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PROBING THE MEMBRANE POLAR REGION WITH 4–(N– HEXADECYLDIMETHYLAMMONIUM)–2,2,6,6–TETRAMETHYLPIPERIDINYL–OXYL BROMIDE SPIN LABEL

BALGAVÝ, P.[1)], GALLOVÁ, J.[1)], ŠVAJDLENKA, E.[1)], Bratislava, KUTEJOVÁ, E.[2)], Bratislava

ESR spectra of the 4-(N-hexadecyldimethylammonium)-2,2,6,6-tetramethylpiperidinyl-oxyl bromide (CAT-16) spin label in oriented and disoriented bilayers prepared from egg yolk phosphatidylcholine (EYPC), $L-\beta,\gamma$-dipalmitoyl-α-phosphatidylcholine (DPPC), or $L-\beta,\gamma$-dipalmitoyl-α-phosphatidyl-ethanolamine (DPPE) are axially symmetric. The CAT-16 paramagnetic N → O group is located in the bilayer — water inferface as indicated by the Heisenberg spin exchange and dipole-dipole broadening induced by the paramagnetic CO^{2+}, Mn^{2+}, Gd^{3+}, and $[Fe(CN)_6]^{3-}$ ions. The ensemble averaged ESR order parameters S_{ii} calculated from the spectra of CAT-16 in oriented EYPC bilayers indicate the preferential orientation of the CAT-16 N → O bond direction and $2p\pi$-orbital axis perpendicular and parallel to the bilayer director, respectively. The ESR spectral parameters are sensitive to the DPPC and DPPE thermotropic phase transitions.

I. INTRODUCTION

Spin labels are frequently used to probe the structure and dynamics of biological membranes and their models. However, the majority of experimental studies has been performed with fatty acid spin labels with a doxyl paramagnetic group located on different carbons or with phospholipids with one acyl chain labelled similarly with the doxyl group [1, 2]. Because these labels are sensitive mainly to the changes in the structure and dynamics of the membrane hydrophobic region, there is a need for labels suitable for studies of the membrane polar region. 4-(N--alkyldimethylammonium)-2,2,6,6-tetramethylpiperidinyl-oxyl bromide spin labels (CAT-n, n=number of carbons in the alkyl chain) could be used for such a purpose. From their chemical structure it follows that their positively charged ammonium group would interact with the negatively charged phosphate groups of membrane phospholipids, and their hydrophobic alkyl chain would penetrate into the hydrophobic region of the membrane [3]. These interactions locate the CAT-n paramagnetic group in the membrane polar region. However, amphiphilic properties of the CAT-n labels influence their partition between the membrane and the aqueous phase. The critical parameter important for this partition is the length of the CAT-n alkyl chain which is primarily responsible for the hydrophobic interactions with the membrane. The CAT-n spin labels with short alkyl chains will partition mainly in the aqueous phase while those with long alkyl chains mainly in the membrane phase. On the other hand this partitioning depends also on the surface potential of the membrane — the more negatively charged membranes bind more CAT-n spin label molecules. Using these partitioning effects, the CAT-n spin labels with intermediate chain lengths (n=8–10), the electron spin resonance (ESR) spectra of which consist of a superposition of aqueous and membrane signals, were used in ESR studies of the membrane suface potential [3–5]. In conclusions, labels with short chains will report on the properties of the aqueous phase, labels with intermediate chains on both the aquoeus phase and the membrane polar region, and labels with long chains ($n \geq 16$) for which the hydrophobic interactions dominate over the surface potential effects should be sensitive exclusively to the membrane polar region structure and dynamics.

In our previous papers [6, 7] we have found that the CAT-16 spin label is suitable for studying perturbation effects of amphiphilic molecules in membranes prepared from lipids isolated from bacteria. In the present communication, we study the ESR spectra of the CAT-16 spin label located in the phosphatidylcholine and in phosphatidylethanolamine model membranes.

II. MATERIAL AND METHODS

Egg yolk phosphatidylcholine (EYPC) was isolated and purified according to [8]. $L-\beta,\gamma$-dipalmitoyl-α- phosphatidylcholine (DPPC), $L-\beta,\gamma$-dipalmitoyl-α-phosphatidylethanolamine (DPPE), and TRIS.HCl were purchased from Fluka (Buchs, Switzerland). 4-(N- hexadecyldimethylammonium)-2,2,6,6-tetramethylpiperidinyl-oxyl bromide (CAT-16) spin label was from Technika (Sofia, Bulgaria). Fatty acid spin labels 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-DSA), 2-(3-carboxydecyl)-4,4,-dimethyl-2-hexyl-3-oxazolidinyloxyl (12-DSA) and (2-(3-carboxytetradecyl)-4,4-dimethyl-2-ethyl-3-oxazolidinyloxyl (16-DSA) were from Syva (Palo ALto, USA). The other chemicals were analytically pure and were purchased from Lachema (Brno, Czechoslovakia).

Oriented EYPC bilayers deposited on a glass surface were prepared by the parallel-beam spattering method described in our following paper [9]. Briefly, the EYPC+CAT-16 mixture dissolved in absolute ethanol was atomized with a stream

[1)] Department of Physical Chemistry, Faculty of Pharmacy, J.A.Comenius University, Odbojárov 10, 832 32 BRATISLAVA, CSFR

[2)] Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta, 842 51 BRATISLAVA, CSFR

of nitrogen gas. The beam of atomized solution then passed through an orifice before deposition on the glass plate. Thereafter, the glass plate in a sample holder was inserted into a glass tube, and the traces of solvent were removed by a diffusion pump evacuation. Finally, the lipid bilayers on the glass plate were hydrated over a saturated NaCl solution at 60 °C for 30 min.

Disoriented EYPC bilayers were prepared as follows: The spin label and lipid (EYPC, DPPC or DPPE) were mixed in a proper molar ratio in ethanol in polypropylene or glass test microtubes. The ethanol was evaporated under a stream of nitrogen gas followed by a diffusion pump evacuation. Thereafter, redistilled water, ion solution or buffer prepared by using redistilled water was added and the lipid was dispersed by sonication in the UC 005 AJ1 Tesla bath sonicator (Tesla, Vráble, Czechoslovakia). DPPC and DPPE were hydrated by heating at temperatures 10-15 °C above the gel-liquid crystal phase transition temperature of the respective lipid for 30 minutes, EYPC was hydrated at room temperature. Hydrated lipid dispersion was filled into a glass capillary and sealed. The final concentration of the lipid was 52.5 μmol/l and the lipid:label molar ratio more than 100:1, except in the specified experiments noted below, where the ESR spectra were studied at different label concentrations.

Electron spin resonance spectra were recorded by an ERS 230 X-band ESR spectrometer (ZWG AdW Berlin, GDR) by using the 100 kHz modulation technique and a GX goniometer (Radiopan, Poznań, Poland). Microwave frequency was measured by using a Ж3-54 frequency meter equipped with a Ш3Ж-43 frequency converter (Moscow, S.U.). The magnetic field was measured by using a nuclear magnetic resonance magnetometer MJ-110 R (Radiopan, Poznań, Poland) calibrated by the Mn^{2+} standard (Mn^{2+} ions in the MgO lattice). The precision of spectral parameters evaluated from the line positions or linewidths was better than 5 per cent. The sample temperature was maintained by using a stream of heated gas from the STT 3 liquid nitrogen temperator (ZWG AdW Berlin, GDR). The precision of temperature setting was ± 0.5 K. All temperature dependence described in the present paper were obtained in heating scans. The ESR spectrum was recorded after equilibration of the sample for 5 min. after each temperature step. In experiments with disoriented DPPC bilayers aimed at studying the effects of subtransition on spin label spectral parameters, the sample capillaries were contained in glass tubes filled with silicon oil for thermal stability. The samples were annealed at 0 °C before being loaded into a pre-cooled cavity of the ESR spectrometer. In the first heating scan the sample was heated from 0 °C to 24 ÷ 30 °C. The second heating scan followed after recooling the sample in the cavity to 0 °C for 30 min.

III. RESULTS AND DISCUSSION

The CAT-16 spin label in disoriented EYPC, DPPC and DPPE bilayers displays axially symmetric power pattern spectra similar to those observed for the *m*-DSA spin labels. The absence of narrow lines typical of the fast isotropic spin label motion in the DPPC (Fig. 1) and EYPC bilayers (not shown) indicates that

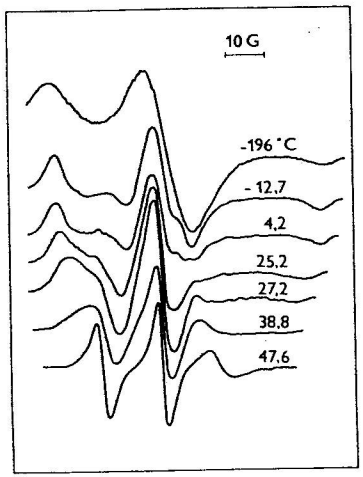

Fig. 1. ESR spectra of the CAT-16 spin label in disoriented DPPC bilayers. The direction of the magnetic field increase is from left to right

practically no label molecules are located in the aqueous phase. Such narrow lines were observed in the DPPE bilayers (Fig. 2) between 0 °C and the gel — liquid crystal $L_\beta \rightarrow L_\alpha$ phase transition temperature of 63.8 °C [10, 11]. These data indicate that the partitioning of the CAT-16 spin label into the gel lipid phase is smaller in phosphatidylethanolamines than in phosphatidylcholines. This may be caused by the tightly packed polar region of phosphatidylethanolamine bilayers in the gel phase [12].

The changes observed in the shapes of spectra with the increase of temperature indicate changes in the spin label molecular motion. To extract an information about this motion from the spectra, it is necessary to exclude the contribution to the spectra caused by the Heisenberg spin-exchange and dipole-dipole interactions between unpaired electrons in different label molecules. At intermediate spin label : lipid molar ratios $w = 2 : 100 \div 6 : 100$, these interactions lead to a broadening of the ESR nitrogen hyperfine lines, which increases with the increase of w [1]. As clearly seen from the results in Fig. 3, the width of the ESR central line

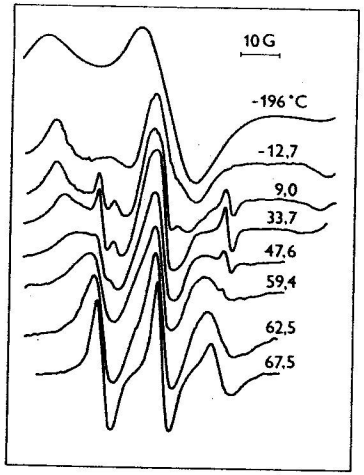

Fig. 2. ESR spectra of the CAT-16 spin label in disoriented DPPE bilayers. The direction of the magnetic field increase is from left to right

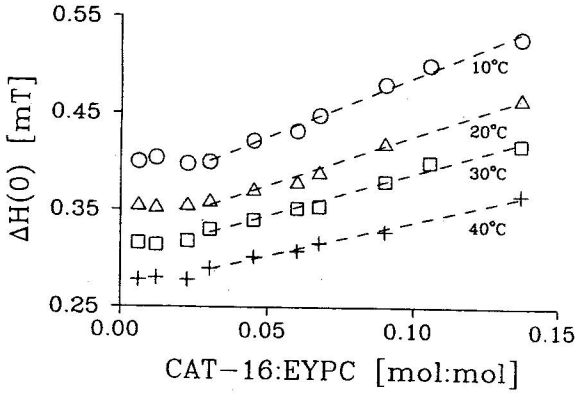

Fig. 3. The dependence of the width $\Delta H(0)$ of the CAT- 16 spin label ESR central line in disoriented EYPC bilayers on the CAT-16:EYPC molar ratio

$\Delta H(0)$ increases with the CAT-16 spin label concentration in the EYPC model membrane. This broadening effect is negligible for the CAT-16:EYPC molar ratio $w < 2.3 : 100$. Therefore, all experiments described below were performed at $w \leq 1 : 100$.

At low temperatures the motion of spin labels is restricted. The value of the nitrogen hyperfine splitting A_{max}^{-196} deduced from the distance of the outer extrema of the ESR spectra recorded at a liquid nitrogen temperature is equal to the A_{zz}

Table 1
The values of A_{max}^{-196} [mT] for different spin labels in phospholipid bilayers.

	EYPC	DPPC	DPPE
CAT-16	3.54	3.650	3.626
5-DSA	3.40	3.410	
12-DSA	3.20		
13-DSA		3.270	
16-DSA	3.24	3.230	

value of the nitrogen hyperfine splitting tensor A [1, 2, 13]. The values of A_{max}^{-196} found in our experiments are shown in Table 1. Since the hyperfine splitting is sensitive to the polarity of the spin label N-oxyl group microenvironment and increases with the increase of the polarity [13], the values in Table 1 indicate that the microenvironment of the CAT-16 spin label $N \rightarrow 0$ group is more polar than in the case of m-DSA spin labels. As mentioned in the Introduction, the positively charged CAT-16 ammonium group interacts with the negatively charged phospholipid phosphate groups in bilayers. Consequently, the CAT-16 spin label $N \rightarrow 0$ group must be located in the aqueous phase — bilayer interface. The relatively high A_{max}^{-196} found for the CAT-16 spin label as compared to those for m-DSA spin labels supports this conclusion.

The location of the $N \rightarrow 0$ group in the interface region can be tested also by the spin label — paramagnetic ion Heisenberg spin exchange and dipole — dipole interactions. It is known from the NMR and diffraction studies [14, 15] that metal ions and their charged complexes are located in the interface — positively charged ions interact with the negatively charged lipid phosphate groups and negatively charged complex ions (e.g. $[Fe(CN)_6]^{3-}$) interact with the positively charged choline or ethanolamine groups. The CAT-16 spin label — paramagnetic ion interactions must thus lead to the broadening of ESR spectral lines if the $N \rightarrow 0$ group were located in the interface. In our experiments we have found that the width of the ESR lines of the CAT-16 spin label in EYPC bilayers increases with the increase of Co^{2+}, Mn^{2+}, Gd^{3+}, or $[Fe(CN)_6]^{3-}$ paramagnetic ion concentration as expected. No broadening of 16-DSA spin label lines has been observed in the comparable paramagnetic ion concentration range (up to 0.1 mol/l) as expected for the 16-DSA spin label $N \rightarrow 0$ group location in the EYPC bilayer hydrophobic region. Noteworthy is the linewidth $\Delta H(0)$ dependence on the concentration of $[Fe(CN)_6]^{3-}$ ions (Fig. 4). In isotropic aqueous solutions $\Delta H(0)$ linearly increases with the increase of $[Fe(CN)_6]^{3-}$ concentration within the same concentration range as used in the present study [16, 17]. In the case of CAT-16 spin label in EYPC bilayers $\Delta H(0)$ increases more steeply at lower $[Fe(CN)_6]^{3-}$ concentrations (≤ 0.01 mol/l) than at higher concentrations (≥ 0.02 mol/l). This

biphasic effect of $[Fe(CN)_6]^{3-}$ might be caused by magnetic interactions of the CAT-16 spin label with $[Fe(CN)_6]^{3-}$ ions bound at two different types of sites in the EYPC bilayers. Obviously, the first type sites would be saturated at lower concentrations of $[Fe(CN)_6]^{3-}$ ions. As a possible site of the first type of $[Fe(CN)_6]^{3-}$ binding one can suggest the positively charged quaternary ammonium group of the CAT-16 spin label itself. The second type sites must then be the choline groups of the EYPC molecules in the bilayer. In conclusion, both the A_{max}^{-196} values and experiments with paramagnetic ions suggest the CAT-16 spin label $N \rightarrow 0$ group location in the interface region.

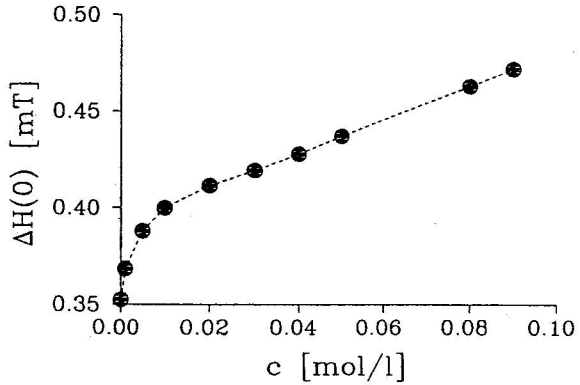

Fig. 4. The dependence of the width $\Delta H(0)$ of the CAT-16 spin label ESR central line in disoriented EYPC bilayers on the $K_3[Fe(CN)_6]$ concentration $c \cdot t = 25\,^{\circ}C$

In the following experiments we studied effects of ionic strength and pH of the equeous phase on the ESR spectra of the CAT-16 spin label at different temperatures. We have found no difference in the distance of the outer extrema $2A_{max}$ of the ESR triplet for samples prepared with aqueous solutions of different ionic strength (redistilled water, KCl $I = 0.2$, KCl $I = 0.6$, KCl $I = 1.0$) at temperatures between 0 °C and 80 °C. However, the A_{max} was observed to be sensitive to the changes in pH (Table 2). We have observed lower values of A_{max} for EYPC

Table 2
The values of A_{max} [mT] for CAT-16 spin label in disoriented EYPC bilayers in different buffers. A: aceteate buffer, $I = 0.05$; P: phosphate buffer, $I = 0.05$; T: TRIS buffer, $I = 0.05$.

pH	buffer	t=5 °C	t=25 °C
4.57	A	2.960	2.440
7.10	T	2.890	2.425
7.56	T	2.860	2.370
9.10	T	2.825	2.325
9.17	P	2.930	2.370

bilayers prepared in the phosphate buffer (pH=9.17, $I = 0.05$) than in the acetate buffer (pH=4.57, $I = 0.05$). Similarly, the A_{max} values observed in the EYPC bilayers in the TRIS buffer ($I = 0.05$) decreased with the increase of the buffer pH. These findings are rather surprising, because there is no chemical group in the EYPC bilayers with the pK_α value within the used pH range. Furthermore, the values of A_{max} in different buffers (phosphate vs. TRIS) are different at comparable pH (9.10÷9.17). There is a possibility that organic ions from the buffer solution interact with charged groups in the bilayer polar region and this is sensed by the CAT-16 spin label. Such an interaction leading to the acyl chain interdigitation has been observed in phosphatidylglycerol bilayers [18].

As mentioned above, the ESR spectra of the CAT-16 spin label in lipid bilayers are axially symmetric (Figs. 1–2). From these spectra as well as from the proposed location of the CAT-16 spin label in the bilayer one may suggest an axially symmetric rotation of the whole CAT-16 spin label molecule about its long axis. The CAT-16 spin label $N \rightarrow 0$ group orientation with respect to the bilayer director can be quantitatively characterized by order parameters S_{ii} defined as the ensemble average over all label molecules

$$S_{ii} = \langle 3\cos^2 \vartheta_i - 1 \rangle : 2, \tag{1}$$

where ϑ_i is the angle between the i-axis of the nitrogen hyperfine splitting tensor A and the bilayer director. The order parameters can be calculated from the experimental time averaged components of the A and g tensors parallel ($g_\parallel, A_\parallel$), and perpendicular ($g_\perp, A_\perp$) to the bilayer director according to the equations

$$S_{11} = [f_g(g_\parallel - g_\perp) + S_{33}(g_{yy} - g_{22})] : (g_{xx} - g_{yy}) \tag{2}$$

$$S_{33} = [f_A(A_\parallel - A_\perp)] : [A_{zz} - (A_{xx} + A_{yy}) : 2] \tag{3}$$

$$S_{22} = -S_{11} - S_{33} \tag{4}$$

$$f_g = (g_{xx} + g_{yy} + g_{zz}) : (2g_\perp + g_\parallel) \tag{5}$$

$$f_A = (A_{xx} + A_{yy} + A_{zz}) : (2A_\perp + A_\parallel), \tag{6}$$

where g_{ii} and A_{ii} are the cartesian principal values of diagonalized g and A tensors, respectively, and f_g and f_A are polarity correction factors (see [1, 2, 9] for details and references).

The principal values of the g and A tensors of the CAT-16 spin label used in the present paper were those found for the 4-oxo- 2,2,6,6,-tetramethylpiperidine-N-oxyl spin label [19, 20]:

$$\begin{aligned} g_{xx} &= 2.0104 & A_{xx} &= 0.52\,\mathrm{mT} \\ g_{yy} &= 2.0074 & A_{yy} &= 0.52\,\mathrm{mT} \\ g_{zz} &= 2.0026 & A_{zz} &= 3.10\,\mathrm{mT} \end{aligned} \tag{7}$$

The principal values of the g and A tensors of m-DSA spin labels used in the present paper were those obtained by Lange et al. [21] from computer simulations of their ESR spectra in oriented phosphatidylcholine bilayers:

$$\begin{aligned} g_{xx} &= 2.0088 \qquad A_{xx} = 0.65\,\mathrm{mT} \\ g_{yy} &= 2.0061 \qquad A_{yy} = 0.58\,\mathrm{mT} \\ g_{zz} &= 2.0027 \qquad A_{zz} = 3.35\,\mathrm{mT} \end{aligned} \tag{8}$$

The $A_\parallel$, $A_\perp$, $g_\parallel$, and $g_\perp$ values can be calculated from the ESR spectra of spin label in an oriented bilayer recorded at different director orientations γ with respect to the magnetic field

$$A(\gamma) = \left[A_\perp^2 \sin^2\gamma + A_\parallel^2 \cos^2\gamma\right]^{1/2}, \tag{9}$$

$$g(\gamma) = g_\perp \sin^2\gamma + g_\parallel \cos^2\gamma \tag{10}$$

using a least-squares method [1, 2, 9]. Fig. 5 demonstrates a typical angular de-

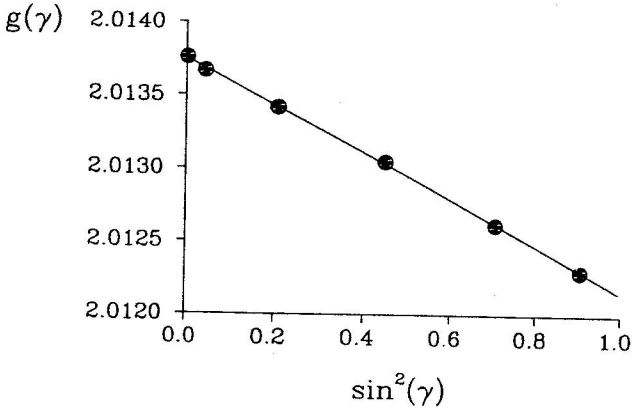

Fig. 5. The angular dependence of the g-factor $g(\gamma)$ for the CAT-16 spin label in oriented EYPC bilayers. $t = 30\,^\circ\mathrm{C}$

pendence of $g(\gamma)$ as obtained with oriented EYPC bilayers. The values of order parameters S_{33} and S_{11} calculated from $A(\gamma)$ and $g(\gamma)$ data at different temperatures are shown in Fig. 6. Relatively small S_{11} values indicate that the principal

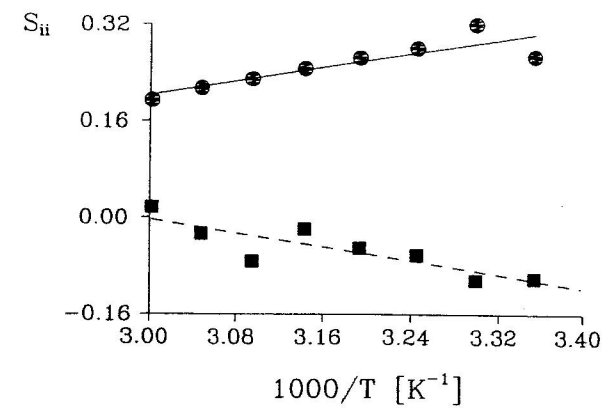

Fig. 6. The temperature dependence of the order parameters S_{33} (circles, full line) and S_{11} (squares, dashed line) for the CAT-16 spin label in oriented EYPC bilayers

x-axis of the g and A tensors (which coincides with the $N \rightarrow 0$ bond direction) lies preferentially within the bilayer plane, while the z-axis (oriented along the axis of the $2p\pi$-orbital of the $N \rightarrow 0$ group) is preferentially oriented parallel to the bilayer director. This suggests a bent-over conformation for the piperidinyl-oxyl moiety which could leave the positively charged ammonium group near the negatively charged lipid phosphate group and place the $N \rightarrow 0$ group closer to the lipid acyl chain carbonyls. Such a conformation has been proposed also by Ellena et al. [22]. They found that the CAT-14 spin label enhances the ^{13}C-NMR spin-lattice relaxation rate at the EYPC acyl OOC-CH_2 position, indicating that the unpaired CAT-14 electron is located near this position.

It is well known that the thermotropic phase behaviour of DPPC bilayers in excess water can be described in the form

$$L_{C'} \rightleftarrows L_{\beta'} \rightleftarrows P_\beta \rightleftarrows L_\alpha, \tag{11}$$

where the three solid-like phases $L_{C'}$, $L_{\beta'}$, and P_β have extended acyl chains predominantly in *trans* configuration, while the lamellar fluid phase L_α has disordered acyl chains due to *trans-gauche* isomerization (see [23–27] for details and references). In the $L_{\beta'}$ and $L_{C'}$ phases, the acyl chains are tilted to the bilayer director, but become perpendicular to the bilayer plane in the P_β phase; the bilayer surface of the P_β phase is rippled [28–32]. The $L_{\beta'} \rightarrow P_\beta$ phase transition is called "pretransition", $P_\beta \rightarrow L_\alpha$ — "main transition", and $L_{C'} \rightarrow L_{\beta'}$ — "subtransition". The subtransition is exclusively slowly reversible upon cooling compared to the other phase transitions in DPPC. Conversion $L_{\beta'} \rightarrow L_{C'}$ occurs only if the DPPC-H_2O system has been annealed at low temperatures for an extended period of time [33–36]. We have studied the effects of the phase transitions

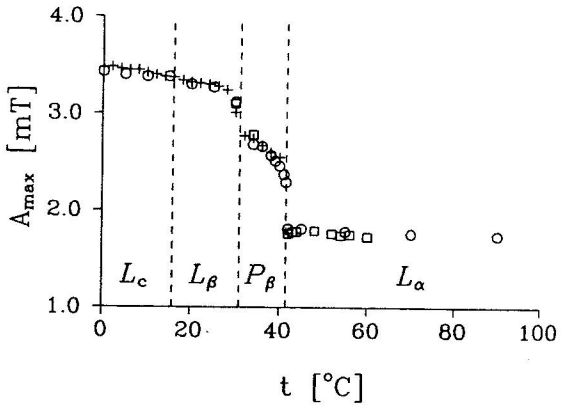

Fig. 7. The temperature dependence of the outer hyperfine splitting A_{max} of the CAT-16 spin label in disoriented DPPC bilayers. Dashed dividing lines separate different DPPC phases. Different symbols – results obtained with different samples

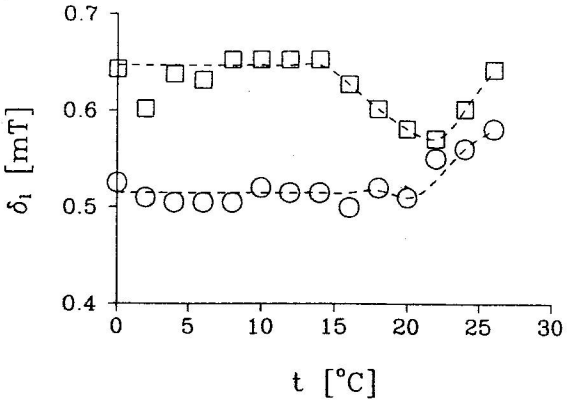

Fig. 8. The temperature dependence of the full width at halfheight of the low field ESR extremum δ_l of the CAT-16 spin label in disoriented DPPC bilayers. Squares: sample annealed at 0 °C for 90 hours, heating scan; circles: heating scan 30 min. after fast recooling from 30 °C to 0 °C

on various ESR spectral parameter of the CAT-16 spin label in the disoriented DPPC bilayers. The temperature dependence of the effective outer splitting A_{max} is shown in Fig. 7. In this dependence two temperature regions (30 °C ÷ 32 °C and 41 °C ÷ 42 °C) can be seen where the A_{max} values sharply decrease. The 40 °C ÷ 42 °C temperature region coincides with the main phase transition temperature observed calorimetrically in the same lot of DPPC at 41.5 °C [37]. The decrease of A_{max} in the temperature region of 30 °C ÷ 32 °C is probably associated with the pretransition. This temperature range is somewhat lower than the pretransition temperature of 35.5 °C found calorimetrically in the same lot of DPPC [37]. This is possibly due to the perturbing effect of the CAT-16 spin label on the bilayer, since the pretransition is known to be particularly sensitive to the presence of impurities. A similar decrease in the pretransition critical temperature has been observed in the presence of the cholestane spin label in the bilayer [38]. The subtransition is accompanied by the onset of the lipid molecules rotation about their long axes (see [39] for references). In our previous paper [39] we found that the A_{max} value of the cholestane spin label decreases at the subtransition, in contrast to the A_{max} of the CAT-16 spin label in the present study. This is in accord with the different orientation of the A tensor with respect to the bilayer director of these two different spin labels. The cholestane spin label is oriented with its long molecular axis preferentially parallel to the bilayer director and thus the x and z-axes of the A tensor lie preferentially within the bilayer plane [1, 2]. Since $A_{zz} : A_{xx} \approx 5 \div 6$ for the cholestane spin label, the A_{max} value of this label is sensitive to the label rotation about its long axis. As found in experiments with oriented EYPC bilayers described above, the x- and y-axes of the CAT-16 spin label A tensor lie preferentially within the bilayer plane, and the z-axis is preferentially parallel to the membrane director, i.e. parallel to the symmetry axis of the label rotation which starts at the subtransition. Since $A_{xx} = A_{yy}$, this rotation cannot affect the observed values of A_{max} appreciably. This is why we have not observed any difference in A_{max} in the annealed and nonannealed samples. However, in contrast to the A_{max} parameter, we have observed small but reproducible differences in the values of the linewidth parameter δ_l (full width at half-height of the low-field extremum). As seen in Fig. 8, the value of δ_l of the CAT-16 spin label in DPPC bilayers annealed for a prolonged period of time are higher than in the nonannealed bilayers in the temperature range of 0 °C ÷ 20 °C. The value of δ_l in the annealed sample decreases with the increase of temperature in the region of 16 °C ÷ 20 °C. A similar effect has been observed in the ESR spectra of the cholestane spin label in DPPC bilayers after annealing [39]. Most probably, a fraction of the spin label molecules forms clusters in the $L_{c'}$ lattice defects during the annealing of samples at 0 °C, and in these clusters the locally increased spin label concentration causes line broadening due to the Heisenberg spin exchange and dipole-dipole interactions. At about 16 °C ÷ 18 °C the lipid and spin label molecules start to rotate at subtransition and this is accompanied by the lateral diffusion and dissolution of label molecules in the host lipid lattice. Since the $L_{c'}$ phase does not form in the nonannealed samples, the $L_{\beta'}$ phase in these samples is overcooled below the subtransition temperature and the linewidth parameter δ_l is not increased — there is no formation of clusters in the $L_{\beta'}$ phase. Above 22 °C the values of δ_l increase in both the annealed and nonannealed samples and do not differ within the experimental error. The change in the linewidth parameter δ_l

in the annealed sample between 16 °C and 18 °C correlates well with the subtransition temperature observed calorimetrically at 17 °C ÷ 18 °C in DPPC bilayers annealed and scanned under conditions close to ours [11, 33, 39–43].

In the temperature range of 24 °C ÷ 90 °C, both the outer and inner effective nitrogen hyperfine splittings $A_{\max}$, $A_{\min}$ could be deduced from the ESR spectra of the CAT-16 spin label in disoriented DPPC bilayers. According to Gaffney [44], the effective splittings are related to the true splittings in case of axially symmetric motion according to

$$A_{\perp} \cong A_{\min} + 0.14[1 - (A_{\max} - A_{\min}) : \{A_{zz} + (A_{xx} + A_{yy}) : 2\}], \quad (12)$$

$$A_{\parallel} \cong A_{\max}. \quad (13)$$

From these splittings, the isotropic nitrogen hyperfine splitting can be calculated

$$A_{iso} = (A_{\parallel} + 2A_{\perp}) : 3 \quad (14)$$

and compared to the isotropic splitting calculated from the principal values of the A tensor and corrected for the bilayer polarity effects using the $A_{\max}^{-196}$ value:

$$A_{iso}^{-196} = A_{\max}^{-196}(A_{xx} + A_{yy} + A_{zz}) : 3A_{zz}. \quad (15)$$

The temperature dependence of A_{iso} is shown and compared with A_{iso}^{-196} (solid line) in Fig. 9. At the main phase transition temperature the A_{iso} falls to a minimum. If the temperature is increased or lowered, the A_{iso} value increases. With the increase of temperature above the main phase transition temperature A_{iso} approaches continuously the A_{iso}^{-196} value. The sharp decrease of A_{iso} at the phase transition temperature could thus indicate fluctuations in the location and/or in the polarity of microenvironment of the CAT-16 spin label paramagnetic $N \rightarrow 0$ group in the bilayer.

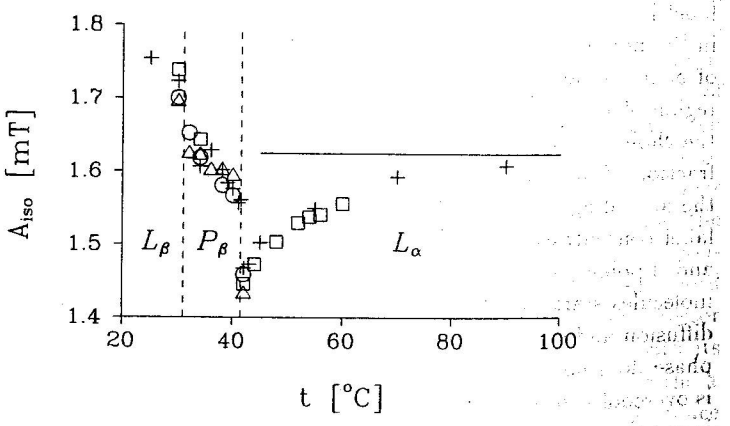

Fig. 9. The temperature dependence of the isotropic hyperfine splitting A_{iso} of the CAT-16 spin label in disoriented DPPC bilayers. Different symbols: calculated from experimental $A_{\max}$, $A_{\min}$ values obtained with different samples; solid line: calculated from the $A_{\max}^{-196}$ and A_{ii} values

A similar temperature dependence of A_{iso} has been observed for the CAT-16 spin label in disoriented DPPE bilayers with a broader minimum at about 50 ÷ 55 °C (Fig. 10). The thermotropic behaviour of fully hydrated DPPE can be

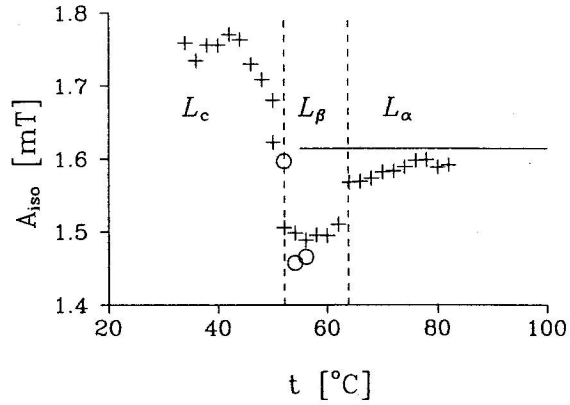

Fig. 10. The temperature dependence of the isotropic hyperfine splitting A_{iso} of the CAT-16 spin label in disoriented DPPE bilayers. Different symbols: calculated from experimental $A_{\max}$, $A_{\min}$ values obtained with different samples; solid line: calculated from the $A_{\max}^{-196}$ and A_{ii} values

described as

$$L_c \rightleftarrows L_\beta \rightleftarrows L_\alpha \rightleftarrows H_{II}, \quad (16)$$

where L_c and L_β are the lamellar subgel and gel phase, respectively, both with extended acyl chains predominantly in *trans* configuration. L_α and H_{II} are the fluid liquid-crystalline phase with disordered acyl chains due to *trans-gauche* isomerization. The L_α phase is a lamellar and H_{II} inverted hexagonal phase (see [45] for references). Unprimed c and β in eqn. 16 denote that the acyl chains in DPPE bilayers are untilted. The $L_\beta \rightarrow L_\alpha$ and $L_\alpha \rightarrow H_{II}$ phase transition have been observed calorimetrically at 63.8 °C [10, 11] and above 115 °C [46, 47], respectively. The dashed arrows in eq. 16 indicate uncertainty in the formation of the subgel phase. Silvius et al. [48] have observed, using calorimetry and Raman spectroscopy, that freshly hydrated $L - \beta, \gamma$-dimyristoyl-α- phosphatidylethanolamine (DMPE) shows a lattice rearrangement without substantial absorption of heat or an increase in the acyl chain *gauche* conformers about 15 °C below the $L_\beta \rightarrow L_\alpha$

phase transition. They suggested that besides the $L_{\beta'}$ L_α and H_{II} phases, hydrated phosphatidylethanolamines can adopt a "subgel-like" phase which we denote L_c in analogy to DPPC. The broad minimum of A_{iso} at $50 \div 55\,^\circ$C might coincide with such a lattice rearrangement in DPPE bilayers indicating changes of the CAT-16 spin label paramagnetic $N \rightarrow 0$ group location and/or microenvironment polarity. With the increase of temperature above 55 °C, the A_{iso} value increases and approaches continuously the A_{iso}^{-196} value at high temperatures. Small change in A_{iso} has been observed also at the $L_\beta \rightarrow L_\alpha$ phase transition temperature.

Griffith and Jost [13] suggested a procedure for the calculation of the S_{33} order parameter from those ESR spectra where only the $A_{\max}$ value is available: First, it is supposed that $A_{iso}^{-196} = A_{iso}$, then $A_\perp$ is calculated from $A_{\max}$ and $A_{\max}^{-196}$ using eqns. 12–14 and the principal values of the A tensor, and, finally, S_{33} is calculated using eqns. 3 and 6. Obviously, this approach can be used for the CAT-16 spin label in DPPC and DPPE bilayers only at the high temperature limit, where A_{iso} obtained from the measured $A_{\max}$ and $A_{\min}$ values is close to the value of A_{iso}^{-196}. Therefore, the S_{33} order parameter of the CAT-16 spin label in disoriented DPPC and DPPE bilayers has been calculated from experimental $A_{\max}$ and $A_{\min}$ values using eqns. 3, 6, 12, 13 and principal components of the A tensor in eqn. 7. The temperature dependence is shown in Figs. 11, 12. In the DPPC bilayers, the S_{33}

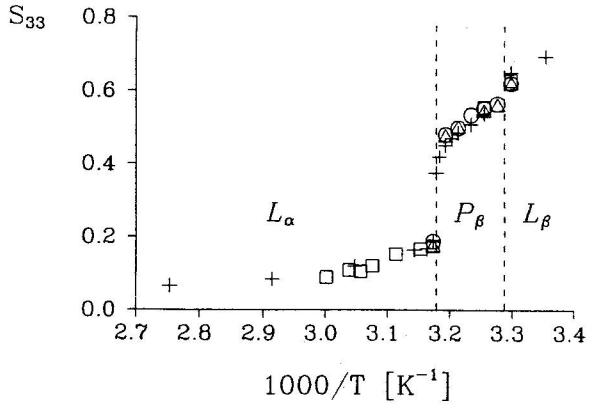

Fig. 11. The temperature dependence of the order parameter S_{33} of the CAT-16 spin label in disoriented DPPC bilayers. Dashed dividing lines separate different DPPC phases. Different symbols – results obtained with different samples

decreases sharply at the $P_\beta \rightarrow L_\alpha$ main phase transition. A rather small change in S_{33} at 30 °C ÷ 32 °C suggests that the drop in the $A_{\max}$ value seen in Fig. 7 at the pretransition is caused probably by the microenvironment polarity effects and not by the orientational and/or motional effects. In the DPPE bilayers, the

S_{33} value of the CAT-16 spin label shows distinct changes at both the $L_\beta \rightarrow L_\alpha$ main phase transition and the supposed $L_c \rightarrow L_\beta$ subtransition.

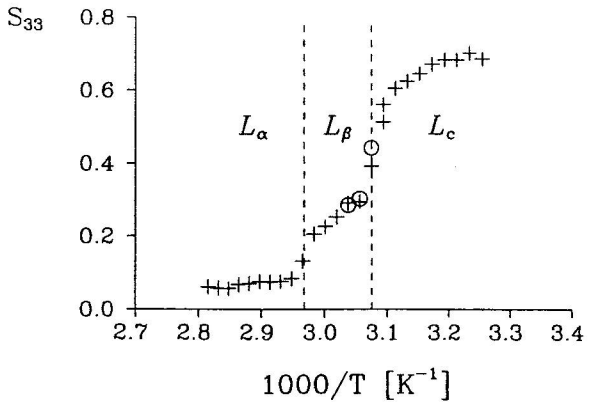

Fig. 12. The temperature dependence of the order parameter S_{33} of the CAT-16 spin label in disoriented DPPE bilayers. Dashed dividing lines separate different DPPE phases. Different symbols – results obtained with different samples

The results of our present study clearly demonstrate that the CAT-16 spin label is suitable for detection of structural and dynamical changes in the lipid bilayer polar region. Its ESR spectral parameters are sensitive to the phase transitions in lipid bilayers. These parameters support the evidence from the Raman studies for the existence of subtransition in hydrated DPPE bilayers.

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ПРОВЕРКА ПОЛАРНОЙ ОБЛАСТИ МЕМБРАНЫ С 4-(*N*-ГЕКСАДЕЦИЛДИМЕТИЛАМОНИУМ)-2,2,6,6-ТЕТРАМЕТИЛПИПЕРИДИНИЛ-ОКСИЛ БРОМА С МЕЧЕНЫМ СПИНОМ

ЭСР спектры 4-(*N*-гексадецилдиметиламониум)-2,2,6,6-тетраметилпиперидинил-оксил брома (CAT-16) с меченым спином в ориентированных и неориентированных двухслойных образцах подготовленых из яйчного желтка фосфатдылхолина (EYPC), *L*-β,γ-дипалмитоил-α-фосфатидилхолина (DPPC), или *L*-β,γ-дипалмитоил-α-фосфатидил-этаноламин (DPPE) оказываются осево-симметричными. Парамагнитная группа $N \rightarrow 0$ CAT-16 размещена на стыке воды и двойного слоя, как показывает спиновый обмен Гейзенберга и дипол-диполное уширение, которое наведено парамагнитными ионами Co^{2+}, Mn^{2+}, Gd^{3+}, и $[Fe(CN)_6]^{3-}$. Параметры порядка S_{ii} усреднения ЭСР расчитаны из спектров CAT-16 в ориентированном EYPC двухслоии, показывают на преобладающую ориентацию CAT-16 $N \rightarrow 0$ направления и $2p\pi$ орбитальной оси перпендикулярно и паралально соответственно, относительно двухслойного образца.