where τ_0 is the shortest value of τ for which ED-spectra were recorded. The dependence of the lineshapes on the echo delay time that is characteristic of librational motion is seen in the intermediate spectral regions at low and high field (see e.g., Dzuba et al., 1992; Dzuba, 1996; and Fig. 1). Corresponding plots of the W-relaxation parameter evaluated from different pairs of ED-spectra recorded at interpulse separations τ_1 and τ_2 are given in Fig. 3 *b*. It is seen that the experimental curves for different pairs of τ essentially coincide to within the noise level. This implies that the relaxation is, in fact, close to exponential, and therefore confirms that the isotropic model is that most appropriate for analyzing the librational dynamics.

Additional experiments were performed at 77 K to distinguish between instantaneous diffusion and residual librational motion (even at this relatively low temperature). This was done by decreasing the amplitude of the microwave pulses, which decreases the spread of spins that are excited, hence decreasing the effect of instantaneous spin diffusion (Toropov et al., 1998). By this method it was possible to determine the residual motional contribution to relaxation of the ED-spectra at 77 K. For instance, a motional contribution to the value of W_{H} of (0.25 ± 0.05) MHz was obtained for 14-PCSL at 77 K. The values of the W-relaxation rate so-obtained at 77 K were added as a correction to those obtained with fixed microwave power at higher temperatures. Limitations of signal/noise ratio did not allow using lower-power selective pulses at temperatures appreciably higher than 77 K.

Temperature dependence

Fig. 4 *a* shows the dependence of the maximum values, W_{L} and W_{H}, of the ED-EPR relaxation rates on temperature, for the 5-PCSL and 14-PCSL spin labels in membranes of DPPC + 50 mol % cholesterol. The temperature profiles of W_{L} and W_{H} are very similar, showing consistency between the low- and high-field regions of the ED-spectrum. Also, the absolute values of W_{L} and W_{H} are mutually consistent, when corrected for the different sensitivities to $\langle\alpha^2\rangle\tau_{\mathrm{c}}$ according to the calibration factors given in Table 1. It is seen from Fig. 4 *a* that, in the temperature range up to 180 K, the intensity of librational motion (i.e., the value of $\langle\alpha^2\rangle\tau_{\mathrm{c}}$) is greater for 14-PCSL, i.e., toward the end of the chain, than it is for 5-PCSL where the spin label is closer to the membrane surface. In addition, the shape of the temperature profile in this region is more complex for 14-PCSL than it is for 5-PCSL. At higher temperatures, the intensity of motion becomes more similar for the labels at the two different chain positions. For both, the temperature dependence becomes much steeper from 180 K onwards.

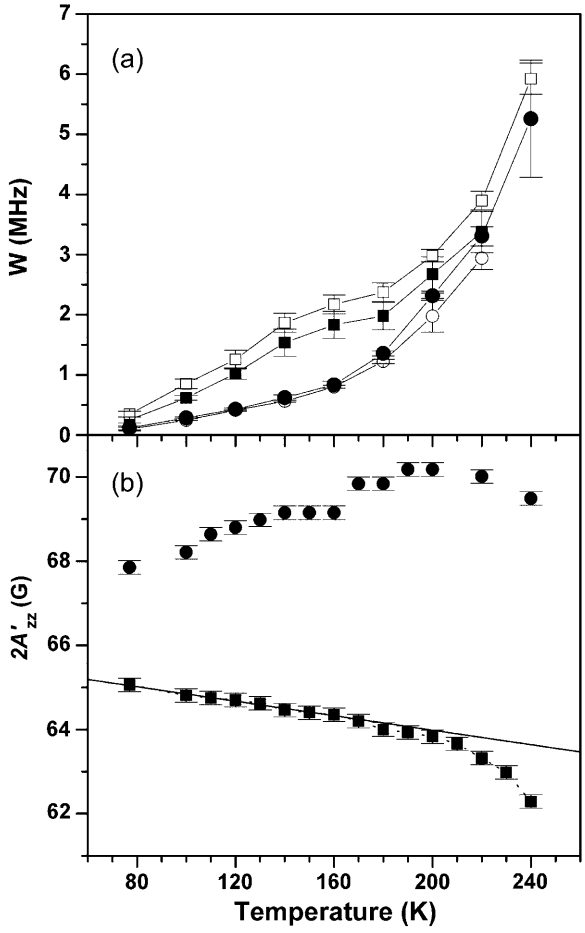

FIGURE 4 (*a*) Temperature dependence of the W_{L} (*solid symbols*) and W_{H} (*open symbols*) ED-EPR relaxation-rate parameters, for 5-PCSL (*circles*) and 14-PCSL (*squares*) in bilayer membranes of DPPC + 50 mol % cholesterol. Determinations of W are for $\tau_1 = 216$ ns and $\tau_2 = 352$ ns (see Eq. 9). The values of W_{L} are increased by a factor of 3.3:1.41 = 2.34 for 5-PCSL, and of 2.9:1.05 = 2.76 for 14-PCSL (see Table 1). (*b*) Temperature dependence of the outer hyperfine splitting, $2A'_{zz}$, in the conventional CW-EPR spectra of 5-PCSL (*circles*) and 14-PCSL (*squares*) in bilayer membranes of DPPC + 50 mol % cholesterol.

Fig. 4 *b* shows the dependence of the outer hyperfine splitting, $2A'_{zz}$, in the conventional CW-EPR spectra on temperature, for 5-PCSL and 14-PCSL spin labels in membranes of DPPC + 50 mol % cholesterol. For small-amplitude fast librations, the mean-square amplitude, $\langle\alpha^2\rangle$, is related to the partially averaged hyperfine splitting, A'_{zz}, by (see Van et al., 1974)

$$A'_{zz} = A_{zz} - (A_{zz} - A_{xx})\langle\alpha^2\rangle, \qquad (11)$$

where the prime indicates the motionally averaged hyperfine tensor of elements A_{xx}, A_{yy}, and A_{zz}. As expected, the values of A'_{zz} for 14-PCSL decrease progressively with increasing temperature, corresponding to an increasing amplitude of libration. The temperature dependence of the apparent hyperfine splitting for 5-PCSL is, instead, anomalous: it

increases with increasing temperature. Most probably this is due to a dominating contribution to the hyperfine splitting from the temperature dependence of hydrogen-bonded water (Johnson, 1981) and the local dielectric permeability (Marsh, 2002). In frozen membranes, both of these latter contributions to the local polarity are important for 5-PCSL, but not for 14-PCSL (Kurad et al., 2003). In addition, the conventional EPR spectra of 5-PCSL are somewhat spin-spin broadened at low temperatures. Unfortunately, therefore, the librational amplitude cannot be determined for the 5-PCSL spin label.

Fig. 5 *a* shows the temperature dependence of the amplitude-correlation time product, $\langle\alpha^2\rangle\tau_c$, for the 14-PCSL spin label in membranes of DPPC + 50 mol % cholesterol. These values are obtained from the measurements of W_L given in Fig. 4 *a*, together with the calibration given in Table 1. Fig. 5 *b* shows the temperature dependence of the mean-square amplitude, $\langle\alpha^2\rangle$, of the librational motion for the 14-PCSL spin label in membranes of DPPC + 50 mol % cholesterol. These values are derived from the data given in Fig. 4 *b*, together with Eq. 11. The rigid-limit hyperfine splitting, $2A_{zz}$, which was used in these calculations was derived by linear extrapolation of these data to zero temperature. As expected, the librational amplitude increases with increasing temperature. The largest value of $\langle\alpha^2\rangle$ (at 240 K) corresponds to a root-mean-square amplitude of 14 ± 1° and the smallest (at 77 K) to 6 ± 1°, which confirms that the small-amplitude approximation is appropriate at low temperatures. Fig. 5 *c* shows correspondingly the temperature dependence of the rotational correlation time, τ_c, for the librational motion of 14-PCSL in membranes of DPPC + 50 mol % cholesterol. Determinations of the amplitude-correlation time product, $\langle\alpha^2\rangle\tau_c$, from Fig. 5 *a* were combined with the amplitude (i.e., $\langle\alpha^2\rangle$) measurements that are given in Fig. 5 *b* to obtain these values for τ_c. Values of τ_c are in the subnanosecond region, which confirms that the librations are in the fast motional regime. The correlation time for 14-PCSL displays a weak dependence on temperature, but with a tendency to decrease with decreasing temperature below 120 K. The librational motion apparently is not a strongly activated process at temperatures above 120 K. It is interesting to note that this behavior is somewhat different from that for the librational motion of small spin labels in polar glassy matrices. The latter display an anomalous temperature dependence, in which τ_c increases monotonically with increasing temperature (Kirilina et al., 2001). Much of the complex temperature dependence of the amplitude-correlation time product that is seen for 14-PCSL in Fig. 4 *a* seems to arise from the nonuniform temperature dependence of the correlation time (compare Fig. 4 *a* and Fig. 5, *a* and *b*).

From the above results for 14-PCSL, it can be inferred that the temperature dependence of the correlation time for 5-PCSL is probably also rather weak. It will be noted that the temperature dependence of W (i.e., of $\langle\alpha^2\rangle\tau_c$) for 5-PCSL in Fig. 4 *a* is rather similar to that of $\langle\alpha^2\rangle$ for 14-PCSL in Fig. 5 *b*, supporting this proposal. If this is the case, most of the temperature dependence of the amplitude-correlation time product for 5-PCSL in Fig. 4 *a* can also be attributed to the expected increase in librational amplitude with increasing temperature.

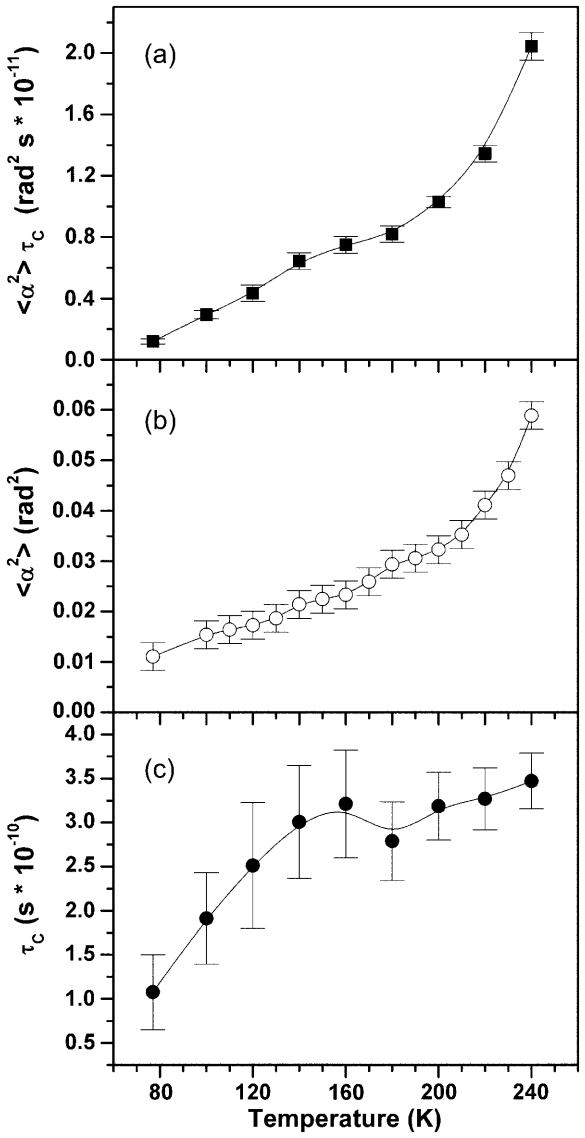

FIGURE 5 (*a*) Temperature dependence of the amplitude-correlation time product, $\langle\alpha^2\rangle\tau_c$, for 14-PCSL in bilayer membranes of DPPC + 50 mol % cholesterol. Values are obtained from measurements of the W_L relaxation-rate parameter, together with the results of spectral simulations that are given in Table 1. (*b*) Temperature dependence of the librational amplitude, $\langle\alpha^2\rangle$, for 14-PCSL in bilayer membranes of DPPC + 50 mol % cholesterol. Values are obtained from the motionally averaged hyperfine splitting, $2A'_{zz}$, together with Eq. 11. (*c*) Temperature dependence of the librational correlation time, τ_c, for 14-PCSL in bilayer membranes of DPPC + 50 mol % cholesterol. Values are obtained from the amplitude-correlation product in *a* and the librational amplitude in *b*.

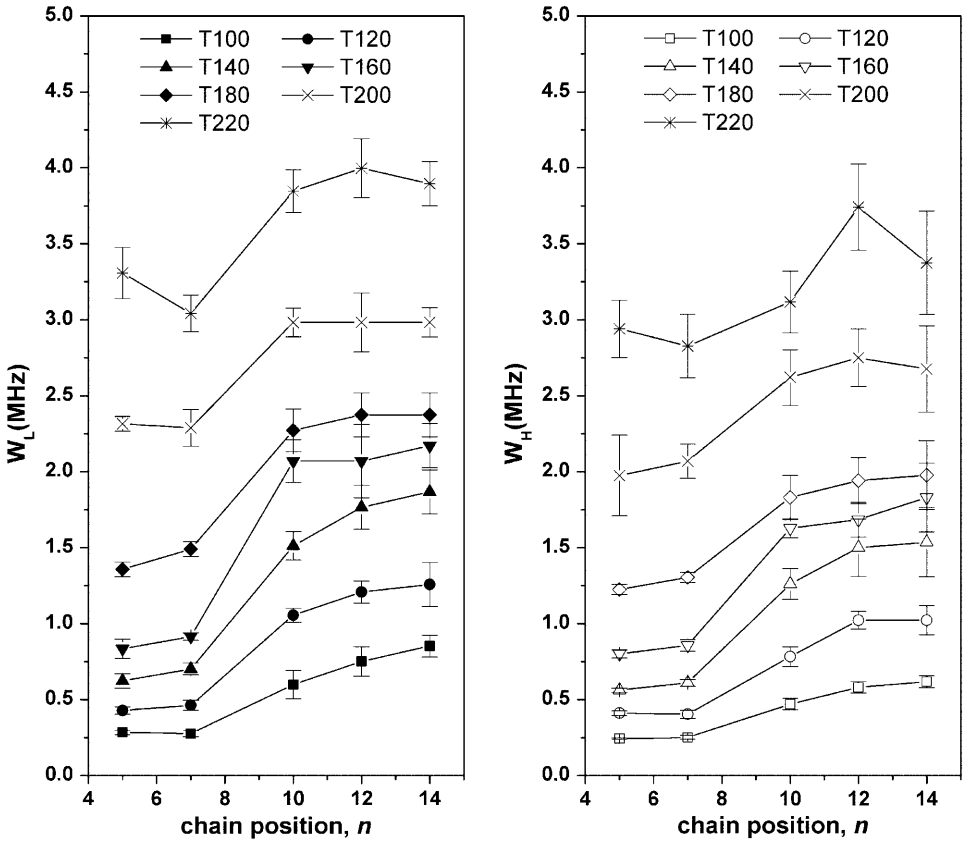

FIGURE 6 Dependence of the W_L (*solid symbols*) and W_H (*open symbols*) ED-EPR relaxation-rate parameters on chain position, n, of spin labeling for the n-PCSL spin probes in bilayer membranes of DPPC + 50 mol % cholesterol, at the temperatures indicated. Determinations of W are for $\tau_1 =$ 216 ns and $\tau_2 = 352$ ns (see Eq. 9). The values of W_L are increased by a factor of 3.3:1.41 = 2.34 for 5- and 7-PCSL, and by a factor of 2.9:1.05 = 2.76 for 10-, 12-, and 14-PCSL (see Table 1).

Dependence on position of chain labeling

Fig. 6 shows the relaxation-rate parameters W_L and W_H for the n-PCSL spin labels in bilayer membranes of DPPC + 50 mol % cholesterol, as a function of the position, n, of chain spin labeling. The positional profiles of the relaxation rate are given for different sample temperatures. At higher temperatures (i.e., above 180 K), the dependence on chain position is slight, as can be inferred also from the relative temperature dependences of 5-PCSL and 14-PCSL in this region (see Fig. 4 *a*). At lower temperatures (180 K and lower), the positional dependence of the librational relaxation becomes more pronounced. It is most evident at ~140–160 K, although the fractional change between 5-PCSL and 14-PCSL remains rather high down to 100 K. At 160 K, the major change in relaxation rate takes place between 7-PCSL and 10-PCSL, and this moves gradually to the region between 10-PCSL and 12-PCSL with decreasing temperature. From the data presented already for 14-PCSL in Fig. 5, it seems likely that the positional profiles of the W-relaxation rates in Fig. 6 reflect mostly changes in the librational amplitude, $\langle\alpha^2\rangle$, rather than in the correlation time, τ_c, although analogy with the temperature dependence is not conclusive on this point. Changes in librational amplitude with chain position can be interpreted in terms of increasing cumulative torsional amplitude toward the end of the chain, in addition to an increase in intrinsic torsional freedom for chain segments closer to the terminal methyl group. The latter may reflect, at least in part, the molecular shape of the cholesterol molecules that are intercalated between the lipid chains. The hydrocarbon tail of cholesterol is smaller in cross-section than is the rigid sterol nucleus that is located adjacent to the upper parts of the chain. As the temperature is reduced, the lipids become more close-packed and the effects of the nonuniform cross-section of cholesterol first become felt at positions lower down the chain. In contrast, at temperatures above 180 K, the packing density is sufficiently reduced that the librational amplitude is similar at all positions down the lipid chain.

Rapid, small-amplitude angular librations are found to be a characteristic feature of the rotational motions of small spin labels in glassy matrices (Dzuba et al., 1992; Kirilina et al., 2001). Glass transitions have been reported in phosphatidylethanolamines at low hydration (Shalaev and Steponkus, 2001, 2003), and are suggested to occur in membranes of lipids with methyl-branched chains (Blöcher et al., 1984, 1985). In all cases, including a dynamical transition in purple membranes (Fitter et al., 1999), the transition takes place in the low-temperature regime, mostly in the region of 200 K. Whether a glass-like transition occurs in membranes containing equimolar cholesterol has yet to be established with certainty, and further appropriate studies are needed to assess this point.

Glass formation by aqueous solutions has been implicated in biological cryopreservation (Fahy, 1986; Fahy et al., 2004). It is equally possible that glassy behavior in lipid membranes may assist in cryopreservation of the embedded integral proteins and their supramolecular complexes. The low-temperature librational motions studied here may be functionally important not only for cryopreservation but also for certain rapid processes of physiological relevance at higher temperatures. More specifically, librational motions may be relevant to the chain dynamics at the high packing densities expected for lipid/cholesterol mixtures in membrane raft domains (Simons and Ikonen, 1997; Simons and Toomre, 2000), particularly the condensed complexes proposed by Radhakrishnan and McConnell (1999, 2000). It should be noted, however, that at higher temperatures, slower large-scale nanosecond rotations may make it difficult to resolve the rapid librational modes explicitly. For this reason, low temperatures are used here to dissect out librational motions, when full-scale rotation is essentially frozen out.

CONCLUSIONS

Both the temperature dependence of the two-pulse ED-EPR spectra and the dependence on spin-label chain position can be interpreted in terms of fast, small-amplitude librational motions occurring in membranes of DPPC + 50 mol % cholesterol at low temperatures. A detailed treatment of the librational motions requires correction of the ED-spectra for instantaneous spin diffusion that arises from spin-spin interactions attendant on the nonvanishing spin-label concentration in the membranes. Simulations with a motional model in which the librational axis is randomly distributed provide a convenient and accessible means for calibrating the dependence on the amplitude correlation-time product, $\langle\alpha^2\rangle\tau_c$, in terms of diagnostic line-height ratios at low and high field, or of relaxation spectra that are obtained from the ratio of ED-spectra recorded at different echo delay times. Measurement of the librational amplitude, $\langle\alpha^2\rangle$, from the partially motionally averaged A'_{zz}-hyperfine splitting for 14-PCSL then allows determination of the librational correlation time, τ_c, from the ED-spectra. The temperature dependence of the correlation time is not large. Most of the variation in echo relaxation rate arises from changes in the librational amplitude. With increasing temperature, a transition takes place from a state with a characteristic segmental dependence of the librational amplitude on chain position to a situation in which the librational motion is independent of the position in the chain.

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REFERENCES

Almeida, P. F., W. L. Vaz, and T. E. Thompson. 1992. Lateral diffusion in the liquid phase of dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis. *Biochemistry*. 31:6739–6747.

Anchordoguy, T., J. H. Crowe, F. J. Griffin, and W. H. Clark, Jr. 1988. Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. *Cryobiology*. 25:238–243.

Bartucci, R., R. Guzzi, D. Marsh, and L. Sportelli. 2003a. Intramembrane polarity by electron spin echo spectroscopy of labeled lipids. *Biophys. J.* 84:1025–1030.

Bartucci, R., R. Guzzi, D. Marsh, and L. Sportelli. 2003b. Chain dynamics in the low-temperature phases of lipid membranes by electron spin-echo spectroscopy. *J. Magn. Reson.* 162:371–379.

Blöcher, D., R. Gutermann, B. Henkel, and K. Ring. 1984. Physicochemical characterization of tetraether lipids from *Thermoplasma acidophilum*. Differential scanning calorimetry studies on glycolipids and glycophospholipids. *Biochim. Biophys. Acta*. 778:74–80.

Blöcher, D., R. Gutermann, B. Henkel, and K. Ring. 1985. Physicochemical characterization of tetraether lipids from *Thermoplasma acidophilum*. Calorimetric studies on miscibility with di-ether model lipids carrying branched or unbranched alkyl chains. *Biochim. Biophys. Acta*. 818:333–342.

Crepeau, R. H., S. Saxena, S. Lee, B. Patyal, and J. H. Freed. 1984. Studies on lipid membranes by two-dimensional Fourier transform ESR: enhancement of resolution to ordering and dynamics. *Biophys. J.* 66:1489–1504.

Demel, F., W. Doster, W. Petry, and A. Schulte. 1997. Vibrational frequency shifts as a probe of hydrogen bonds: thermal expansion and glass transition of myoglobin in mixed solvents. *Eur. Biophys. J.* 26:327–335.

Di Pace, A., A. Cupane, M. Leone, E. Vitrano, and L. Cordone. 1992. Protein dynamics—vibrational coupling, spectral broadening mechanism and anharmonicity effects in carbonmonoxy heme proteins studied by the temperature-dependence of the Soret band lineshape. *Biophys. J.* 63:475–484.

Dzuba, S. A., Y. D. Tsvetkov, and A. G. Maryasov. 1992. Echo-detected EPR spectra of nitroxides in organic glasses: model of orientational molecular motions near equilibrium position. *Chem. Phys. Lett.* 188:217–222.

Dzuba, S. A., H. Watari, Y. Shimoyama, A. G. Maryasov, Y. Kodera, and A. Kawamori. 1995. Molecular motions of the cholestane spin label in a multibilayer in the gel phase studied using echo-detected EPR. *J. Magn. Reson.* 115:80–86.

Dzuba, S. A. 1996. Librational motion of guest spin probe molecules in glassy media. *Phys. Lett. A*. 213:77–84.

Erilov, D., R. Bartucci, R. Guzzi, D. Marsh, S. A. Dzuba, and L. Sportelli. 2004. Echo-detected EPR spectra of spin-labelled lipids in membrane model systems. *J. Phys. Chem. B*. 108:4501–4507.

Fahy, G. M. 1986. Vitrification: a new approach to organ cryopreservation. *Prog. Clin. Biol. Res.* 224:305–335.

Fahy, G. M., B. Wowk, J. Wu, J. Phan, C. Rasch, A. Chang, and E. Zendejas. 2004. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology*. 48:157–178.

Fitter, J., R. E. Lechner, and N. A. Dencher. 1999. Interactions of hydration water and biological membranes studied by neutron scattering. *J. Phys. Chem. B*. 103:8036–8050.

Frauenfelder, H., G. A. Petsko, and D. Tsernoglou. 1979. Temperature-dependent x-ray diffraction as a probe of protein structural dynamics. *Nature*. 280:558–563.

Gabel, F., D. Bicout, U. Lehnert, M. Tehei, M. Weik, and G. Zaccai. 2002. Protein dynamics studied by neutron scattering. *Quart. Rev. Biophys.* 35:327–367.

Johnson, M. E. 1981. Apparent hydrogen bonding by strongly immobilized spin labels. *Biochemistry.* 20:3319–3328.

Kar, L., E. Ney-Igner, and J. H. Freed. 1985. Electron spin resonance and electron-spin-echo study of oriented multilayers of Lα-dipalmitoylphosphatidylcholine water systems. *Biophys. J.* 48:569–595.

Kirilina, E. P., S. A. Dzuba, A. G. Maryasov, and Y. D. Tsvetkov. 2001. Librational dynamics of nitroxide molecules in a molecular glass studied by echo-detected EPR. *Appl. Magn. Reson.* 21:203–221.

Kurad, D., G. Jeschke, and D. Marsh. 2003. Lipid membrane polarity profiles by high-field EPR. *Biophys. J.* 85:1025–1033.

Libertini, L. J., and O. H. Griffith. 1970. Orientation dependence of the electron spin resonance spectrum of di-t-butyl nitroxide. *J. Chem. Phys.* 53:1359–1367.

Marsh, D., and A. Watts. 1982. Spin-labeling and lipid-protein interactions in membranes. *In* Lipid-Protein Interactions, Vol. 2. P.C. Jost and O.H. Griffith, editors. Wiley-Interscience, New York. 53–126.

Marsh, D. 2002. Membrane water-penetration profiles from spin labels. *Eur. Biophys. J.* 31:559–562.

McConnell, H. M., and A. Radhakrishnan. 2003. Condensed complexes of cholesterol and phospholipids. *Biochim. Biophys. Acta.* 1610:159–173.

McConnell, H. M., and M. Vrljic. 2003. Liquid-liquid immiscibility in membranes. *Annu. Rev. Biophys. Biomol. Struct.* 32:469–492.

Millhauser, G. L., and J. H. Freed. 1984. Two-dimensional electron spin echo spectroscopy and slow motions. *J. Chem. Phys.* 81:37–48.

Nocek, J. M., E. D. A. Stemp, M. G. Finnegan, T. I. Kosly, M. K. Johnson, E. Margoliash, A. G. Mauk, M. Smith, and B. M. Hoffman. 1991. Low-temperature, cooperative conformational transition within [Zn-cytochrome *c* peroxidase, cytochrome *c*] complexes: variation with cytochrome. *J. Am. Chem. Soc.* 113:6822–6831.

Radhakrishnan, A., and H. M. McConnell. 1999. Cholesterol-phospholipid complexes in membranes. *J. Am. Chem. Soc.* 121:486–487.

Radhakrishnan, A., and H. M. McConnell. 2000. Chemical activity of cholesterol in membranes. *Biochemistry.* 39:8119–8124.

Rasmussen, B. F., A. M. Stock, D. Ringe, and G. A. Petsko. 1992. Crystalline ribonuclease A loses function below the dynamic transition at 220 K. *Nature.* 357:423–424.

Saxena, S., and J. H. Freed. 1997. Two-dimensional electron spin resonance and slow motions. *J. Phys. Chem. A.* 101:7998–8008.

Schroeder, R. J., S. N. Ahmed, Y. Zhu, E. London, and D. A. Brown. 1998. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. *J. Biol. Chem.* 273:1150–1157.

Shalaev, E. Y., and P. L. Steponkus. 2001. Phase behavior and glass transition of 1,2-dioleoylphosphatidylethanolamine (DOPE) dehydrated in the presence of sucrose. *Biochim. Biophys. Acta.* 1514:100–116.

Shalaev, E. Y., and P. L. Steponkus. 2003. Glass transition of a synthetic phospholipid in the lamellar phase. *J. Phys. Chem. B.* 107:8734–8737.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* 387:569–572.

Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1:31–41.

Steinbach, P. J., and B. R. Brooks. 1996. Hydrated myoglobin's anharmonic fluctuations are not primarily due to dihedral transitions. *Proc. Natl. Acad. Sci. USA.* 93:55–59.

Toropov, Y. V., S. A. Dzuba, Y. D. Tsvetkov, V. Monaco, F. Formaggio, M. Crisma, C. Toniolo, and J. Raap. 1998. Molecular dynamics and spatial distribution of TOAC spin-labeled peptaibols studied in glassy liquid by echo-detected EPR spectroscopy. *Appl. Magn. Reson.* 15:237–246.

Van, S. P., G. B. Birrell, and O. H. Griffith. 1974. Rapid anisotropic motion of spin labels. Models for motion averaging of the ESR parameters. *J. Magn. Reson.* 15:444–459.

Vist, M. R., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures. ^{2}H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.