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Nuclear magnetic resonance studies of lecithin bimolecular leaflets with incorporated fluorescent probes*

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ABSTRACT **Pulse Fourier transform proton magnetic resonance techniques have been used to determine (*i*) the precise location of four membrane fluorescent probe molecules in lipid bilayer model membranes above the gel-to-liquid crystal transition temperature, t_m; (*ii*) the dynamical perturbation of the host lipid molecules induced by incorporation of the probe into the bilayer. The regions of maximal perturbation induced by these probes do not necessarily coincide with their actual location in the bilayer.**

Due to the dependence of their spectroscopic response to the microenvironment around the chromophore, fluorescent probes have been used in several studies with the intention of clarifying problems associated with membrane-related phenomena (1, 2). The limitations and validity of this technique appear however strictly dependent on the knowledge of (*i*) the location of the probe in the membrane; (*ii*) the extent of the structural and dynamical perturbation induced by the probe within the host membrane system.

The recent application of Fourier transform and proton magnetic resonance (PMR) techniques (3) to model membrane systems (4, 5) may prove useful for independently assessing both molecular organization and dynamical structure modifications induced in phospholipid bilayers by incorporated probes, above the gel-to-liquid crystal transition temperature (t_m). Useful information can, in particular, be obtained from chemical shift and proton magnetic relaxation measurements carried out on lecithin vesicles of comparable size, differing only in the introduction of an additional molecular component (6, 7).

The present work deals in particular with the location of (*i*) 1-anilinonaphthalene 8-sulfonate (ANS); (*ii*) 12-(9-anthroyl) stearic acid ([12,9]AS); (*iii*) 2-(9-anthroyl) palmitic acid ([2,9]AP) and (*iv*) pyrene (PY), in dipalmitoyl lecithin bimolecular leaflets; differential structural and dynamical perturbations induced by the probes in the host lipid-bilayer membranes are also discussed. These results have been partly reported elsewhere*.

MATERIALS AND METHODS

Synthetic β, γ-dipalmitoyl L-(α) lecithin (DPL) was purchased from Calbiochem Laboratories. ANS was obtained commercially from K & K Laboratories, Inc. (Plainview, N.Y.) and recrystallized twice with activated charcoal and twice from water. 12-(9-Anthroyl) stearic acid was a gift of A. S. Waggoner and L. Stryer (8). 2-(9-Anthroyl) palmitic acid was a gift of G. K. Radda (Oxford University). Pyrene was obtained from Eastman Chemical Co. (Rochester, N.Y.) and was recrystallized twice from hot ethanol and water.

Single-wall vesicles of pure DPL were prepared by low power sonication for 2 min under N_2 of a degassed dispersion of lecithin (37 mM) in a D_2O solution (D_2O 99.8% isotopic purity, 45 mM NaCl, 30 mM sodium acetate, 5 mM sodium phosphate, pH 7.6) with a Sonifier Cell Disruptor (model W185-Ultrasonic, Inc.) at 20 KHz. Short intervals of sonication were alternated with waiting intervals sufficiently long to reestablish the thermal equilibrium between the sample and the temperature control bath. Free palmitic acid (PA) was added to the DPL dispersions before sonication (PA/DPL, 3% wt/wt) to give the lecithin bilayer a defined electric surface charge (9). The same amount of palmitic acid was added to the vesicles containing fluorescent probes. Samples (mole ratios) of DPL/ANS (30:2 and 3:2), DPL/[12,9]AS (4:1), DPL/[2,9]AP (4:1), DPL/PY (3:1, 5:1, and 10:1) were prepared by dissolving the appropriate amounts of DPL and the probes in distilled organic solvents, then drying the solutes onto the walls of a Pyrex tube under vacuum, and finally sonicating the sample in D_2O buffer.

Nuclear magnetic resonance spectra and pulsed measurements of the spin-lattice relaxation rates (T_1^{-1}) were obtained at 220 MHz by using both continuous wave (10) and Fourier transform (3) modes as described (7, 11, 12). The estimated precision of T_1 rates is 5% for the choline *N*-methyl and the chains' $-(CH_2)_n$-groups, and 10–15% for the chains' $-CH_3$ groups.

Graphical analyses were performed on the alkyl chains' resonance band profile for measuring line widths and areas of the various signals arising from the chains' methylene and terminal methyl groups. In the bilayers DPL, DPL/ANS, and DPL/[2,9]AP, the CH_3 signal was isolated from the chains' methylene resonance band by assuming that the $-(CH_2)_{4-15}$ band shape was generally symmetric. In DPL/[12,9]AS and DPL/PY, analogous criteria of symmetry were assumed for the main component of the chain methylene groups' band based on the shape of its lower-field components and for the coalesced CH_3 triplet in order to extract that signal due to the upfield shifted CH_2 unit resonances. Line widths and peak areas were measured on continuous wave spectra, recorded under slow passage conditions, at radio frequency levels well below the saturation of all the lecithin resonances. The estimated precision on peak areas was ±10%.

RESULTS

Spectra (220 MHz) recorded on DPL vesicles above t_m have been already reported (12–14). T_1 rates and line widths were determined in these systems for various proton groups in the temperature range 45–92° (12).

DPL/ANS. As shown in Fig. 1 and in Table 1, the introduction of ANS into DPL vesicles at a DPL/ANS mole ratio of 3:2 caused significant upfield shifts on the resonances of the choline *N*-methyl group (0.20 ppm), *N*-methylene group (0.18

Abbreviations: PMR, proton magnetic resonance; t_m, gel-to-liquid crystal transition temperature; ANS, 1-anilinonaphthalene 8-sulfonate; [12,9]AS, 12-(9-anthroyl) stearic acid; [2,9]AP, 2-(9-anthroyl) palmitic acid; PY, pyrene; DPL, β, γ-dipalmitoyl L-(α) lecithin; PA, palmitic acid; SA, stearic acid.

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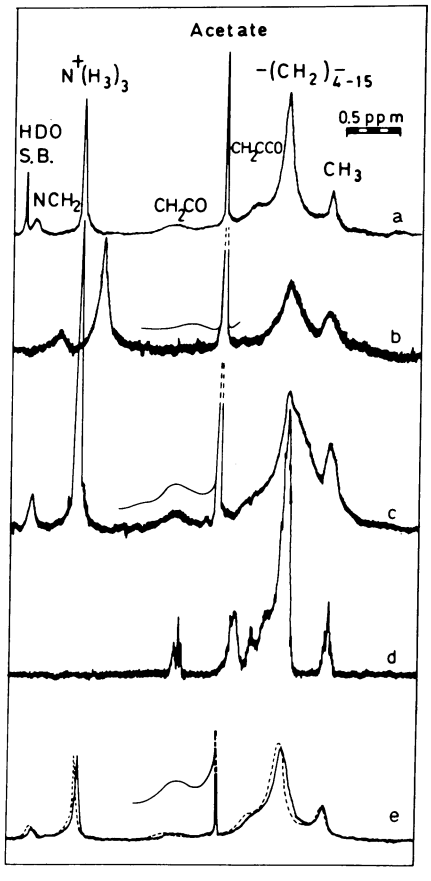

FIG. 1. Continuous-wave PMR spectra at 220 MHz of sonicated aqueous dispersions of (a) dipalmitoyl lecithin at 72°; (b) DPL/ANS (3:2) at 70°; (c) DPL/[2,9]AS (4:1) at 47°. Spectrum (d) was obtained from a solution of 12-(9-anthroyl) stearic acid in C^2HCl_3 at 20°. Spectra (e) were obtained from sonicated aqueous disperions of DPL/[2,9]AP (4:1) (—) and DPL (- - - -) at 60°. S.B. denotes a sideband of $^1H^2HO$.

ppm), and the first methylene group of the alkyl chains (0.18 ppm). Within experimental errors, the spectral positions of the other proton resonances were practically unaltered.

The resonance line widths and the temperature dependence of the spin-lattice relaxation rates of the main lecithin signals in DPL and DPL/ANS (mole ratios of 3:2 and 30:2) are shown in Table 2 and Fig. 2.

DPL/[12,9]AS. Chemical shifts (Fig. 1), resonance linewidths (Table 2) and the temperature dependence of spin-lattice relaxation rates (Fig. 3) were measured for DPL/[12,9]AS vesicles (mole ratio of 4:1) and compared to those of DPL/stearic acid (SA) (2:1)*. The chemical shifts of the various proton resonances were generally maintained unaltered under incorporation of [12,9]AS, with the only exception of some of the interior methylene groups within the alkyl chains. In fact, the $(CH_2)_{4-15}$ band profile, symmetrical in pure DPL vesicles, appeared strongly modified on its upfield side in the DPL/[12,9]AS system. The upfield shoulder of this band profile could not be assigned to any of the probe resonances because the anthroyl moiety is only able to induce down-field shifts on the neighboring [12,9]AS methylene groups. The area of the mentioned upfield shoulder of this profile, as determined by graphical analysis, corresponded to about four methylene groups shifted an average of +0.20 ppm.

DPL [2,9]AP. Chemical shifts (Fig. 1, Table 1), resonance line widths (Table 2) and spin-lattice relaxation rates (Fig. 3) were measured for DPL/[2,9]AP 4:1 and compared to those of DPL/PA mole/ratio of 2:1*, at 60°. The presence of the probe

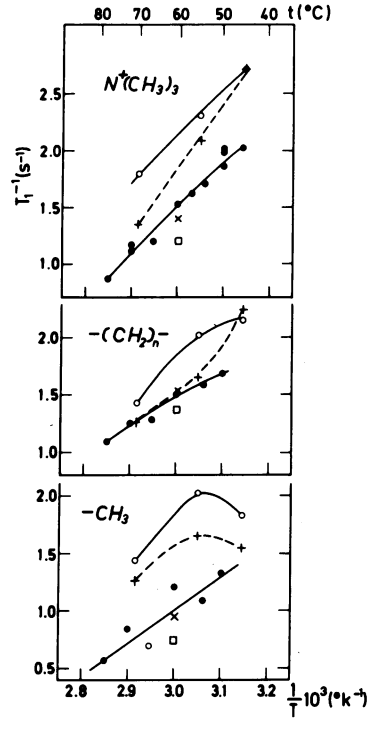

FIG. 2. Proton spin-lattice relaxation rates at 220 MHz from sonicated aqueous dispersions of DPL (●); DPL/ANS at 30:2 (+) and 3:2 (○); DPL/ at 10:1 (×) and 3:1 (□). The results obtained for pure lecithin vesicles have been already reported elsewhere (12).

in the bilayer induced slight but significant upfield shifts in the positions of the choline *N*-methyl and *N*-methylene resonances (0.03 ppm) and rather larger upfield shifts for the CH_2COO groups (about 0.10 ppm) and CH_2CCOO groups (about 0.05 ppm) of the chains. The main resonance band of the chains' methylene groups was slightly shifted as a whole, while prac-

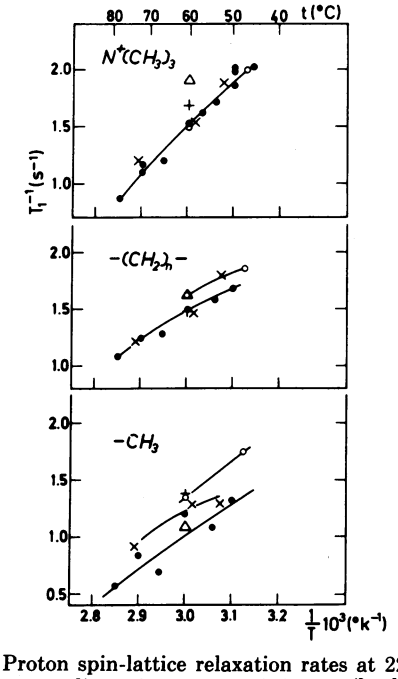

FIG. 3. Proton spin-lattice relaxation rates at 220 MHz from sonicated aqueous dispersions of DPL (●); DPL/[2,9]AS at 4:1 (○); DPL/SA at 2:1 (×); DPL/[2,9]AP at 4:1 (△); DPL/PA at 2:1 (+). The results obtained for pure lecithin, lecithin/palmitic acid, and lecithin/stearic acid vesicles have been already reported elsewhere (7, 12).

Table 1. Proton chemical shifts (ppm) for dipalmitoyl lecithin vesicles containing fluorescent probes*

System (mole ratio)	Resonance				
	$N^+(CH_3)_3$	CH_2N	CH_2COO	$(CH_2)_{4-15}$	CH_3
DPL†	−1.34 (±0.02)	−1.75 (±0.02)	−0.46 (±0.02)	0.63 (±0.02)	1.01 (±0.03)
DPL/ANS (30:2)	−1.32 (±0.03)	−1.75 (±0.03)	−0.39 (±0.03)	0.63 (±0.03)	1.04 (±0.03)
DPL/ANS (3:2)	−1.14 (±0.03)	−1.57 (±0.03)	−0.28 (±0.03)	0.67 (±0.03)	1.04 (±0.03)
DPL/[2,9]AP (4:1)	−1.31 (±0.02)	−1.72 (±0.02)	0.36 (±0.04)	0.64 (±0.02)	1.01 (±0.03)
DPL/PY‡ (10:1)	−1.34 (±0.02)	−1.75 (±0.02)	−0.39 (±0.02)	0.63 (0.77)§ (±0.02)	1.00 (±0.03)
DPL/PY‡ (5:1)	−1.34¶ (±0.02)	−1.75 (±0.02)	−0.37 (±0.02)	0.64 (0.79)§ (±0.02)	1.01 (±0.03)
DPL/PY‡ (3:1)	−1.34¶ (±0.02)	−1.75 (±0.02)	−0.33 (±0.02)	0.66 (0.85) (±0.02)	1.01 (±0.03)

* DPL/ANS between 45 and 70°; DPL/[2,9]AP at 60°; DPL/PY at 60°. The values in parentheses are ± SD.

† Peak assignments follow those by Finer *et al.* (14). The chemical shifts, referred to the acetate methyl peak, are positive in the direction of increasing electron screening.

‡ Due to an upfield chemical shift induced by the probe, the CH_2COO signal, barely detected as a shoulder of the main $(CH_2)_{4-15}$ band in DPL/pyrene (10:1), became buried under the same band in DPL/pyrene (5:1 and 3:1).

§ The chemical shift of the maximum of the resonance band is reported. The values in parentheses are those of some methylene units (see *text*).

¶ Upon increasing the probe occupancy, the choline methyl signals from the inner and outer surface of the vesicle appeared more resolved. The average value of the two chemical shifts is reported.

tically no variations were observed at the level of the chains' terminal methyl groups.

DPL/PY. Chemical shifts measured at 60° from PMR spectra of dipalmitoyl lecithin vesicles containing pyrene at various occupancy levels are reported in Table 1. Upfield chemical shifts were induced by the probe only at the level of some of the chains' methylene groups, all other resonances of the lecithin headgroup and the chains' terminal CH_3 groups being maintained unaltered. In the DPL/PY system for a mole ratio of 3:1, the first methylene unit, CH_2COO, was shifted upfield by 0.13 ppm; about three of the chains' internal methylene groups (per DPL molecule) were shifted (on the average) by 0.22 ppm, as obtained by graphical analysis of the resonance band profile. Smaller effects were induced by lower probe occupancy levels (Table 1).

Spin-lattice relaxation rates and resonance linewidths were measured on the DPL/PY system (10:1 and 3:1 mole ratios) (Fig. 2 and Table 2).

DISCUSSION

The chemical shift variations of the various proton group resonances of lecithin, induced by the incorporation of fluorescent probes into the lecithin bilayer, are interpreted on the basis of local effects induced on neighbor protons by the strong diamagnetic anisotropy of the probes' aromatic rings (15). It is known that the shielding effects associated with the secondary magnetic field generated by the ring system can only be significant within distances of a few angstroms from the center of the ring system itself (15). In the systems DPL/ANS, DPL/[12,9]AP, and DPL/PY, various lecithin resonances are shifted upfield by the presence of the probe. The presence of only one resonance for each proton species whose resonance appears shifted indicates that the lecithin molecules experience fast exchange conditions between sites respectively close to and far from the probe. This is taken as evidence of "ideally mixed" lecithin/probe bilayers on the PMR time scale. In the case of the system DPL/[12,9]AS, the upfield shift experienced by a fraction of the chains' $(CH_2)_{4-15}$ groups might in principle be interpreted either on the basis of an "ideally mixed" bilayer (where only certain methylene groups on all the chains come into contact with the probe) or on the basis of the coexistence of "laterally" separated phases between which only some lecithin chains experience slow exchange conditions. However the "ideal mixing" already found in the DPL/[2,9]AP system suggests that the first interpretation should be considered the most reasonable.

It is known that proton magnetic resonance linewidths in lecithin vesicles may depend on the vesicle size (7, 12, 16, 17). It is also known that the ultimate vesicle size in lipid dispersions produced by sonication depends on the composition of the bilayer (18). However, it should also be emphasized that a modified vesicle size is not sufficient to explain *selective* linewidth changes, namely, those occurring at the level of only some of the lecithin chemical groups. In this respect, the comparison of two vesicular systems formed from the same host lecithin and differing only in the incorporation of one additional molecular component may provide information about their different intramolecular structure and dynamics. In particular modifications of spin-lattice relaxation rates and selective line widths of the resonances of the various proton groups of lecithin induced by incorporation of the probe into the lecithin bilayer are useful to detect possible changes in the motional anisotropy of these proton groups (12, 19, 20). A useful although simplified interpretation of these relaxation phenomena in terms of the intramolecular dynamical structure of a lecithin bilayer can be provided in terms of the rotations of the proton pairs about C–C chemical bonds (correlation times $\tau_c' \lesssim 10^{-9}$–10^{-10} s) and reorientations of these rotor axes, i.e., these C–C bonds, occurring through coupled *trans-gauche* interconversions along the alkyl chains (19) and the choline group (correlation times $\tau_c'' \simeq (10^3 - 10)\tau_c'$). Intramolecular rotation around the C–C bonds is expected to affect primarily the spin-lattice relaxation, while the slower segmental reorientation of the C–C bond axes mainly affects the spin–spin relaxation and hence resonance

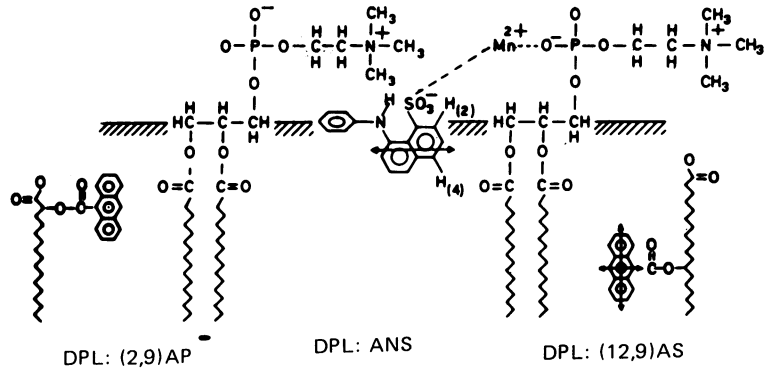

FIG. 4. Models for the relative locations in a dipalmitoyl lecithin bilayer of the fluorescent probes ANS (either above or below the alkyl chains transition temperature, t_m); AS (either above or below t_m); AP (above t_m).

line width. T_1^{-1} and line width measurements can therefore provide information not only about the local changes induced by the probe on the rotational and segmental motions of a particular proton group, but also on the changes undergone by the average value of the ratio τ_c''/τ_c', namely, by the degree of motional anisotropy of these particular proton groups whose resonances are observable (i.e., $\tau_c''/\tau_c' = 1$ implies isotropic motion for a particular proton group while increasing $\tau_c''/\tau_c' > 1$ implies an increasing motional anisotropy for that proton group). In particular, broader (narrower) line widths accompanied by smaller (larger) T_1^{-1} values indicate a higher (lower) degree of motional anisotropy of a particular proton group (7, 12). On the other hand, increased line widths paralleled by only slightly enhanced T_1^{-1} values (few percent rate increments) may still be interpreted in terms of an increased τ_c''/τ_c' ratio; however, when such T_1^{-1} increments become more significant, it should be taken as an indication of an increased local microviscosity hindering both types of motion (see refs. 7 and 12 for further discussion).

Table 2. Proton magnetic resonance line widths (Hz) for dipalmitoyl lecithin vesicles containing fluorescent probes

System (mole ratio)	Temperature (°C)	Resonance $N^+(CH_3)_3$	Resonance $(CH_2)_{4-15}$	Resonance CH_3*
DPL	45–70	11–7 (±1)	45–28 (±2)	25–16 (±2)
DPL/ANS (30:2)	45–70	12–8 (±2)	75–50 (±4)	25–25 (±4)
DPL/ANS (3:2)	45–70	27–18 (±2)	80–50 (±4)	36–30 (±4)
DPL/[12,9]AS (4:1)	47–60	10–8 (±2)	72–54 (±4)	27–24 (±4)
DPL/SA (2:1)	59–73	7.5–5 (±1)	45–35 (±2)	20–20 (±2)
DPL/[2,9]AP (4:1)	60	10 (±1)	33 (±2)	16 (±2)
DPL/PA (2:1)	59–73	10 (±1)	50 (±2)	25 (±2)
DPL/PY (10:1)	60	—†	44‡ (±4)	18 (±4)
DPL/PY (3:1)	60	—†	54‡ (±4)	18 (±4)

Values in parentheses are ±SD.

* The line width refers to that of the unresolved triplet.

† The line width of the choline *N*-methyl signal could not be correctly evaluated due to its "doublet" nature arising from the "inner" and "outer" headgroups in the vesicles (22). At higher pyrene occupancy levels the "doublet" appeared better resolved.

‡ These values refer to the line width of the symmetrical main component of the $(CH_2)_{4-15}$ resonance band.

DPL/ANS. The chemical shift measurements indicate that ANS is located anisotropically in the bilayer in a region close to the surface and necessarily penetrates a short distance between the chains of the hydrocarbon core: the center of an aromatic ring penetrates at least to the level of the chains' initial carbonyl group, as shown schematically in Fig. 4. The orientation of the probe has been selected on the basis of polarized optical absorption spectra obtained below t_m on oriented multilayers, and indicates that the 3800 Å transition dipole moment of ANS lies predominantly in the plane of the bilayer (see ref. 6 for the detailed description of the methods).

The local dynamical structure of the lecithin molecules in the bilayer appears perturbed by the presence of ANS, even at rather low probe occupancies, not only at the level of the probe location (the polar headgroup region), but also in the interior region of the hydrocarbon core of the bilayer. The selective line broadening observed for the $(CH_2)_n$ resonance band in DPL/ANS (30:2) indicates that the probe induces a severe restriction of the segmental motion of the lecithin chains.‡ The general enhancements induced by the probe on the spin-lattice relaxation rates of the main lecithin resonances can be attributed to a restriction of the rotational motions about the C–C and N–C bonds, and possibly also to a more effective intermolecular dipole–dipole interaction between neighboring molecules in the mixed bilayers. Both of these mechanisms of T_1 rate enhancements point to the same interpretation of an increased local microviscosity identified with a tighter packing of the lecithin molecules induced in the bilayer by the presence of the probe, as described at the end of the introduction to this *Discussion* section.

DPL/[12,9]AS. The upfield chemical shifts exhibited by only some methylene groups along the alkyl chains in the DPL bilayers containing [12,9]AS, together with the lack of chemical shift variations of the other lecithin resonances strongly suggest that the anthroyl moiety is located in the bilayer as schematically indicated in Fig. 4. For the orientation of the anthroyl moiety, as assessed by polarized optical absorption spectra carried out below t_m, the 2500 Å absorption transition moment is predominantly normal to the plane of the bilayer, while the 3500 Å transition moment lies parallel to this plane.

The thermal behavior of the spin-lattice relaxation rates indicates that either [12,9]AS or SA incorporated into the bilayer structure produces similar effects on the choline *N*-methyl groups and maintains the spin-lattice relaxation of these groups

‡ At larger probe occupancies, a larger vesicle diameter might further contribute to the line broadening of all the peaks in the PMR spectrum.

essentially unaltered. Small but significant effects are observed under incorporation of [12,9]AS (but not of the free stearic acid) on the average methylene groups' T_1 relaxation for the chains, with the rotational mobility of these groups about C–C bonds appearing therefore slightly perturbed. The strongest T_1 rate increment observed in the region of the chains' terminal methyl groups in the DPL/[12,9]AS versus the DPL/SA system is taken as evidence of a greater perturbation of the dynamical structure of the bilayer induced by the anthroyl moiety on the proton groups nearer to the center of the hydrocarbon core. The selective line broadenings observed in the mixed bilayers (DPL/[12,9]AS versus DPL/SA and DPL) indicate that the segmental motion of the chains' methylene and terminal methyl groups is considerably reduced by the presence of the probe. In particular, the strong line broadening of the chains' methylene group resonance (paralleled by only a slight T_1 rate enhancement) point to a considerable increase in the degree of motional anisotropy of the chains' methylene groups.

DPL/[2,9]AP. The chemical shift measurements carried out on the DPL/[2,9]AP system indicate that the anthroyl moiety of this probe is mainly located at the level of the lecithin carbonyl group (Fig. 4), a region where its diamagnetic anisotropy induces the strongest effects on the first and second methylene groups of the fatty acid chains, and progressively lesser effects on the more distal methylene groups of the alkyl chains. The orientation of the choline group is such that the *N*-methyl groups are also affected by the diamagnetic anisotropy of the aromatic moiety.

The rotational mobility of the lecithin *N*-methyl groups (about N-C bonds) is hindered to an extent by the anthroyl moiety located at the C-2 position of the fatty acid chain, as indicated by the different extent of the T_1 rate increments undergone by these groups in DPL/[2,9]AP versus DPL/PA. Resonance linewidths and T_1 rate measurements indicate that different effects are induced on the degree of motional anisotropy of the chains' proton groups when the anthroyl moiety is located differently in the bilayer. In fact, in the case where this group is located close to the lecithin carbonyl group (DPL/[2,9]AP), the degree of motional anisotropy of the chains' methylene groups is slightly decreased (i.e., decreased linewidth and increased T_1 rate) with respect to that of the bilayer incorporating the corresponding free fatty acid chains (PA). The opposite effect is induced by the anthroyl group of [12,9]AS which, being located nearer the center of the bilayer, can more likely hinder segmental reorientations along the lecithin chains.

DPL/PY. Proton chemical shifts measured in DPL vesicles containing high concentrations of pyrene indicate that this probe is mainly located in the hydrocarbon core of the bilayer (21). The effect of its diamagnetic anisotropy is most significant at the level of some interior methylene groups of the chains, but considerable effects are also observed on positions 2 and 3 in the chains' CH_2 groups. The average "ring current" effect induced by pyrene is negligible at the level of the terminal methyl groups, a fact that might in principle be interpreted either on the basis of a much lower probe occupancy at this level of the bilayer or of a more rapid relative motion of the probe near the center of the bilayer.

The reduced proton spin-lattice relaxation rates observed at high probe occupancy levels for both the choline *N*-methyl and the chain's terminal methyl groups suggest that both the headgroup and the chains' terminal CH_3 regions can experience higher rotational mobility about bonds due to the location of the probe within each monolayer. The slightly smaller T_1 rates observed for the symmetrical main resonance component of the chains' methylene groups suggests that the corresponding CH_2 groups more distal to the glycerol moiety have a higher rotational mobility about C–C bonds. This observation together with the considerable broadening exhibited by the chains' methylene resonance band in the DPL/pyrene systems, indicates an increase in the degree of motional anisotropy of the chains' methylene groups in this region induced by the presence of the probe.

CONCLUSIONS

This paper together with our earlier x-ray diffraction studies (9) shows that fluorescent probe molecules can be precisely located within host lipid-bilayer membrane structures to within a few angstroms. In addition, this work also clearly indicates the danger in associating the region of maximal structural and/or dynamical perturbation in the host lipid bilayer (or membrane) with the actual location of the perturbant probe molecule. Finally, we suggest that such perturbations of the host lipid bilayer induced by the probes might be similar to local perturbations induced by these probes at much lower probe occupancies of the model membrane.

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