An Electron Spin Resonance Study of Interactions Between Phosphatidylcholine and Phosphatidylserine in Oriented Membranes

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ABSTRACT A detailed electron spin resonance (ESR) study of mixtures of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and phosphatidylserine (POPS) in oriented multilayers in the liquid crystalline phase is reported with the purpose of characterizing the effects of headgroup mixing on the structural and dynamical properties of the acyl chains. These studies were performed over a range of blends of POPC and POPS and temperatures, utilizing the spin-labeled lipids 16-phosphatidylcholine and 5-phosphatidylcholine as well as cholestane (CSL). The ESR spectra were analyzed by nonlinear least-squares fitting using detailed spectral simulations. Whereas CSL shows almost no variation in ordering and rotational dynamics versus mole fraction POPS, (i.e. x_{PS}), and 5-PC shows small effects, the weakly ordered end-chain labeled 16-PC shows large relative effects, such that the orientational order parameter, S is at a minimum for $x_{PS} = 0.5$ where it is about one-third the value observed for $x_{PS} = 0$ and 1. This is directly reflected in the ESR spectrum as a substantial variation in the hyperfine splitting with x_{PS}. The least-squares analysis also shows a reduction in rotational diffusion coefficient, $R_\perp$ by a factor of 2 for $x_{PS} = 0.5$ and permits the estimation of S_2, the ordering parameter representing deviations from cylindrically symmetric alignment. These results are contrasted with ^{2}H NMR studies which were insensitive to effects of mixing headgroups on the acyl chains. The ESR results are consistent with a somewhat increased disorder in the end-chain region as well as a small amount of chain tilting upon mixing POPC and POPS. They demonstrate the high sensitivity of ESR to subtle effects in chain ordering and dynamics.

INTRODUCTION

Although much information is accumulating on the structure, conformation, and dynamics in the lipid hydrocarbon chain region, less is known about the interactions between different head-groups and how they influence the acyl chain conformation and dynamics. An understanding of the effects of interactions between different head-groups on the structure and dynamics of the hydrocarbon chain region is essential for interpreting how membrane proteins or peptides affect the structure and dynamics of mixed lipid membranes (Devaux et al., 1986; Roux et al., 1989; Dempsey et al., 1989). It is also important to better understand the nonideal mixing of different lipid species (Swanson and Feigenson, 1990; Huang et al., 1993) and the phase structure of mixed membranes.

Diacylphosphatidylcholines and diacylphosphatidylserines are two lipid species commonly found in biological membranes. They are found to form homogeneous solutions at all compositions in the liquid crystal phase, that are, in general nonideal (Swanson and Feigenson, 1990; Huang et al., 1993). ^{2}H and ^{31}P NMR studies have shown that although the interactions between negatively charged phosphatidylserine (PS) head-groups and the interactions between zwitterionic phosphatidylcholine (PC) head-groups are quite different, the order parameter profiles of the acyl chain in these two lipids are quite similar (Browning and Seelig, 1980). It is known that the conformation of the dimyristoylphosphatidylcholine (DMPC) head-group is changed when dimyristoylphosphatidylserine (DMPS) is incorporated (Roux et al., 1989; Christopher et al. 1989). Also, the strong interactions between the DMPS head-groups due to intermolecular electrostatic forces or to hydrogen bonding is removed by dilution with DMPC (Browning and Seelig, 1980). Nevertheless, the ^{2}H NMR spectra of DMPC-d_{54} (i.e., fully deuterated acyl chains) in pure DMPC and in DMPC/DMPS mixtures are indistinguishable (Devaux et al., 1986).

Our previous studies in this laboratory have shown that the ESR spectra from spin labels dissolved in macroscopically oriented lipid bilayers, either pure or containing cholesterol or a membrane peptide, such as gramicidin A′, are very sensitive to the ordering of the acyl chain (Kar et al., 1985; Tanaka and Freed, 1984, 1985; Shin and Freed, 1989a, 1989b; Shin et al., 1993). Given this sensitivity, a worthy goal would be to determine whether the effect of the interactions between different head-groups on the ordering and dynamics of the hydrocarbon chains in mixed lipid bilayers was amenable to study by these methods. In this paper, we report on the results of an ESR study of POPC/POPS (the 1-palmitoyl-2-oleoyl forms of PC and PS), in which the rotational diffusion rates and order parameters of the hydrocarbon chains were studied. We utilized the spin probes 5-PC, in which the nitroxide moiety is near the head-group, 16-PC, with the label close to the end of the chain, and cholestane (CSL), a rigid cholesterol-like molecule that senses the overall chain behavior (Kar et al., 1985; Tanaka and Freed, 1984; 1985; Shin and Freed, 1989a, 1989b). We are able to show a significant effect from the mixing of POPC

Received for publication 15 November 1993 and in final form 10 February 1994.

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0006-3495/94/05/1515/07 $2.00

and POPS on the hydrocarbon chain, particularly for the end-chain behavior sensed by 16-PC.

MATERIALS AND METHODS

Material and sample preparations

The lipids POPC, POPS (sodium salt), and the spin-labeled lipids 5-PC, 16-PC were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). CSL was purchased from Sigma Chemical Co. (St. Louis. MO). All materials were used without further purification. The appropriate amounts of POPC, POPS, or POPC/POPS mixture and the spin label, dissolved in chloroform stock solutions, were mixed thoroughly, and the solvent was evaporated in a nitrogen stream. In the present work, all the samples used were fully hydrated as described by Shin (1990). The detailed procedure of sample alignment by pressure annealing is given by Shin (1990) and Shin and Freed (1989a,b). However, the annealing temperature utilized was increased with the concentration of POPS to maintain it above the main transition temperature. That is, annealing was performed at 20°C for pure POPC and at 35°C for pure POPS with intermediate values used for the mixtures.

ESR spectroscopy and simulations

Immediately after the samples were aligned, ESR spectra were taken at several temperatures in the liquid crystal phase with a Bruker ER 200 ESR spectrometer equipped with a Varian temperature control unit. At each temperature, spectra were obtained for $\Psi = 0°$ and $\Psi = 90°$, where Ψ is the tilt angle of the normal membrane plane with the direction of the DC magnetic field.

The ESR spectra obtained for $\Psi = 0°$ and $\Psi = 90°$ at a given temperature were fit simultaneously using the nonlinear least-squares methods developed in this laboratory (Crepeau et al., 1987; Shin and Freed, 1989a) in order to obtain the rotational diffusion constants $R_\perp$ and $R_\parallel$, which are the principal components of the rotational diffusion tensor and the dimensionless ordering potential coefficients ϵ_0^2 and ϵ_2^2 from which the order parameters S and S_2 may be calculated (Schneider and Freed, 1989). These ordering terms are defined as follows. First, the orientational distribution of the spin-labeled molecules is determined by a potential $U(\Omega)$, which may be expanded in a series of Wigner rotational matrix elements, D^L_{0K} as:

$$-U(\Omega)/kT = \sum_{L,K} \epsilon^L_K D^L_{0K}(\Omega), \quad (1)$$

where $\Omega = (\alpha, \beta, \gamma)$ are the Euler angles specifying the orientation of the principal axes of molecular diffusion and ordering with respect to the mean ordering axis of the membranes. Also, k is Boltzmann's constant and T is the temperature. The most commonly used order parameter, S is defined by:

$$S = \langle D^2_{00} \rangle = \left\langle \frac{1}{2}(3\cos^2\theta - 1) \right\rangle = \frac{\int d\Omega \exp(-U/kT) D^2_{00}(\Omega)}{\int d\Omega \exp(-U/kT)}, \quad (2)$$

and the order parameter $S_2 = \langle D^2_{00} + D^2_{0-2} \rangle$ is defined in a similar manner. The S_2 parameter represents the deviation from cylindrical symmetry of the molecular alignment relative to the main alignment axis.

The magnetic parameters used for 5-PC and 16-PC were $g_x = 2.0089$, $g_y = 2.0058$, $g_z = 2.0021$, and $A_x = A_y = 4.9$ G, $A_z = 33.0$ G (Tanaka and Freed, 1984). For CSL, the g tensor components were the same as that for 5-PC and 16-PC, and the A tensor components used were: $A_x = A_y = 5.0$ G, $A_z = 33.8$ G (Tanaka and Freed, 1984).

The spectral fitting procedure for the 5-PC spin probe was somewhat different from that for either CSL or 16-PC. The combined data for $\Psi = 0°$ and $\Psi = 90°$ could not be fit without introducing a significant diffusion tilt angle, β_d. It is the angle between the magnetic z axis of the molecule, i.e., the axis of the nitrogen p-orbital of the nitroxide moiety and the main axis of a lipid chain in the all-*trans* configuration. A value of $\beta_d = 30.8°$ was obtained after individual best fits were averaged. The value of β^d for a doxyl nitroxide moiety attached to a kink is 34° (Ge and Freed, 1993). Thus the value of 30.8° may be regarded as an average of kink versus all-*trans* conformations of the acyl chain near the head-group.

TABLE 1 Potential coefficients ϵ_0^2, ϵ_2^2, order parameters S, S_2, and rotational diffusion constant, $R_\perp$ of 16-PC in POPC/POPS mixtures in the liquid crystal phase

x_{PS}	T(°C)	S	S_2	ϵ_0^2	ϵ_2^2	$R_\perp \times 10^9$ s^{-1}
0.00	35.0	0.118	−0.206	0.67	−0.65	0.41
	43.0	0.118	−0.203	0.66	−0.64	0.54
	51.0	0.116	−0.201	0.65	−0.63	0.62
	59.0	0.115	−0.213	0.66	−0.67	0.78
	67.0	0.115	−0.219	0.66	−0.69	0.82
0.10	35.0	0.111	−0.151	0.58	−0.46	0.42
	43.0	0.107	−0.139	0.55	−0.42	0.50
	51.0	0.104	−0.174	0.57	−0.53	0.61
	59.0	0.103	−0.085	0.50	−0.25	0.75
	67.0	0.099	−0.092	0.49	−0.27	0.94
0.20	35.0	0.098	−0.158	0.52	−0.47	0.30
	43.0	0.095	−0.123	0.49	−0.38	0.37
	51.0	0.097	−0.200	0.59	−0.61	0.50
	59.0	0.096	−0.203	0.57	−0.62	0.65
	67.0	0.091	−0.219	0.54	−0.66	0.79
0.40	35.0	0.072	−0.234	0.47	−0.69	0.28
	43.0	0.071	−0.222	0.45	−0.65	0.34
	51.0	0.070	−0.234	0.47	−0.70	0.42
	59.0	0.068	−0.230	0.44	−0.67	0.54
	67.0	0.068	−0.241	0.46	−0.71	0.73
0.50	35.0	0.036	−0.274	0.34	−0.78	0.20
	43.0	0.035	−0.262	0.32	−0.74	0.25
	51.0	0.035	−0.271	0.33	−0.77	0.31
	59.0	0.038	−0.271	0.34	−0.77	0.35
	67.0	0.034	−0.256	0.31	−0.72	0.48
0.60	35.0	0.045	−0.256	0.36	−0.73	0.21
	43.0	0.042	−0.254	0.34	−0.72	0.29
	51.0	0.035	−0.250	0.30	−0.70	0.39
	59.0	0.035	−0.256	0.31	−0.72	0.49
	67.0	0.036	−0.243	0.30	−0.68	0.56
0.70	35.0	0.087	−0.192	0.50	−0.57	0.26
	43.0	0.078	−0.182	0.45	−0.53	0.32
	51.0	0.073	−0.314	0.59	−0.98	0.40
	59.0	0.071	−0.269	0.51	−0.81	0.48
	67.0	0.067	−0.308	0.56	−0.95	0.59
0.80	35.0	0.129	−0.171	0.68	−0.54	0.43
	43.0	0.117	−0.203	0.66	−0.64	0.49
	51.0	0.110	−0.212	0.63	−0.66	0.56
	59.0	0.100	−0.241	0.62	−0.75	0.61
	67.0	0.094	−0.249	0.60	−0.77	0.69
0.90	35.0	0.133	−0.097	0.64	−0.30	0.48
	43.0	0.125	−0.141	0.64	−0.44	0.58
	51.0	0.114	−0.181	0.62	−0.56	0.66
	59.0	0.104	−0.213	0.61	−0.66	0.81
	67.0	0.096	−0.231	0.59	−0.71	0.96
1.00	35.0	0.147	−0.128	0.72	−0.41	0.55
	43.0	0.135	−0.143	0.68	−0.45	0.64
	51.0	0.124	−0.123	0.62	−0.38	0.70
	59.0	0.122	−0.136	0.62	−0.42	0.80
	67.0	0.115	−0.157	0.60	−0.48	0.89

RESULTS AND DISCUSSION

In Tables 1, 2, and 3 we present the results for $R_\perp$, $R_\parallel$, ϵ_0^2, ϵ_2^2 and S, S_2 for 16-PC, 5-PC, and CSL, respectively, for five or six different temperatures ranging between 21° and 68°C.

In Fig. 1 we present ESR spectra obtained for 16-PC in an aligned POPC/POPS membrane of POPS mole fraction $x_{PS} = 0.6$, for both $\Psi = 0°$ and $\Psi = 90°$ at temperatures ranging from 35° to 67°C. The high quality of the fits, represented by dotted lines, is apparent from this figure. One readily finds that in the case of 16-PC the ^{14}N hyperfine

TABLE 2 Potential coefficients ϵ_0^2, ϵ_2^2, order parameters S, S_2, and rotational diffusion constant, $R_\perp$ of 5-PC in POPC/POPS mixtures in the liquid crystal phase

x_{PS}	T(°C)	S	S_2	ϵ_0^2	ϵ_2^2	$R_\perp \times 10^8$ s^{-1}
0.00	21.0	0.398	−0.121	1.98	−0.11	0.25
	36.0	0.417	−0.133	2.03	−0.78	0.34
	45.0	0.418	−0.130	2.03	−0.76	0.34
	51.0	0.434	−0.191	2.32	−1.27	0.42
	61.0	0.444	−0.204	2.45	−1.44	0.52
	68.0	0.442	−0.234	2.45	−1.75	0.62
0.25	25.0	0.520	−0.090	2.54	−0.71	0.28
	36.0	0.539	−0.115	2.76	−0.99	0.54
	45.0	0.542	−0.093	2.70	−0.79	0.78
	51.0	0.542	−0.106	2.75	−0.92	0.87
	61.0	0.542	−0.113	2.78	−0.99	1.10
	68.0	0.534	−0.118	2.73	−1.00	1.30
0.50	21.0	0.451	−0.068	1.98	−0.12	0.40
	35.0	0.478	−0.082	2.16	−0.71	0.40
	44.0	0.466	−0.117	2.18	−0.93	0.60
	51.0	0.480	−0.134	2.32	−1.10	0.69
	61.0	0.500	−0.153	2.51	−1.36	0.86
	68.0	0.489	−0.166	2.53	−1.44	0.94
0.75	25.0	0.569	−0.094	2.88	−0.78	0.39
	35.0	0.571	−0.091	2.85	−0.66	0.59
	45.0	0.560	−0.104	2.73	−0.47	0.85
	51.0	0.554	−0.118	2.70	−0.50	1.02
	61.0	0.552	−0.121	2.66	−0.35	1.23
	68.0	0.541	−0.137	2.63	−0.55	1.38
1.00	24.0	0.441	−0.168	2.27	−1.10	0.39
	34.0	0.439	−0.192	2.36	−1.31	0.44
	45.0	0.442	−0.227	2.58	−1.67	0.55
	51.0	0.441	−0.249	2.72	−1.92	0.62
	61.0	0.437	−0.251	2.70	−1.93	0.72
	68.0	0.433	−0.240	2.58	−1.75	0.78

* $N = R_\parallel/R_\perp \sim 4$; $\beta_d = 30.8°$ (for 5-PC).

splitting is a sensitive function of x_{PS}, as we show in Fig. 2 for three temperatures. In Fig. 3 the order parameter, of 16-PC, $S_{16\text{-PC}}$ is plotted as a function of x_{PS} for all five temperatures. Whereas the ordering of this end-chain label is low ($S_{16\text{-PC}} \sim 0.1$), nevertheless a very substantial relative variation is observed versus x_{PS} with a minimum in $S_{16\text{-PC}}$ at $x_{PS} = 0.5$. The values of $S_{16\text{-PC}}$ in pure POPC and pure POPS are seen to be comparable, but for 1:1 mixtures, $S_{16\text{-PC}}$ is seen to have decreased to about one-third of this value. This significant variation of $S_{16\text{-PC}}$ clearly reflects the observed variation of ^{14}N hf splitting, in Fig. 2. The rotational diffusion coefficient, $R_\perp$ for 16-PC is plotted versus x_{PS} for the five temperatures in Fig. 4. Here we see that, whereas $R_\perp$ is comparable in the pure solvents, it is about a factor of two smaller in the 1:1 mixture. Note that the ESR simulations were not sensitive to $R_\parallel$ for 16-PC as previously reported (Shin and Freed, 1989a).

We show in Figs. 5 and 6, respectively, the order parameters S and $R_\perp$ for the spin labels 5-PC and CSL versus x_{PS} for all the temperatures. Fig. 5 shows that 5-PC and CSL are more highly ordered, as expected, since 5-PC reflects the ordering near the head-group, whereas CSL reflects the overall ordering of the hydrocarbon chain. They are found to have comparable ordering, and to exhibit only a modest change with composition. Nevertheless, it is interesting that $S_{5\text{-PC}}$ shows an increase as POPS (POPC) is added to the pure POPC (POPS) by as much as 25% for the 3:1 mixtures followed by a slight decrease for the 1:1 mixture. Similarly $R_\perp$ does not show much variation with x_{PS} for these spin labels. For CSL, $R_\perp$ hardly changes for the three compositions studied. For 5-PC, $R_\perp$ tends to increase as POPS (POPC) is added to the pure POPC (POPS) (by as much as a factor of 1.5–2.0) followed by a small decrease for the 1:1 mixture.

In general, we note that $R_\perp$ increases with increasing S for the labeled lipids. The fact that small increases in S can enhance the rotational mobility of the lipid chains has been noted previously (Shin et al. 1993). One must consider the complex internal modes of motion of the chain, as well as the overall molecular reorientation in any interpretation of $R_\perp$ (Ferrarini et al., 1989). An enhanced ordering probably enables the internal modes to be less encumbered by the neighboring lipid molecules. The reduced order parameter and $R_\perp$ experienced by 16-PC for the 1:1 mixtures most likely indicates increased disorder at the ends of the lipid chains which interferes with the internal modes of motion that dominate the reorientation of the end chain.

We are not aware of any previous study that showed as significant a change in the ordering and dynamics of the

TABLE 3 Potential coefficients ϵ_0^2, ϵ_2^2, order parameters S, S_2, and rotational diffusion constant, $R_\perp$, $R_\parallel$ of CSL in POPC/POPS mixtures in the liquid crystal phase

x_{PS}	T(°C)	S	S_2	ϵ_0^2	ϵ_2^2	$R_\perp \times 10^8$ s^{-1}	$R_\parallel \times 10^9$ s^{-1}
0.00	35.0	0.474	−0.046	2.20	−0.30	0.33	0.34
	43.0	0.465	−0.045	2.15	−0.29	0.39	0.46
	51.0	0.438	−0.038	2.06	−0.23	0.49	0.56
	59.0	0.437	−0.014	1.99	−0.08	0.57	0.58
	67.0	0.419	−0.023	1.90	−0.13	0.64	0.58
0.50	35.0	0.459	−0.050	2.12	−0.31	0.21	0.39
	43.0	0.428	−0.051	1.96	−0.29	0.26	0.52
	51.0	0.391	−0.055	1.79	−0.29	0.28	0.85
	59.0	0.367	−0.069	1.68	−0.34	0.33	0.97
	67.0	0.348	−0.043	1.57	−0.20	0.38	1.00
1.00	35.0	0.487	−0.057	2.29	−0.39	0.26	0.50
	43.0	0.460	−0.058	2.13	−0.36	0.30	0.56
	51.0	0.421	−0.064	1.94	−0.36	0.34	0.73
	59.0	0.402	−0.058	1.84	−0.31	0.37	1.50
	67.0	0.380	−0.050	1.72	−0.25	0.48	2.30

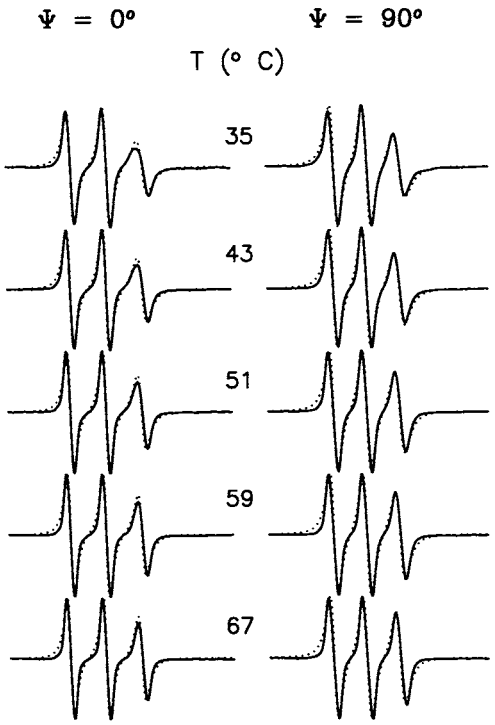

FIGURE 1 ESR spectra of 16-PC in an aligned POPC/POPS membrane with POPS mole fraction x_{PS} = 0.6 for tilt angles Ψ = 0° and Ψ = 90° at various temperatures.

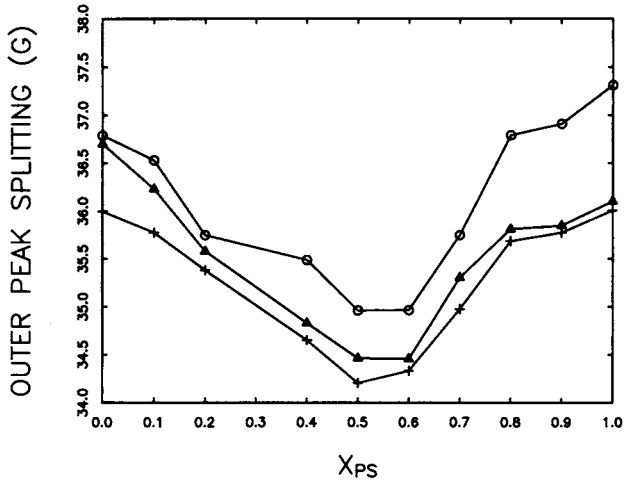

FIGURE 2 Plot of outer peak separations of ESR spectra from 16-PC in aligned POPC/POPS membranes (Ψ = 0°) as a function of mole fraction of POPS, x_{PS} at three different temperatures; +, 35°; △, 51°; ○, 67°.

hydrocarbon chain regions resulting from mixing lipids with different head-groups. For example, Devaux et al. (1986) found that the ^{2}H spectra of deuterated DMPC in the liquid crystal phase of pure DMPC are "essentially indistinguishable" from those observed in mixtures of DMPC and DMPS. Our ESR results on aligned samples would appear to provide enhanced resolution to these subtle changes. There is, however, a ^{2}H NMR study of hydrocarbon chain ordering in a substantially different system with results qualitatively simi-

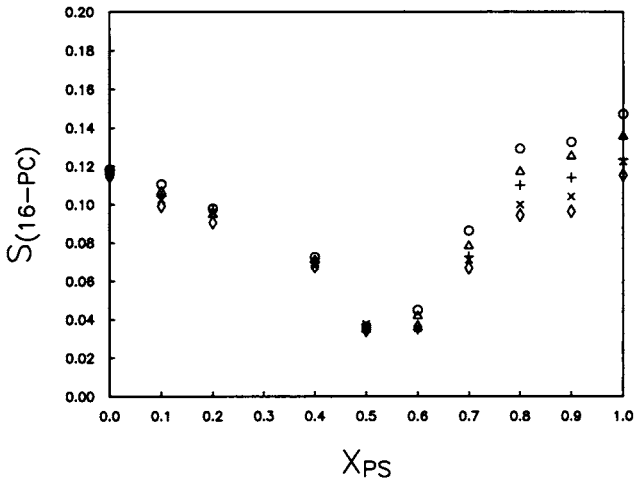

FIGURE 3 Plot of order parameter S of 16-PC as a function of mole fraction of POPS, x_{PS}, in POPC/POPS mixtures at various temperatures. ○, 35°; △, 43°; +, 51°; X, 59°; ◇, 67°.

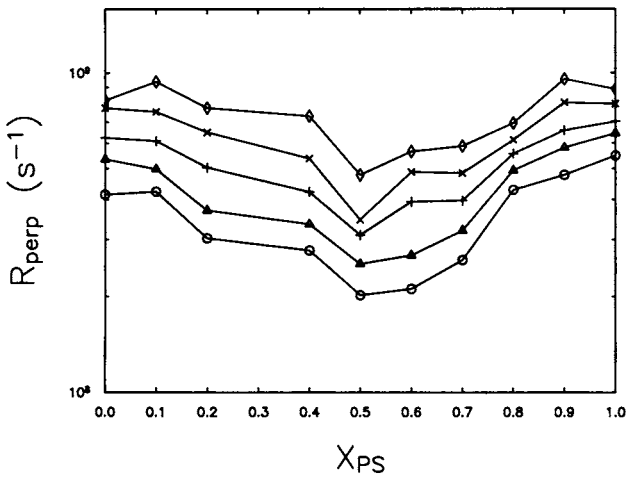

FIGURE 4 Semi-log plot of $R_\perp$ for 16-PC as a function of mole fraction of POPS, x_{PS}, in POPC/POPS mixtures at various temperatures. ○, 35°; △, 43°; +, 51°; ×, 59°; ◇, 67°.

lar to ours. De Boeck and Zidovetzki (1992) studied the interactions of a series of saturated diacylglycerols (DAGs) with DPPC. In the case of DAGs with shorter fatty acid chain length (6 or 8), they noted that an appreciable mole fraction of DAG affects primarily the lipid side chain segments close to the center of the bilayer by causing a disordering of that part of the bilayer. The lipid side chains close to the head-group experience an increase in the average quadrupole splittings, indicating increased ordering. They concluded that the DAGs intercalate between the bulky PC head-groups, promoting a tighter contact between PC side chains close to the head-group that increases their ordering. But free volume is created below the level of DAG penetration allowing for more disorder in that region of the side-chains. Of course, insertion of DAGs, which have no head-group is a major perturbation on the local bilayer structure, and that is why prominent effects are seen in the ^{2}H NMR. It would seem

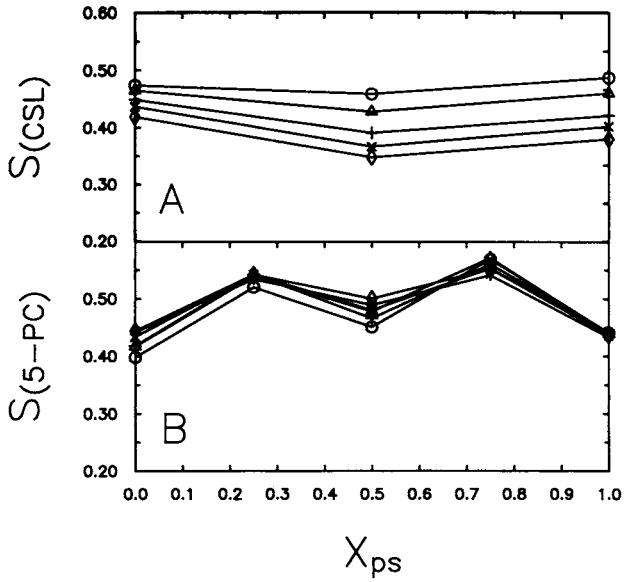

FIGURE 5 Plots of order parameters S of 5-PC and CSL as a function of mole fraction of POPS, x_{PS}, in POPC/POPS mixtures at various temperatures. (A) CSL. ○, 35°; △, 43°; +, 51°; ×, 59°; ◇, 67°. (B) 5-PC. ○, 25°; △, 35°; +, 45°; ×, 51°; ◇, 61°; *, 68°.

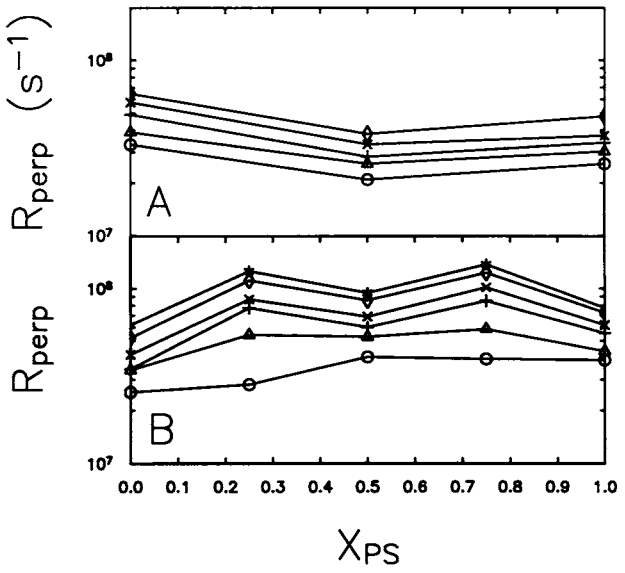

FIGURE 6 Semi-log plot of $R_\perp$ of CSL as a function of mole fraction of POPS, χ_{PS}, in POPC/POPS mixtures at various temperatures. (A) CSL. ○, 35°; △, 43°; +, 51°; ×, 59°; ◇, 67°. (B) 5-PC. ○, 25°; △, 35°; +, 45°; ×, 51°; ◇, 61°; *, 68°.

from our results that the admixture of the PS and PC head-groups leads to structural changes that modestly enhance packing in the upper chain region near the head-group but these slight constraints must be such as to induce larger effects lower down the chains. We do not envision an increased free volume there, especially as $R_\perp$ is observed to decrease in the mixture. The precise nature of the head-group interactions that lead to such effects on the hydrocarbon chains is not so obvious.

There have been several NMR studies with ^2H labels on the PS and PC head-groups in PS/PC mixtures as noted in the Introduction (Browning and Seelig, 1980; Seelig et al., 1987; Roux et al., 1989; Dempsey et al., 1989). First of all we note that NMR studies have shown that pure DPPC and DPPS have the same average conformation of the glycerol moiety and their hydrocarbon chains have the same order profile. Our ESR result that the ordering of each of the probes 16-PC, 5-PC, and CSL are very similar in pure POPC and POPS is consistent with this, although we see a slightly higher ordering in pure POPS relative to pure POPC.

The ^2H NMR studies of DMPC/DMPS mixtures show that the PC head-group does change its average orientation upon addition of PS in a manner that is described as a rotation of the head-groups relative to the bilayer interface. Thus, the NMR results are not consistent with a general disordering of the head-group. Significant effects on the PS head-group are also found to arise from addition of PC, although no specific models for the conformational change were developed for this case. Browning and Seelig (1980) do, however, conclude that there are strong intermolecular interactions between PS head-groups, and that these interactions are suppressed by addition of PC. Moreover, Dempsey et al. (1989) find that the effects of PC on the PS head-group are maximal in 1:1 mixtures of DMPC and DMPS. Further dilution of DMPS with DMPC has little effect on the DMPS head-group quadrupolar splittings. Thus there appears to be a saturating effect.

Our ESR results from 16-PC are consistent with a maximum effect at 1:1 mixtures of POPC and POPS. We do see an end-chain effect that is maximized for this composition (cf. Fig. 3). Given the sharply reduced values of S for the 1:1 mixtures, one might expect that effects which are maximal at this composition, such as the suppression of interactions between PS head-groups, or alternatively the twisting of PC head-groups, are also disordering the end-chains. However, further insight is provided by the additional order parameter, S_2, which represents the deviation from cylindrical symmetry of the molecular alignment, which we were able to obtain by ESR. The values of S_2 given in Tables 1, 2, and 3 are seen to have somewhat more scatter than those of S. (Note that S is closely associated with the accurately measured hf splittings (cf. Fig. 2), whereas the discrimination between S and S_2 required the full line shape analysis that yields ϵ_0^2 and ϵ_2^2.) Nevertheless, some clear trends do emerge. For 16-PC at 43.0°C, S_2 varies from −0.21 (x_{PS} = 0) to −0.27 (x_{PS} = 0.5), to −0.12 (x_{PS} = 1.0), with similar results at the other temperatures. We can better determine the implications of these results by considering the potential function of Eq. 1. This may be rewritten as

$$u(\theta, \phi) \equiv U(\theta, \phi)/kT = \left(\frac{\epsilon_0^2}{2}\right)(3\cos^2\theta - 1) + \sqrt{\frac{3}{2}}\epsilon_2^2 \sin^2\theta \cos 2\phi, \quad (3)$$

where θ and ϕ represent the polar and azimuthal angles of

the main chain axis, which is the principal axis of alignment, in the molecular axis system defined by the magnetic tensor of the nitroxide moiety. That is the molecular x, y, and z axes are defined relative to the doxyl ring (Schneider and Freed, 1989). The x axis is along the N—O bond, the z axis is along the $2p$ orbital of the nitrogen, and the y axis is perpendicular to the other two. In an all-*trans* stearoyl chain the molecular z axis lies parallel to the main chain axis, and the y axis points from the labeled chain to the other chain of the *sn*-2 pair (Kar et al., 1985).

We seek the aligning potential associated with the magnetic x, y, and z axes relative to the main chain axis, z'. Given the simplicity of the two parameter potential of Eq. 3 one finds that the potential minimum must always lie along one of these principal axes. More sophisticated potential functions with more terms in the expansion of Eq. 1 could also include a potential minimum tilted relative to these axes. Our analysis is necessarily limited by the limited experimental resolution which permitted only an estimate of the two leading terms in the expansion as an approximation to Eq. 1. Nevertheless we can hope to get somewhat better insight than from a single potential parameter, ϵ_2^0 (or alternatively just S). One finds from the data of Table 1 that $u(\theta, \phi)$ is at a minimum for $z \| z'$ for all values of x_{PS} (and temperatures) making it the preferred orientation, as we expect. Similarly, the $x \| z'$ orientation always has the maximum value of $u(\theta, \phi)$, making it the least preferred orientation, and the value for $y \| z'$ is intermediate. Thus a dynamic bending of the end-chain would favor tilting the y axis to be more parallel to z' than the x axis. For $x_{PS} = 0$ and 1 the values of u along each of the three axes are nearly identical (e.g., for 43°C, $u_z = -0.66$ (−0.68), $u_x = 1.1(0.9)$, and $u_y = 0.33(0.34)$ for $x_{PS} = 0(1)$). But for $x_{PS} = 0.5$ there is a small but significant change (e.g., for 43°C $u_z = -0.32$, $u_x = 1.1$, and $u_y = 0.16$), which corresponds to a reduced alignment of the z axis relative to z' but slightly enhanced alignment of the y axis relative to z' and no change for the x axis. This limited analysis indicates that the significant effects sensed by 16-PC are due, at least in part, to a small orientation change, but is there increased disorder at the end of the chain? We shall use as a criterion for this: $\Delta u \equiv u_{max} - u_{min}$, i.e., the difference between the maximum and minimum values of u. One finds $\Delta u = 1.76$, 1.42, and 1.58 for $x_{PS} = 0$, 0.5, and 1, respectively. Thus, according to this criterion there is a rather small disordering induced. (If instead of using $u_{max} = u_x$ we used $u_{avg} = \frac{1}{2}(u_x + u_y)$, then a somewhat greater disorder is found, i.e., the redefined Δu has values 1.38, 0.95, and 1.30, respectively).

When we consider the other two spin-labeled molecules we first note that for CSL, S_2 remains close to zero for all values of x_{PS}, representing its expected cylindrically symmetric alignment along the main chain axes. Also, 5-PC shows small but significant values for S_2 (≈ -0.1 to -0.2) relative to its S values (0.4 to 0.6). The variation of S_2 with x_{PS} is less clear than that of S (and similarly for ϵ_2^2 versus ϵ_0^2), but there would appear to be a small amount of twisting at the 5-C position as a result of mixing PC and PS. This twisting would be relative to the average tilt of $\beta_d = 30.8°$ of the doxyl moiety attached to the 5-carbon atom of the chain.

Given the above analysis, it is not clear at present to what extent the decrease in $R_\perp$ with decreasing S (especially for 16-PC) may be interpreted as due to the increased disordering of the chain or to its small tilting.

We now wish to comment on possible reasons why the ^{2}H NMR studies did not appear to show the changes in ordering at the end of the chain upon mixing PC and PS that we have measured by ESR. First of all we note that those studies used deuterated lipid dispersions (liposomes) which yield a superposition of powder spectra from the deuterons on all the CD_2 segments, and the terminal CD_3, and this yields significantly poorer resolution than may be achieved from a macroscopically aligned sample with a specific deuterium label near the end of the chain. The absolute change in order parameter that we see by ESR is less than 0.1, and this may well be difficult to see with the reduced resolution of the NMR experiments. Also, we would like to call attention to the fact that it may not be entirely appropriate to compare our ESR results with the ^{2}H NMR results on the acyl chain, wherein no effects of mixing were discerned. First of all, we have studied the 1-palmitoyl-2-oleoyl chains with an unsaturated bond on the oleoyl chain, whereas in the NMR work lipids with dimyristoyl chains were used. The extra tilt of the oleoyl chain could possibly enhance any effects of headgroup on the chains. Secondly, we cannot rule out that the doxyl ring, with its added bulk to the end-chain, is necessarily more affected than an unlabeled chain. However, even if that were the case, it would necessarily be amplifying an effect that was already present in the bilayer.

In addition, we would like to point out that estimates of ϵ_2^2 and S_2 could, in principle, be improved by accurate measurements of the g-shifts in the spectrum, but at conventional ESR frequencies (9.5 GHz) and fields (3.3 kG) they are small, especially for weakly ordered 16-PC (Lin and Freed, 1979). The advent of high-frequency, high-field ESR (e.g., 250 GHz and 90 kG) has made the measurement of g values much more accurate (Budil et al., 1989, 1993; Earle et al., 1994); moreover, some of the internal chain motions that lead to spectral averaging at conventional ESR frequencies may appear slow-motional at high frequency. These features should permit much more subtle orientational alignment effects to be discerned by high-frequency ESR.

Additionally, well-resolved ^{2}H NMR results (e.g., from macroscopically aligned and/or selectively deuterated samples) could complement the ESR data on ordering. The ESR hf tensor is nearly cylindrically symmetric along the molecular z axis, which is parallel to the main chain axis, z', for an all-*trans* chain, as noted above. The ^{2}H NMR quadrupole coupling tensor is cylindrically symmetric about the C-D bond, which is oriented perpendicular to z'. Thus ^{2}H NMR would be useful for estimates of ϵ_2^2 and S_2 in conjunction with the ESR measurements. However, one must be careful of the fact that ESR order parameters result from motional averaging on a significantly shorter timescale than

those from NMR, which would tend to make the ESR values larger. But for relatively rapid end-chain motions, this is probably not important at X-band frequencies (Tanaka and Freed, 1984).

In summary, we note that ESR spin-label studies utilizing well-aligned multilayers are very sensitive to even subtle changes in the hydrocarbon chain ordering and dynamics. In particular, we find that, whereas the overall ordering and fluidity in the chain region monitored by CSL does not appear to be affected by mixing POPC and POPS, the end-chain monitored by 16-PC shows significant relative effects which appear to imply some reduction in ordering, a possible enhanced tilting of the end-chain, and reduced rate of rotational diffusion upon mixing POPC and POPS.

We wish to thank David Hanauer with assistance in preparing the 5-PC samples used in this study.

This work was supported by National Institutes of Health (NIH) Grant GM25862. D.E.B. acknowledges support from NIH National Research Service Award GM12924. Computations were performed at the Cornell National Supercomputer Facility.

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