

Thermodynamics and Dynamics of Phosphatidylcholine-Cholesterol Mixed Model Membranes in the Liquid Crystalline State: Effects of Water

Yeon-Kyun Shin,* David E. Budil, and Jack H. Freed

Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853 USA

ABSTRACT A method for obtaining the thermodynamic activity of each membrane component in phosphatidylcholine (PC)/cholesterol mixtures, that is based upon ESR spin labeling is examined. The thermodynamic activity coefficients, γ_{PC} and γ_{chol} , for the PC and cholesterol, respectively, are obtained from the measured orientational order parameters, S_{PC} and S_{chol} , as a function of cholesterol content for a spin-labeled PC and the sterol-type cholestane spin probe (CSL), respectively, and the effects of water concentration are also considered. At water content of 24 weight%, the thermodynamics of DMPC/cholesterol/water mixtures in the liquid-crystalline state may be treated as a two-component solution ignoring the water, but at lower water content the role of water is important, especially at lower cholesterol concentrations. At lower water content (17 wt%), γ_{chol} decreases with increasing cholesterol content which implies aggregation. However, at higher water content (24 wt%), γ_{chol} is found initially to increase as a function of cholesterol content before decreasing at higher cholesterol content. This implies a favorable accommodation for the cholesterol in the membrane at high water and low cholesterol content. Good thermodynamic consistency according to the Gibbs-Duhem equation was obtained for γ_{PC} and γ_{chol} at 24 wt% water. The availability of γ_{chol} (and γ_{PC}) as a function of cholesterol concentration permits the estimate of the boundary for phase separation.

The rotational diffusion coefficients of the labeled PC and of CSL were also obtained from the ESR spectra. A previously proposed universal relation for the perpendicular component of the rotational diffusion tensor, R_{\perp} , for CSL in PC/cholesterol mixtures (i.e., $R_{\perp} = R_{\perp}^0 \exp(-AS_{chol}^2/RT)$) is confirmed. A change in composition of cholesterol or of water for DMPC/cholesterol/water mixtures affects R_{\perp} only through the dependence of S_{chol} on the composition. In particular, the amount of water affects the membrane fluidity, monitored by R_{\perp} for CSL, solely by the structural changes it induces in the membrane for the compositions studied. Rotational diffusion for the labeled PC is found to be more complex, most likely due to the combined action of the internal modes of motion of the flexible chain and of the overall molecular reorientation.

INTRODUCTION

Thermodynamics

The thermodynamics of model membranes and biomembranes has been an important subject of membrane research. Studies include the polymorphic behaviors of lipids (Shipley, 1973; Seelig and Seelig, 1980; Makowski and Li, 1984; Sackman, 1983), hydration effects (Parsegian et al., 1979; Rand and Parsegian, 1989), lateral phase separation (Lee, 1977; Marsh et al., 1976; Shimshick et al., 1973; Ikeda et al., 1990), protein-lipid interactions (Mouritsen and Bloom, 1984; Peschke et al., 1987; Sackman et al., 1984; Owicki and McConnell, 1978), and transverse asymmetry in membranes (Bretscher, 1972; Devaux, 1991; Hubbell, 1990). Information from these studies has played a significant role in elucidating basic membrane structures and their relation to membrane functions.

Biomembranes are composed of a diversity of lipids, cholesterol, and proteins. There also exists asymmetry in membrane compositions in different cell membranes. Thus, the thermodynamics of lipid mixtures is crucial for a better understanding of biomembranes. However, except for phase

separations in a number of cases, little is known concerning the mixing properties between membrane components. Classical thermodynamics suggests that the most important thermodynamic quantity in the study of mixtures is the activity of each component. From thermodynamic activities of the components, almost all the mixing properties may be deduced.

A fundamental difficulty in the study of mixed membranes has been that there has not been a practical method to measure the thermodynamic activities of membrane components. Consequently, it has only been possible to characterize the nonideal behavior of mixed membranes indirectly: for example, by comparing the phase diagram with that predicted using an appropriate theory (Lee, 1977; Freire and Snyder, 1980; Jan et al., 1984). However, tractable theories are necessarily very approximate, and reliable data on thermodynamic activities in membranes would also enable one to accurately test and refine theoretical models. Feigenson has developed a reliable experimental method based on solubility products (Feigenson, 1989). This method is, however, only applicable to systems containing phosphatidylserines.

A method to obtain the thermodynamic activity of each membrane component, which is, in principle, applicable to any mixed membranes, has been proposed (Shin and Freed, 1989a, 1989b). This is based on the ESR spin labeling method. There has been initial success in relating the activity

Received for publication 1 February 1993 and in final form 21 May 1993.

*Present address: Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, CA 90024.

© 1993 by the Biophysical Society

0006-3495/93/09/1283/12 \$2.00

of each component to the orientational order parameter for model systems based on phosphatidylcholine/cholesterol mixtures.

Since cholesterol is one of the major components in biomembranes, the thermodynamics of membranes containing cholesterol is important not only in elucidating phospholipid-cholesterol interactions but also in understanding the structure of membranes. Thus, there has been much attention to the thermodynamic behavior of PC/cholesterol mixed membranes. Studies have, however, focused mainly on the phase behavior because of the inaccessibility of the thermodynamic activities of components. For PC/cholesterol mixed model membranes a basic controversy arose from identifying the number of phases and phase boundaries related to the liquid crystalline state (Shimshick and McConnell, 1973; Hui and He, 1983; Ipsen et al., 1987; Knoll et al., 1985; Vist and Davis, 1990; Shankaram and Thompson, 1991; Thewalt and Bloom, 1992). Once experimental activities are available, it becomes practical to predict phase boundaries and to confirm whether single phase behavior is observed, as we have previously shown for the case of phase separation at high cholesterol concentrations (Shin and Freed, 1989b).

In this work we present further studies on the thermodynamics of PC/cholesterol mixed model membranes. We focus on two issues. One relates to a careful check of the thermodynamic consistency of our method. The second addresses the effect of water on the activities of the cholesterol and PC components. We shall find that these two issues are actually somewhat interrelated.

By obtaining experimental data on the activities for each component over a range of compositions, it is possible to test whether the data satisfy the Gibbs-Duhem equation of thermodynamics. This is a necessary requirement for a consistent thermodynamic treatment of mixed solutions. In our previous work, this matter was only partially dealt with. In our most careful previous study, the results on spin-labeled cholesterol (Shin and Freed, 1989b), obtained by us from oriented multilayers were compared with results using a labeled stearic-acid (instead of a phospholipid) obtained in another study using dispersion samples (Kusumi et al., 1986). The comparisons were partially satisfactory. It was suggested that deviations could be due to (i) flaws in the simplified analysis of spectra arising from vesicle dispersions, (ii) the use of labeled stearic acid rather than a labeled PC, (iii) implicit neglect of the effects of the third component, water, or (iv) to other differences between oriented multilayers and vesicles.

In the present work, performed on cholesterol/DMPC mixtures at higher water content (24 wt% water as opposed to the 17 wt% water used in previous studies) over an extensive range of compositions, both CSL and 7,6-PC probes (cf. below) were utilized. We are able to show that there is good agreement with the Gibbs-Duhem equation and demonstrate that previously noted discrepancies are largely due to the effects of water. It is further possible to learn about the effects of water on the activities of cholesterol and lipid in the mixed

model membranes from this data, a matter we discuss in some detail. In fact the water concentration is found to affect the thermodynamic activity of cholesterol significantly.

Rotational Dynamics

ESR is also useful in the study of molecular reorientational modes in membranes, which reflects directly on the membrane fluidity. In our recent work on PC/cholesterol mixed model membranes we have presented evidence for a new relationship between the rotational dynamics of the rigid cholesterol-like CSL probe and the thermodynamic activity as reflected by the orientational order parameter (Shin and Freed, 1989a, 1989b; Shin et al., 1990). In particular we found that for the perpendicular component of the rotational diffusion tensor, R_{\perp} (Shin and Freed, 1989a; Shin et al., 1990):

$$R_{\perp} = R_{\perp}^0 \exp \left[- \frac{AS^2}{\mathcal{R}T} \right], \quad (1)$$

where S is the orientational order parameter, A and R_{\perp}^0 are constants for a given type of mixture, and \mathcal{R} is the universal gas constant. That is, the activation energy for rotational diffusion: AS^2 is a function of S^2 , corresponding to an enhanced activation energy as the ordering of the membrane is increased by addition of cholesterol. Furthermore, the only dependence of R_{\perp} on composition is through its dependence on S . (A very similar relationship has been found for the lateral diffusion coefficients, which may be studied by dynamic imaging of diffusion ESR (Shin and Freed, 1989a; Shin et al., 1990).) Correlations such as expressed by Eq. 1 provide an important insight into the properties of PC/cholesterol mixtures, viz. that the effect of cholesterol on the dynamical properties of PC model membranes is mainly due to the structural change in the membrane induced by cholesterol, which is directly related to the order parameter.

In the present work we provide extensive further support for Eq. 1, and we also examine the effects of water concentration on this relationship. These results are then analyzed in terms of our free volume theory for the dynamics of mixed model membranes, which describes how the enhanced local ordering resulting from addition of cholesterol decreases the free volume needed for reorientation.

EXPERIMENTAL METHODS

Materials

1,2-Dimyristoyl-*sn*-glycerophosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and was used without any further purification. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO) and recrystallized in ethanol. The 3-doxyl derivative of cholestan-3-one (CSL) was purchased from Syva Co. (Palo Alto, CA) and also recrystallized in ethanol. The 1-palmitoyl-2-(8-doxylpalmitoyl)phosphatidylcholine (7,6-PC) was a gift from Prof. G. Feigenson in the department of Biochemistry at Cornell University.

Sample preparation

The well-aligned homeotropic multilayer plate samples were prepared by a modified hydration-evaporation technique described elsewhere (Shin et al., 1990; Shin, 1990). The samples were first mixed in chloroform solution, deposited on glass slides, and evacuated to remove traces of the solvent. Samples with various water contents could be prepared by placing each sample in a closed vessel with a reservoir containing salt solutions of different concentrations for a period of 24–48 h (Shin, 1990; Shin et al., 1990). When pure deionized water was used for the hydration, the sample was found to have about 24% water by weight. The water contents of the samples were determined by weighing after the ESR measurement.

The samples were prepared with a spin probe concentration of 0.5 mol% for both CSL and 7,6-PC. The ESR spectra from these samples did not show any lineshape broadening due to Heisenberg spin exchange at these concentrations.

Nonlinear least square spectral simulation

A Varian Associates, Inc. (Palo Alto, CA) E-12 spectrometer was used for the experiments. Spectra were obtained under standard conditions (Shin and Freed, 1989a, 1989b; Shin et al., 1990). All spectra were digitized to 1024 points and had 100 G sweep widths. They were analyzed to obtain the ordering and rotational dynamics utilizing ESR spectral simulation methods (Freed, 1976; Meirovitch et al., 1982; Schneider and Freed, 1989). The ESR simulations were performed utilizing nonlinear least square fitting to obtain the optimum parameters (Crepeau et al., 1987; Shin and Freed, 1989a).

The potential $U(\Omega)$ determining the orientational distribution of the spin probe molecules around the ordering axis in the uniaxially ordered lipid multilayers can be expanded in a series of generalized spherical harmonics: $D_{KM}^L(\Omega)$, e.g.,

$$-U(\Omega)/kT = \lambda D_{00}^2(\Omega) + \rho [D_{0-2}^2(\Omega) + D_{02}^2(\Omega)] + \dots, \quad (2)$$

where Ω represents the Euler angles specifying the relative orientation between the principal rotational diffusion axis, which is assumed to coincide with the molecular long axis and the ordering axis. The values of the magnetic tensor \mathbf{A} used were $A_z'' = 33.0$ G, $A_x'' = A_y'' = 4.9$ G for 76PC and $A_z''' = 33.8$ G, $A_x''' = 5.6$ G, and $A_y''' = 5.3$ G for CSL (Tanaka and Freed, 1984). Those of \mathbf{g} were $g_x''' = 2.0081$, $g_y''' = 2.0061$, and $g_z''' = 2.0024$ (Korstanje et al., 1989). One can specify the angle ψ between the ordering axis and the applied magnetic field (H_0). All spectra of CSL that were simulated had $\psi = 0^\circ$, whereas those of 7,6-PC had $\psi = 90^\circ$.

The first step in the nonlinear least squares simulation was to choose reasonable starting values for the four parameters: λ , ρ , and the perpendicular and parallel components of the rotational diffusion tensor, R_\perp and R_\parallel . (Actually, we varied R_\perp and the ratio $N = (R_\perp/R_\parallel)$ (Shin and Freed, 1989a).)

The fitting process was iterated by a Marquardt-Levenberg algorithm until a minimum in the least squares was achieved. To insure that the global minimum was obtained, and to guard against spurious local minima, we restarted the algorithm several times with a range of different seed values. Once the potential coefficients ρ and λ were determined, the orientational order parameter S was obtained by integrating (Shin and Freed, 1989a)

$$S = \langle D_{00}^2(\Omega) \rangle = \frac{\int d\Omega D_{00}^2(\Omega) \exp\left[-\frac{U(\Omega)}{kT}\right]}{\int d\Omega \exp\left[-\frac{U(\Omega)}{kT}\right]} \quad (3)$$

where $D_{00}^2(\Omega) = \frac{1}{2}(3 \cos^2 \theta - 1)$.

RESULTS AND ANALYSIS

We obtained the orientational order parameter S and the rotational diffusion coefficient R_\perp for both CSL and 7,6-PC spin probes in DMPC/cholesterol mixtures at 24 wt% over a range of compositions of cholesterol. In particular, for CSL,

S_{CSL} and $R_{\perp, \text{CSL}}$ were obtained for 10 different compositions: 0, 3.4, 5.1, 6.6, 9.6, 15.1, 21.4, 25.0, 30.0, and 40.0 mol% cholesterol (cf. Table 1); for 7,6-PC, S_{PC} and $R_{\perp, \text{PC}}$ were obtained for the nine compositions: 0, 2.0, 4.0, 10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 mol% cholesterol (cf. Table 2). The results of our previous study of CSL on DMPC/cholesterol mixtures at lower water content (17 wt% water) were also utilized for the analysis.

Ordering and thermodynamics

We show in Fig. 1 *a* the data for S_{CSL} plotted vs. x the mole fraction of cholesterol for the results on 24 wt% water at five different temperatures and in Fig. 1 *b* for 17 wt% water at three temperatures. One sees that S_{CSL} increases fairly rapidly for small x and then tends to level off. The lower water content samples have characteristically higher values of S_{CSL} for a given value of x and T and tend to level off to their maxima for lower x (~ 0.2) than for the higher water content ($x \sim 0.3$). Another key difference is the appearance of a sigmoidal shape at $x \leq 0.1$ for the higher water content, most notably at the higher temperatures. The results for S_{PC} are plotted in Fig. 2 *a* vs. x for 24 wt% water at three temperatures. They show a more gradual increase in S_{PC} vs. x compared to S_{CSL} .

The nonlinear behavior of S_{CSL} and S_{PC} shows that DMPC and cholesterol form a nonideal solution, and the tendency toward saturation in S_{CSL} and S_{PC} at higher x is a clear indication for the self-association of cholesterol in the DMPC bilayers, i.e., cholesterol and lipids are tending to segregate (Shin and Freed, 1989a, 1989b). Of course, the occurrence of a two-phase region for high cholesterol concentration (Shimshick and McConnell, 1973; Kusumi et al., 1986) is a clear indication of nonideality.

The order parameter for the i^{th} component in a mixed membrane, $S_i(\{x\}, T)$ is an intensive thermodynamic property somewhat analogous to the partial pressure (Shin and Freed, 1989b; Prausnitz et al., 1986). (Here, the symbol $\{x\}$ refers to the collection of mole fractions defining the membrane composition.) Thus, one can postulate that $S_i(\{x\}, T)$ will be linear with the mole fraction x_i if the solution is ideal, otherwise it should deviate from linear behavior. Now, when $S_i(\{x\}, T)$ varies nonlinearly with x_i one may expect that the replacement of x_i by the activity of the i^{th} component would "relinearize" the functional dependence, just as it does other intensive thermodynamic properties of a solution (e.g., partial pressure). This is equivalent to writing (Shin and Freed, 1989b):

$$S_i(x_i, T) - S_i(0, T) = ba_i(\{x\}, T) = bx_i \gamma_i(\{x\}, T), \quad (4)$$

where a is the activity of the i^{th} component, γ_i is its associated activity coefficient, and b is a constant of proportionality.

We regard CSL simply as a labeled cholesterol molecule such that S_{CSL} and S_{chol} are comparable. Thus S_{chol} may be obtained from Eq. 4 in which we expand S_{chol} about its value for $x_{\text{chol}} \equiv x = 0$ corresponding to pure lipid. In the case of the labeled lipid we again expand about the limit of pure

TABLE 1 Data for the CSL spin probe in DMPC/cholesterol mixtures at 24 wt% water

T	S	λ	ρ	R_{\perp}	R_{\parallel}
$^{\circ}\text{C}$				10^7 s^{-1}	10^8 s^{-1}
0 mol% cholesterol ($x = 0.0$)					
35	0.600	2.964	0.07	2.18	6.55
45	0.558	2.682	0.192	2.83	5.66
55	0.524	2.473	0.141	3.52	7.03
63	0.507	2.380	0.285	3.74	3.74
69	0.476	2.210	0.281	4.59	2.29
3.4 mol% cholesterol ($x = 0.034$)					
35	0.616	3.079	0.036	1.83	18.10
45	0.573	2.772	-0.036	3.09	30.91
55	0.528	2.490	0.148	3.83	38.27
63	0.522	2.457	0.169	4.25	42.46
69	0.508	2.374	0.176	4.84	48.42
5.1 mol% cholesterol ($x = 0.051$)					
35	0.646	3.332	-0.038	1.20	11.81
45	0.605	3.012	-0.217	2.30	23.0
55	0.567	2.734	-0.028	3.04	30.4
63	0.535	2.534	0.128	3.43	34.3
69	0.507	2.377	0.213	3.38	33.8
6.6 mol% cholesterol ($x = 0.066$)					
35	0.690	3.762	0.132	0.98	9.87
45	0.626	3.169	0.184	1.77	17.7
55	0.597	2.947	0.141	2.29	22.9
63	0.583	2.842	0.013	2.52	25.2
69	0.574	2.783	0.187	2.94	29.4
9.6 mol% cholesterol ($x = 0.096$)					
35	0.711	4.004	0.294	0.75	7.5
45	0.645	3.328	0.029	1.50	15.0
55	0.623	3.138	0.066	2.06	20.6
63	0.614	3.067	0.00	3.07	30.7
69	0.576	2.819	0.368	2.85	28.5
15.1 mol% cholesterol ($x = 0.151$)					
35	0.796	5.468	0.659	0.36	3.6
45	0.722	4.130	0.559	0.73	7.3
55	0.673	3.614	0.452	1.11	11.1
63	0.649	3.388	0.434	1.38	13.8
69	0.628	3.232	0.498	1.92	19.2
21.4 mol% cholesterol ($x = 0.214$)					
35	0.835	6.906	1.895	0.22	2.2
45	0.818	5.994	0.499	0.38	3.8
55	0.774	5.028	0.788	0.49	4.9
63	0.744	4.528	0.784	0.64	6.4
69	0.733	4.291	0.284	0.95	9.5
25.0 mol% cholesterol ($x = 0.250$)					
35	0.886	9.545	2.269	0.14	1.4
45	0.852	7.359	1.353	0.23	2.3
55	0.807	5.947	1.494	0.31	3.1
63	0.788	5.439	1.252	0.41	4.1
69	0.773	5.200	1.396	0.41	4.1
30.0 mol% cholesterol ($x = 0.300$)					
35	0.893	9.959	1.877	0.14	1.4
45	0.868	8.083	1.698	0.19	1.9
55	0.835	6.751	1.501	0.26	2.6
63	0.824	6.472	1.687	0.27	2.7
69	0.807	5.978	1.567	0.31	3.1
40 mol% cholesterol ($x = 0.400$)					
35	0.901	11.792	4.666	0.05	0.5
45	0.899	11.403	1.233	0.11	1.1
55	0.885	9.354	1.770	0.14	1.4
63	0.872	8.480	1.693	0.15	1.5
69	0.857	7.64	1.481	0.21	2.1

TABLE 2 Data for 7,6-PC spin probe in DMPC/cholesterol mixtures at 24 wt% water

T	S	λ	ρ	R_{\perp}
$^{\circ}\text{C}$				10^8 s^{-1}
0 mol% cholesterol ($x = 0.0$)				
35	0.270	1.74	1.55	1.50
45	0.272	1.61	1.30	2.18
56	0.237	1.46	1.30	2.07
2 mol% cholesterol ($x = 0.02$)				
35	0.278	1.72	1.45	1.71
45	0.275	1.66	1.37	2.70
55	0.264	1.48	1.11	3.31
4 mol% cholesterol ($x = 0.04$)				
35	0.287	1.82	1.56	1.92
45	0.280	1.78	1.54	3.13
56	0.275	1.58	1.23	3.47
10 mol% cholesterol ($x = 0.10$)				
35	0.310	2.21	2.04	1.82
45	0.308	1.93	1.60	2.95
56	0.282	1.62	1.22	3.57
15 mol% cholesterol ($x = 0.15$)				
35	0.360	2.42	2.02	1.77
45	0.340	2.22	1.85	2.50
56	0.324	1.97	1.55	2.92
20 mol% cholesterol ($x = 0.20$)				
35	0.403	2.66	2.10	1.32
45	0.377	2.50	2.04	2.30
56	0.356	2.22	1.72	2.74
25 mol% cholesterol ($x = 0.25$)				
35	0.466	2.99	2.16	1.00
45	0.427	2.65	1.91	1.87
56	0.396	2.37	1.69	2.34
30 mol% cholesterol ($x = 0.30$)				
35	0.494	3.17	2.22	1.27
45	0.451	2.71	1.84	1.88
55	0.414	2.53	1.82	2.65
35 mol% cholesterol ($x = 0.35$)				
35	0.498	3.02	1.95	1.10
45	0.450	2.74	1.84	1.86
56	0.452	2.54	1.52	2.60

lipid. If the solution were ideal, we would expect $S_{\text{PC}}(x, T) - S_{\text{PC}}(0, T) = b'[1 - x_{\text{PC}}] = b'x_{\text{chol}}$. Then we let $x_{\text{PC}} \rightarrow a_{\text{PC}}$ to obtain (Shin and Freed, 1989b):

$$\begin{aligned} S_{\text{PC}}(x, T) - S_{\text{PC}}(0, T) &= b'[1 - a_{\text{PC}}(x, T)] \\ &= b'[1 - \gamma_{\text{PC}}(x, T)(1 - x)]. \end{aligned} \quad (4')$$

Note that in the limit of infinite dilution, i.e., $x \rightarrow 0$, Eq. 4 for S_{chol} implies $\gamma_{\text{chol}} \rightarrow 1$, which is the Henry's Law convention for solute, whereas Eq. 4' for S_{PC} implies $\gamma_{\text{PC}} \rightarrow 1$ corresponding to Raoult's Law convention.

In order to obtain the activity coefficients γ_{chol} and γ_{PC} according to Eqs. 4 and 4' we need to utilize an interpolation function to compensate for the finite data set and its inherent uncertainties. In our previous study (Shin and Freed, 1989b) we utilized a fairly complicated function which was an amalgam of regular solution theory with a "saturating function." Although we find that this function is able to provide good

interpolations to our present data, there is considerable non-uniqueness, stemming in part from its many adjustable parameters. Instead, we have found a simpler interpolation function that is widely used in the thermodynamics of liquid mixtures (Prausnitz et al., 1986), which uniquely fits the data with fewer parameters. That is, for a two component solution one assumes that $\ln \gamma_1$ and $\ln \gamma_2$ may be expanded in a power series in x_2 and x_1 , respectively, according to:

$$\mathcal{R}T \ln \gamma_1 = a_1 x_2^2 + b_1 x_2^3 + c_2 x_2^4 + \dots \quad (5a)$$

and

$$\mathcal{R}T \ln \gamma_2 = a_2 x_1^2 + b_2 x_1^3 + c_2 x_1^4 + \dots \quad (5b)$$

In our case $\gamma_1 = \gamma_{\text{chol}}$ and $\gamma_2 = \gamma_{\text{PC}}$, $x_1 = x_{\text{chol}} = x$, $x_2 = x_{\text{PC}} = (1 - x)$.

When the Gibbs-Duhem equation (Prausnitz et al., 1986),

$$x_1 d \ln \gamma_1 + x_2 d \ln \gamma_2 = 0, \quad (6)$$

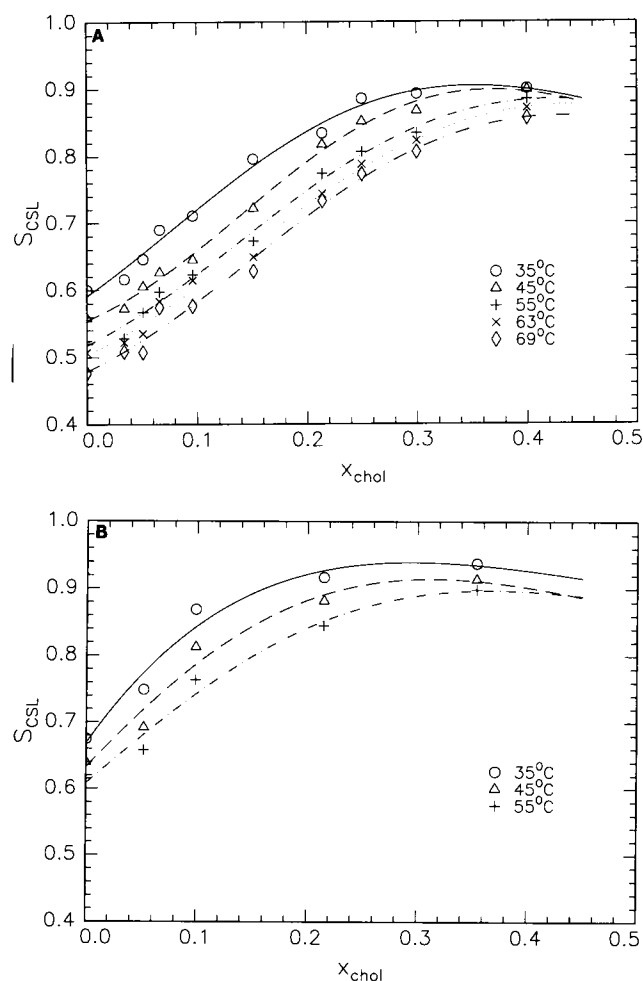


FIGURE 1 Plots of the order parameter S_{CSL} for DMPC/cholesterol mixtures at different temperatures: (a) 24 wt% water: \circ , 35°C; Δ , 45°C; $+$, 55°C; \times , 63°C; and \diamond , 69°C. (b) 17 wt% water: \circ , 35°C; Δ , 45°C; and $+$, 55°C, from Shin et al. (1990). The curves represent the best fits to the data utilizing a three-suffix Margules function: in (a), (—) $a_1 = 3.60$, $b_1 = 1.57$; (---) $a_1 = 3.89$, $b_1 = 2.21$; (- · -) $a_1 = 2.71$, $b_1 = 1.53$; (····) $a_1 = 2.51$, $b_1 = 1.48$; (- · ·) $a_1 = 2.62$, $b_1 = 1.46$; in (b) (—) $a_1 = 3.07$, $b_1 = 0.549$; (---) $a_1 = 3.52$, $b_1 = 1.07$; (- · -) $a_1 = 2.93$, $b_1 = 0.982$.

is taken into account, one finds that the coefficients in Eqs. 5a and 5b are not independent but are interrelated according to:

$$\begin{aligned} a_1 &= A + 3B + 5C & a_2 &= A - 3B + 5C \\ b_1 &= -4(B + 4C) & b_2 &= 4(B - 4C) \\ c_1 &= 12C & c_2 &= 12C \end{aligned} \quad (7)$$

The number of independent parameters (A , B , C , ...) needed will depend upon the complexity of the functional forms of the γ_i , the quality of the data, and the number of data points available. Typically, two or at most three constants are utilized. When only A is nonzero, then one obtains the form for regular solution theory, so Eqs. 5 may be regarded as a power series expansion such that the leading term yields a regular solution. Another virtue of this expansion is that the excess Gibbs-free energy for the binary solution is given

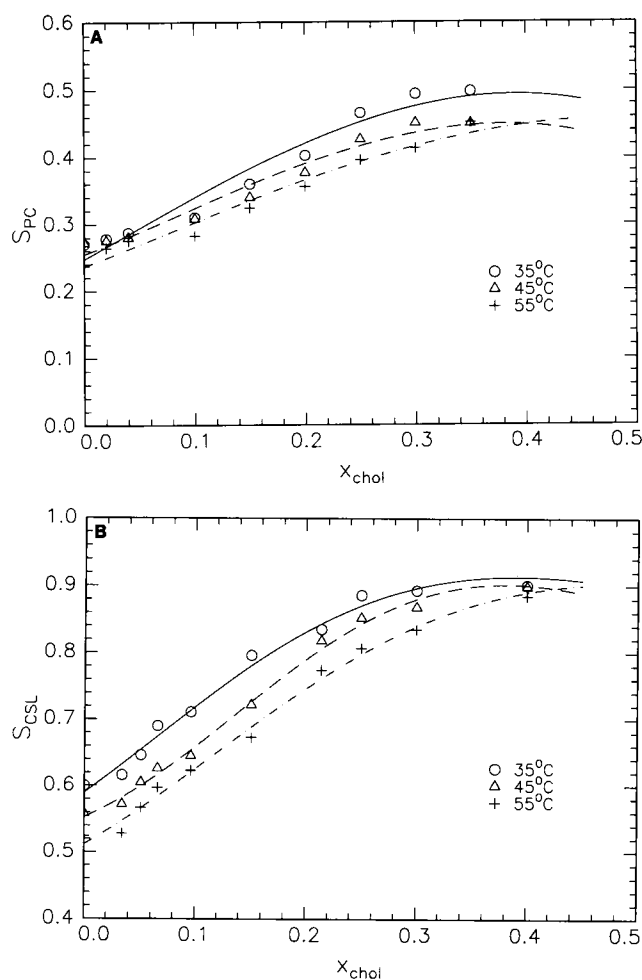


FIGURE 2 Universal fit, according to the Gibbs-Duhem Equation, of the combined data on (a) S_{PC} for 7,6-PC and (b) S_{CSL} for 24 wt% water and the temperatures 35°C (\circ), 45°C (Δ), and 55°C ($+$). The curves represent the best fits to the data utilizing a three-suffix Margules function: (—) $a_1 = 2.87$, $b_1 = 1.18$; (---) $a_1 = 3.52$, $b_1 = 1.89$; (- · -) $a_1 = 2.07$, $b_1 = 1.07$.

by (Prausnitz et al., 1986):

$$g^E = x_1 x_2 [A + B(x_1 - x_2) + C(x_1 - x_2)^2 + \dots]. \quad (8)$$

Initially, we tested this functional form for γ_{chol} on our data for S_{CSL} for both water concentrations. We found our data could be fit rather well with just the two parameters, A and B (in addition to the $S_{CSL}(0, T)$ and b parameters from Eq. 4). Inclusion of C provided no significant improvement in the fits. This two-parameter fit is known as the three-suffix Margules function (Prausnitz et al., 1986). The curves shown in Fig. 1 were obtained in this manner. One finds rather good global fits to the data. The sigmoidal shape suggested by the data at lower values of x for the higher water concentration could however be fit somewhat better by increasing the relative weight of these points, but at the expense of the global fit.

A principal check on the validity of our method is to determine whether the Gibbs-Duhem equation applies for the data on γ_{chol} and γ_{PC} obtained according to Eqs. 4 and 4'.

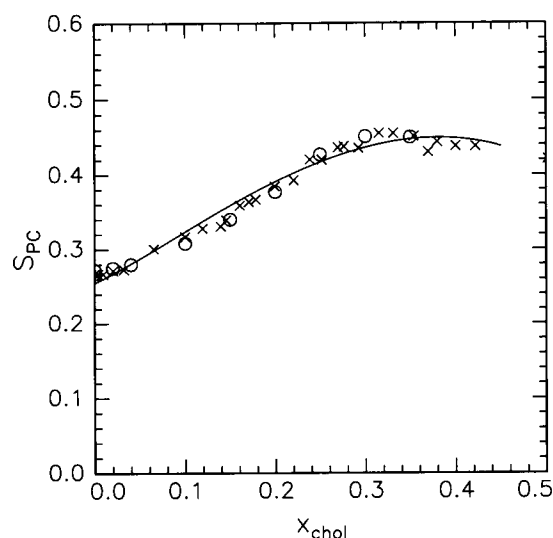


FIGURE 3 The order parameter S_{PC} for 7,6-PC in DMPC/cholesterol/24 wt% water mixtures (O) compared with results from 5-stearic acid in vesicles with excess water (X) at 45°C. The latter are arbitrarily scaled to overlap with the former. The solid curve is from Fig. 2.

As noted above, for the three-suffix Margules function, this is equivalent to simultaneously applying Eqs. 5a and 5b with Eq. 7 (and $C = 0$). We have therefore simultaneously fit the data for both CSL and 7,6-PC to Eqs. 4 and 4' with a single set of parameters A and B . However, one adjustment must be made in Eqs. 5, because they imply that both γ_{PC} and γ_{chol} are treated according to Raoult's Law convention (e.g., $\gamma_{chol} \rightarrow 1$ as $x \rightarrow 1$). To recalibrate γ_{chol} we note that the Raoult's Law convention yields from Eq. 5a $\lim_{x \rightarrow 0} \mathcal{R}T \ln \gamma_{chol}^{RL} = A - B$. Thus for the Henry's Law convention, $\mathcal{R}T \ln \gamma_{chol}^{HL}$ is obtained by subtracting $(A - B)$ from the right-hand side of Eq. 5a.

We show in Fig. 2, *a* and *b*, our results of the simultaneous fit of the S_{PC} and S_{CSL} data. The fit to the S_{CSL} data is comparable in quality to that given in Fig. 1 *a*, and the coefficients A and B agree within the estimated uncertainties. Overall, the S_{PC} data are reasonably well fit by these same coefficients, A and B , although some discrepancies exist at the lowest temperature, 35°C. (There is, of course, some scatter in the data. In our analysis, the ρ term in the orienting potential for 7,6-PC (cf. Eq. 2) was found to have a large uncertainty. When we recalculated a modified order parameter \hat{S}_{PC} utilizing only the accurately determined λ term for 7,6-PC, and then employing Eq. 4' for \hat{S}_{PC} , the agreement of Fig. 2 was markedly enhanced.)

As a further test, we have utilized data on 5-stearic acid spin probe in vesicles of DMPC/cholesterol (Kusumi et al., 1986) which yield macroscopically dispersed samples. The outer splittings of the spectra have been measured for 45°C, and they afford an approximate measure of the true thermodynamic order parameter provided that the rotational motion is indeed fast enough to average out the time dependent part of the spin Hamiltonian (otherwise incomplete motional av-

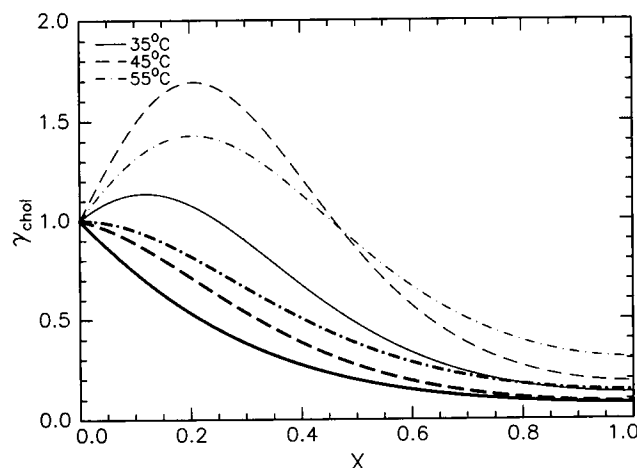


FIGURE 4 Plot of the activity coefficient of cholesterol γ_{chol} versus x for the 17 wt% water samples (heavier lines) and the 24 wt% water samples (lighter lines) at temperatures: (—) 35°C, (---) 45°C, and (- · -) 55°C.

eraging would cause significant deviations). We plot the approximate order parameters of 5-stearic acid in Fig. 3 along with the results of S_{PC} for DMPC/cholesterol 24% H₂O multilayers at 45°C. These two sets of results are seen to be in excellent agreement, indicating first that the outer splittings primarily reflect ordering and not motional effects; i.e., the rotational motion is fast enough to average out the time-dependent Hamiltonian. (The motional rate of 5-stearic acid is expected to be much faster than that of 7,6-PC, for which R_{\perp} ranges from 2×10^8 s⁻¹ to 3×10^8 s⁻¹ at 45°C (see Table 2), depending on cholesterol concentration. This is supported by the fact that much narrower ESR linewidths are observed for the 5-stearic acid spin probe than for 7,6-PC. In fact, for such fast motional rates, the order parameter calculated from the spectral splitting has been found to approximate the true order parameter to within a few percent error (Korstanje et al., 1989).) Second, this shows that both spin probes are sensing the same variation in ordering as a function of cholesterol concentration.

We also show in Fig. 3 the prediction from the approach based on the Gibbs-Duhem equation described above. The overall agreement is very good, and it is, we believe, a good demonstration of the thermodynamic consistency of the measured γ_{chol} and γ_{PC} . The actual data do, however, show a small sigmoidal departure from the prediction. Since a similar feature appears in the S_{CSL} data (cf. Fig. 1 *a*), this small residual discrepancy could, at least in part, be due to the limitations of the three-suffix Margules fitting function. The good agreement obtained at high water content also explains the significant discrepancy previously reported by us for the Gibbs-Duhem comparison at low values of x . That comparison utilized 17 wt% water samples containing CSL versus stearic acid label results (Kusumi et al., 1986) from dispersions in excess water. Fig. 1 displays the substantial difference between S_{CSL} at 24 vs. 17 wt% water, especially for low values of x .

By utilizing the fits to the three-suffix Margules function we can obtain γ_{chol} from Eq. 5a, and these results are displayed in Fig. 4 for both wt% of water. (Similar results are, of course obtained for γ_{PC} from Eq. 5b). These results are based upon the Henry's Law convention as described above such that $\gamma_{\text{chol}} \rightarrow 1$ as $x \rightarrow 0$. The results for 17 wt% water are very similar to those derived previously using a more complex fitting function for the data. They are characterized by $\gamma_{\text{chol}} < 1$ for all x and for all temperatures. This implies that as cholesterol is added to the membrane it has a tendency to associate to form cholesterol-rich domains. Note that this effect is enhanced at lower temperatures.

However, for 24 wt% water one observes a somewhat different result. There is an initial increase in γ_{chol} above unity until about $x \sim 0.2$, after which it decreases. This implies that at higher water content and lower x there is an actual (but weak) preference for cholesterol to associate with the lipids. This effect is most pronounced at the higher temperatures where the bilayer is most expanded and steric packing effects are less effective. At the lowest temperature, 35°C, γ_{chol} hardly increases above unity and appears more similar to the values for 17 wt% water. This might explain the limited success of our global fit for the case of 35°C (cf. Fig. 2).

A useful application of the activities is the prediction of phase boundaries (Shin and Freed, 1989b). Here one applies the thermodynamic stability conditions for binary mixtures. It follows from thermodynamics (Prausnitz et al., 1986) that a binary mixture is in stable equilibrium (i.e., a single phase) if the chemical potential of a component increases as its mole fraction increases:

$$\left(\frac{\partial \mu_i}{\partial x_i} \right)_{T,P} > 0 \quad (9)$$

where μ_i is the chemical potential of the i th component. However, μ_i reaches its maximum at the phase boundary. This enables one to predict the phase boundary directly from the measurement of the orientational order parameter S_i , according to (Shin and Freed, 1989b):

$$\frac{\partial \ln |\Delta S_i(x, T)|}{\partial x_i} = 0 \quad (10)$$

where $\Delta S_i(x, T) = S_i(x, T) - S_i(0, T)$. Eq. 10 is obtained using Eq. 4 and the standard thermodynamic equation $\mu_i(x, T) = \mu_i^0(\mathcal{R}T) + \mathcal{R}T \ln a_i$.

We have utilized the results of the three-suffix Margules equation fit to the CSL data for both 17 and 24 wt% water to predict this phase boundary (cf. Fig. 5). For compositions to the right of the boundary, there is phase separation of cholesterol and lipid, whereas to the left there is a single phase. Note that the phase boundary shifts to higher x_{chol} with increase in water content at the lower temperatures, i.e., extra water tends to stabilize the single phase region. These predictions of the phase boundary for DMPC/cholesterol mixed membranes agree rather well with other experimental results based on TEMPO partitioning (Shimshick and McConnell, 1973). We should mention that the determination of the maxi-

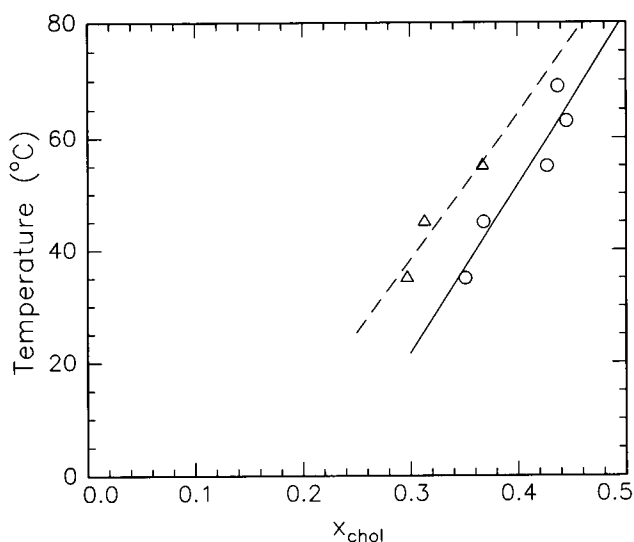


FIGURE 5 The temperature composition phase diagram for DMPC-cholesterol mixtures for (O) 24 wt% water and (Δ) 17 wt% water.

mum in ΔS_{CSL} according to Eq. 10 involves an extrapolation of the data and is therefore somewhat sensitive to the choice of fitting function, as well as the usual noise in the data. This is particularly true in the present case where the quantity ΔS_{CSL} appears to exhibit a fairly broad maximum.

Rotational dynamics and structure

The results on the rotational diffusion tensor component R_{\perp} for CSL in DMPC/cholesterol mixtures is shown in a semilog graph versus $1/T$ in Fig. 6 for all compositions (including 24 and 17 wt% water). Each composition obeys simple Arrhenius behavior, but there are very substantial differences depending upon composition. In general, the higher the cholesterol content (i.e., x) and the lower the water content, the lower is R_{\perp} . When we apply Eq. 1 to the complete set of 65 data points we obtain a single universal curve with activation energy AS_{chol}^2 where $A = 4.019 \pm 0.064$ kcal/mol and $R_{\perp}^0 = (2.02 \pm 0.014) \times 10^8 \text{ s}^{-1}$ shown in Fig. 7. That this extensive data set can be fit with a single Eq. 1 represents further strong confirmation of its basic validity. Furthermore, the fit here, which appears equally good for the 17 and 24 wt% water data, shows that the effect of water on the dynamics of the PC model membranes can readily be accounted for just in terms of the structural change in the membrane induced by the water, as reflected in the way S_{chol} changes with water composition. Thus the effect of composition changes on the fluidity of the membrane as monitored by $R_{\perp, \text{chol}}$, whether due to cholesterol content or to water content, is simply determined by the structural changes. (Note that there is one point corresponding to 24 wt% water at $x = 0.4$ and $T = 35^\circ\text{C}$ that falls below the universal curve. Since the phase transition is predicted to occur at lower x for this temperature (cf. Fig. 5), this deviation is not surprising.)

When we consider the data on R_{\perp} for 7,6-PC we find that they do not exhibit simple well-defined behavior such as

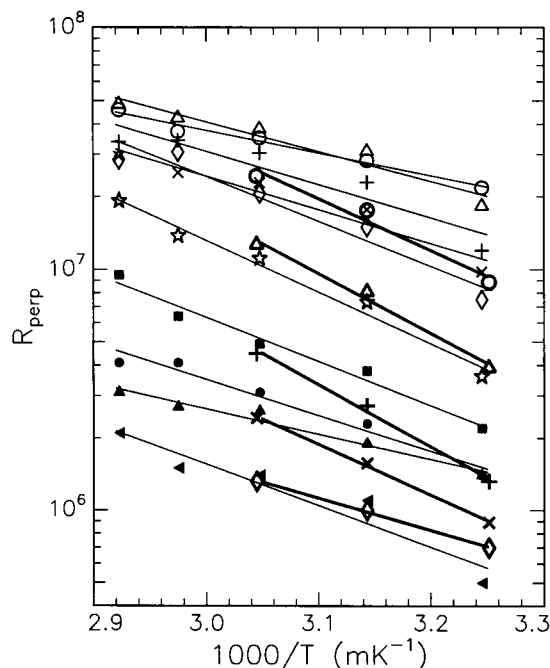


FIGURE 6 Semilog plot of R_{\perp} for CSL versus $1/T$ for 10 different values of x_{chol} containing 24 wt% water and for nine different values of x_{chol} containing 17 wt% water. Cholesterol contents at 24 wt% water (light lines) are 0.0% (\circ), 3.4% (Δ), 5.1% ($+$), 6.6% (\times), 9.6% (\diamond), 15% ($*$), 21% (\blacksquare), 25% (\bullet), 30% (\blacktriangle), and 40% (\blacktriangleleft); cholesterol contents at 17 wt% water (heavy lines) are 0.0% (\circ), 5.2% (Δ), 9.9% ($+$), 21% (\times), and 35% (\diamond).

expressed by Eq. 1. (Our results and analysis were found to be less sensitive to the anisotropy ratio, N , which was typically of order 10.) In fact, we observed that R_{\perp} actually increased with increasing x , reaching a maximum at $x \sim 0.1$ before decreasing with further increase in x . Since the 7,6-PC is nonrigid, unlike CSL, 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). It would appear that the enhanced alignment induced by small amounts of cholesterol enables the internal modes to be less encumbered by the neighboring lipid molecules. But large increases in ordering from substantial amounts of cholesterol lead to increased packing effects, which reduce the chain and overall mobility of the lipid as well as the overall motion of the CSL.

DISCUSSION

Effects of water: ordering and thermodynamics

The ordering of model membranes in the liquid crystalline state is strongly dependent on water concentration. It has been observed for PC multilayers that the S_{CSL} is significantly reduced by an increase of water content (e.g., for DPPC multilayers S_{CSL} diminishes by 0.2 upon going from 7 to 15 wt% water (Tanaka and Freed, 1984)). We also find a substantial decrease of S_{CSL} for DMPC/cholesterol mixtures (by as much as 0.1 at $x < 0.05$) upon increasing water

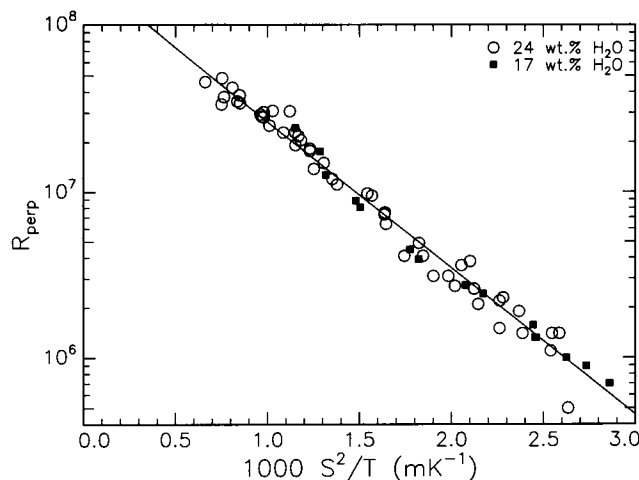


FIGURE 7 Semilog plot of R_{\perp} for CSL versus S_{CSL}^2/T for all the data shown in Fig. 6.

content from 17 to 24 wt%. Our results are consistent with the observation from x-ray diffraction studies (Parsegian et al., 1979) that the bilayers deform to become thicker and to bring together molecules on the same surface when water is removed from the lattice.

In contrast, our results show that the change in S_{CSL} with increasing water content is reduced for high cholesterol concentration (e.g., by ≤ 0.04 at $x = 0.35$). This indicates that the effect of water on the ordering of the bilayers is less significant at high cholesterol concentration. Thus, it would appear that the thermodynamic properties of DMPC/cholesterol are less sensitive to the variation of water content at high x than they are at low x . Consistent with this conclusion is our observation that the change in S_{PC} with x predicted via the Gibbs-Duhem relation using the data on CSL for 17 wt% water mixtures agrees rather well with the experimental results for PC in 24 wt% water mixtures at high x even though the agreement is not so good at low x (cf. also Shin and Freed, 1989b (Fig. 3)). In our analysis so far, we have implicitly neglected the thermodynamic role of water in the phospholipid bilayer by utilizing the Gibbs-Duhem equation for just two components, lipid and cholesterol. Now we wish to treat the water explicitly as a third component of the lipid phase in the following analysis.

For three-component mixtures of DMPC, cholesterol, and water, the Gibbs-Duhem equation interrelating the activity coefficients of the components may be given by

$$x_{\text{chol}} \frac{\partial \ln \gamma_{\text{chol}}}{\partial x_{\text{chol}}} + x_{\text{PC}} \frac{\partial \ln \gamma_{\text{PC}}}{\partial x_{\text{chol}}} + x_{\text{w}} \frac{\partial \ln \gamma_{\text{w}}}{\partial x_{\text{chol}}} = 0 \quad (11)$$

where x_{w} and γ_{w} are the mole fraction and the activity coefficient of water respectively. The mole fraction x_i of the i th component in three component mixtures is defined by

$$x_i = \frac{N_i}{N_{\text{chol}} + N_{\text{PC}} + N_{\text{w}}} \quad (12)$$

where N_i is the number density of component i . In the bilayers we expect $N_w \ll (N_{PC} + N_{chol})$.

We have observed (cf. Fig. 4) that at high water content the Gibbs-Duhem equation for a binary mixture of phospholipid and cholesterol (Eq. 6) properly interrelates the behavior of γ_{PC} and γ_{chol} ; that is, the bilayer phase behaves as a two-component solution. This implies that the third term in Eq. 11 is much smaller than the first two terms (which are then approximately equal in magnitude). Thus

$$\frac{x_w}{x_{chol}} \frac{\partial \ln \gamma_w}{\partial x_{chol}} \ll \frac{\partial \ln \gamma_{chol}}{\partial x_{chol}} \quad \text{and} \quad \frac{x_w}{x_{PC}} \frac{\partial \ln \gamma_w}{\partial x_{chol}} \ll \frac{\partial \ln \gamma_{PC}}{\partial x_{chol}} \quad (13)$$

for all x_{chol} less than that at the phase boundary. These equations suggest that, at high water content x_w , γ_w is a much weaker function of x_{chol} than either γ_{chol} or γ_{PC} .

Eqs. 13 may be rationalized in another way. For PC/cholesterol mixtures with high enough water content to contain bulk free water, we may consider that the bulk water is in equilibrium with the water phase in the multilayer, since the samples have been hydrated by equilibration with bulk water. Thus, the chemical potential of water in the multilayer must be, in principle, the same as that in bulk water regardless of the cholesterol content in the bilayer phase. However, the water phase in the multilayer is also in equilibrium with trace amounts of water in the bilayer phase, so that the chemical potential of water in the bilayer should also remain constant regardless of cholesterol content. In this case, the third term in Eq. 11 vanishes, which is fully consistent with our experimental observations.

For lower water contents, in order to examine more clearly the effects of water on the activity coefficient of each component, we may rewrite the three-component Gibbs-Duhem equation as:

$$x_{chol} \frac{\partial \ln \gamma_{chol}}{\partial x_w} + x_{PC} \frac{\partial \ln \gamma_{PC}}{\partial x_w} + x_w \frac{\partial \ln \gamma_w}{\partial x_w} = 0 \quad (14)$$

The experimental results are consistent with $\partial \ln \gamma_{chol}/\partial x_w$ being significant at low x_{chol} ; that is, γ_{chol} is sensitive to the water content at low x_{chol} , whereas it is less so at high x_{chol} . The question remains of the sensitivity of γ_{PC} to the water content. A comparison of the variation of S_{PC} obtained previously for 17 wt% water in POPC (for $x_{chol} \leq 0.2$) with our current results for 24 wt% water in DMPC shows that they are rather similar. This would seem to imply that γ_{PC} is less sensitive to the water content than γ_{chol} . If this is true, then it would have to follow that γ_w has some sensitivity to the water content, in this range, in order to preserve the equality of Eq. 14. Such a conclusion would have interesting implications regarding the influence of interbilayer interactions on the water in the multilayer phase.

In summary, it appears that water can play a significant role in the thermodynamics of cholesterol at low x_{chol} only if there is no excess water between the bilayers. As long as

free water exists, then PC/cholesterol/water mixtures show no explicit dependence on the water. Thus, in the presence of excess water, the thermodynamics of PC/cholesterol mixtures can be described by the Gibbs-Duhem equation for binary mixtures. As is well-known, there is water penetration into the bilayer (Griffith et al., 1974, Simon et al., 1982) but this effect must have been saturated in the presence of excess water.

Dynamics and structure

In the free volume theory for R_\perp previously presented (Shin et al., 1990), the dependence of the activation energy on S^2 was obtained. We present here the simple form when this S^2 dependence is dominant:

$$R_\perp(T, S_{ch}) = g^{R_\perp} \exp \left\{ - \frac{\xi \theta_{ch} S_{ch}^2 + (1 - \xi) \theta_{lp} S_{lp} S_{ch}}{T - T_0} \right\} \quad (15)$$

Here g^{R_\perp} is a geometric factor equal to $\tilde{g}(v^*)e^{-\lambda}$ where $\tilde{g}(v^*) > 1$ is essentially the ratio of the small-scale diffusion coefficient (referring to a cage with the critical free volume v^* , just large enough to permit a substantial displacement) to the diffusion coefficient in the absence of a cage. The "interaction factor" λ scales the importance of the ratio v^*/v^f , where v^f is the average free volume per molecule in the reorientation, i.e., one finds in general $R_\perp \propto e^{\lambda v^*/v^f}$.

The activation energy depends on two terms, weighted by ξ , the average fractional number of cholesterol molecules in the neighborhood of the CSL probe. The first term gives the contribution from the neighboring cholesterol molecules, whereas the second term gives that from the neighboring phospholipid molecules. \bar{T}_0 is an effective glass transition temperature for the lipid-cholesterol mixture; based on the experimental results, it is appropriate to ignore \bar{T}_0 in comparison to T . The two terms in the activation energy are: $\theta_{ch} = \lambda_{ch} k_{ch} \rho_0(P, T) b_{ch}/2 \langle \alpha \bar{v} \rangle$ and $\theta_{lp} = \lambda_{lp} k_{lp} \rho_0(P, T) b_{lp}/2 \langle \alpha \bar{v} \rangle$. Here k_i is the isothermal compressibility of the i th type of molecule, while $\rho_0(P, T)$ is the mean number density of the mixture, and $\langle \alpha \bar{v} \rangle$ is a mean of the product of the thermal expansion coefficient and the average molecular volume. The parameters b_{ch} and b_{lp} are measures of the mean field orienting potential experienced by a cholesterol molecule due to surrounding cholesterol and lipid molecules respectively. That is:

$$U_{ch} = -\rho_0 [\xi b_{ch} S_{ch} + (1 - \xi) b_{lp} S_{lp}]^{1/2} (3 \cos^2 \theta - 1). \quad (16)$$

Also λ_{ch} is the intermolecular interaction factor between cholesterol molecules and λ_{lp} is that between the cholesterol probe and the phospholipid neighborhood.

Note that the activation energy terms in Eq. 15 arise from the decrease in average free volume available to the molecule as a result of the molecular ordering in the lipid bilayer. In order for a molecule to gain the critical free volume v^* for reorientation, the cage has to expand by the amount by which it was reduced by the molecular ordering (and then, in addition, by an amount characteristic of the isotropic fluid,

which is neglected in Eq. 15 due to its presumed smallness but is given elsewhere (Shin et al., 1990)).

Our observation of Eq. 1 that the activation energy goes as AS_{CSL}^2 indicates that the other thermodynamic factors in θ_{ch} and/or θ_{lp} are hardly changed when the composition is changed by adding cholesterol or water or raising the temperature compared to the change in S_{CSL}^2 . The dominant effect is the free volume change expressed by the change in ordering.

There is ambiguity that remains from the form of Eq. 15 (or Eq. 16) about the relative importance of neighboring cholesterol versus lipid molecules. One may argue that the tendency of cholesterol molecules to aggregate should make the term in S_{chol}^2 predominate, which is certainly consistent with our experimental results. We have also considered whether our experimental data could be fit by an activation energy of form $BS_{\text{lp}}S_{\text{chol}}$, since both order parameters increase in a similar (although not identical) fashion with x_{chol} . We do not obtain a satisfactory fit to our data by this form. Thus, in order to be consistent with our experimental results at lower cholesterol concentrations, we require $\theta_{\text{ch}} \gg \theta_{\text{lp}}$. Indeed, given the structural rigidity of the cholesterol molecule relative to that of a lipid molecule, we would expect both $b_{\text{ch}} > b_{\text{lp}}$ and $\lambda_{\text{ch}} > \lambda_{\text{lp}}$ so this requirement appears quite reasonable.

We now wish to comment further on our observation that the primary effect of water on the fluidity of the membrane as monitored by $R_{\perp, \text{chol}}$ is simply due to the structural change reflected in S_{chol} . In the context of the free volume theory of Eq. 15, we have noted that this relates to the free volume change with the ordering. Apparently the small amount of water penetration, which is in fact reduced by the presence of cholesterol (Simon et al., 1982), is not playing a significant role in inducing frictional changes which would modify $g^{R_{\perp}}$ in Eq. 15. The overall effect of water on the bilayer structure is well-known, and is largely due to interactions of the headgroups with the water. In fact, Lis et al. (1982) have shown that the cross-sectional area per lipid molecule is nearly proportional to the water content for PC bilayers until excess water amounts are reached. This is also true for PC/cholesterol mixtures, and it appears from their work that the proportionality constant is nearly the same for all the mixtures they studied. It is this expanding cross-sectional area which would increase the free volume (and also reduce S_{chol}^2).

CONCLUSIONS

(a) At 24% by weight water, good thermodynamic consistency according to the Gibbs-Duhem equation is achieved between the activity coefficients for cholesterol and DMPC in the mixed model membranes, when these activity coefficients are inferred from the orientational order parameters.

(b) The functional dependences of the order parameters and activities for the phospholipid with respect to cholesterol content that are obtained from oriented multilayers with 24% by weight water are in good agreement with those obtained from vesicles in excess water.

(c) At water content of 24% weight (or greater) the thermodynamics of DMPC/cholesterol/water mixtures in the liquid crystalline state may be treated as a two component solution, but at lower water content (e.g. 17% by weight) the role of water becomes important, especially at lower cholesterol concentrations.

(d) At lower water content (17 wt%) the activity coefficient for cholesterol, λ_{chol} decreases monotonically with increasing cholesterol content, implying aggregation; at higher water content (24 wt%) there is an initial increase in λ_{chol} for the lower cholesterol concentrations indicating a greater accommodation for the cholesterol in the membrane at this higher water content.

(e) Further confirmation was obtained of the universal relation for the R_{\perp} of CSL in phosphatidylcholine/cholesterol mixtures, whereby the activation energy shows an S_{CSL}^2 dependence, and it is only in this way that R_{\perp} is affected by the composition.

(f) This S_{CSL}^2 dependence of the activation energy for $R_{\perp, \text{CSL}}$ has been shown to apply also to the effects of water content from a comparison of results for 17 and 24% by weight water. Thus the membrane fluidity, as monitored by $R_{\perp, \text{CSL}}$ appears to be affected by the water simply in terms of the structural changes induced by the water.

This work was supported by National Institutes of Health grant GM25862. Computations were performed at the Cornell National Supercomputer Facility.

REFERENCES

- Bretscher, M. S. 1972. Asymmetry of the lipid bilayer structure for biological membranes. *Nature, New Biol.* 236:11–12.
- Crepeau, R. H., S. Rananavare, and J. H. Freed. 1987. Automated least square fitting of slow motional ESR spectra. In *Abstracts of the 10th International EPR Symposium*. Denver, CO.
- Devaux, P. F. 1991. Static and dynamic lipid asymmetry in cell membranes. *Biochemistry*. 30:1163–1173.
- Feigenson, G. W. 1989. Calcium ion binding between lipid bilayers: the 4-component system of phosphatidylserine, phosphatidylcholine, calcium chloride, and water. *Biochemistry*. 28:1270–1278.
- Ferrarini, A., P.-L. Nordio, G. J. Moro, R. H. Crepeau, and J. H. Freed. 1989. A theoretical model of phospholipid dynamics in membranes. *J. Chem. Phys.* 91:5707–5721.
- Freed, J. H. 1976. The theory of slow tumbling ESR spectra for nitroxides. In *Spin Labeling: Theory and Applications*. L. J. Berliner, editor. Academic Press, Inc., New York. 53–132.
- Freire, E., and B. Snyder. 1980. Estimation of the lateral distribution of molecules in two-component lipid bilayers. *Biochemistry*. 19:88–94.
- Griffith, O. H., P. J. Dehlinger, and S. P. Van. 1974. Shape of the hydrophobic barrier of phospholipid bilayers. (Evidence for water penetration in biological membranes). *J. Membr. Biol.* 15:159–192.
- Hubbell, W. L. 1990. Transbilayer coupling mechanism for the formation of lipid asymmetry in biological membranes. *Biophys. J.* 57:99–108.
- Hui, S. W., and N.-B. He. 1983. Molecular organization in cholesterol-lecithin bilayers by x-ray and electron diffraction measurement. *Biochemistry*. 22:1159–1164.
- Ikeda, T., H. Yamaguchi, and S. Tazuke. 1990. Phase separation in phospholipid bilayers induced by biologically active polycations. *Biochim. Biophys. Acta*. 1026:105–112.
- Ipsen, J. H., K. G. Karlstrom, O. E. Mouritsen, H. Wennerstrom, and M. J. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta*. 905:162–172.

- Jan, N., T. Lookman, and D. A. Pink. 1984. On computer simulation methods used to study models of two-component lipid bilayers. *Biochemistry*. 23:3227-3232.
- Knoll, W., G. Schmidt, K. Ibel, and E. Sackmann. 1985. Small angle neutron scattering study of lateral phase separation in dimyristoyl-phosphatidylcholine-cholesterol mixed membranes. *Biochemistry*. 24:5240-5246.
- Korstanje, L. J., E. E. van Faassen, and Y. K. Levine. 1989. Slow motional ESR study of order and dynamics in oriented lipid multilayers: effect of unsaturation and hydration. *Biochim. Biophys. Acta*. 980:225-233.
- Kusumi, A., W. K. Subczynski, M. Pasenkiewicz-Gierula, J. S. Hyde, and H. Merkle. 1986. Spin-label studies on phosphatidylcholine-cholesterol membranes: effect of alkyl chain length and unsaturation in the fluid phase. *Biochim. Biophys. Acta*. 854:307-317.
- Lee, A. G. 1977. Lipid phase transitions and phase diagrams. I. Lipid phase transitions. *Biochim. Biophys. Acta*. 472:237-281.
- Lis, L. J., M. McAlister, N. Fuller, R. P. Rand, and V. A. Parsegian. 1982. Interactions between neutral phospholipid bilayer membranes. *Biophys. J.* 37:657-666.
- Makowski, L., and J. Li. 1984. X-ray diffraction and electron microscope studies of the molecular structure of biological membranes. *Biomembrane Structure and Function*. D. Chapman, editor. Verlag Chemie, Basel. 43-166.
- Marsh, D., A. Watts, and P. F. Knowles. 1976. Evidence for phase boundary lipid. Permeability of tempo-choline into dimyristoyl phosphatidylcholine vesicles at the phase transitions. *Biochemistry*. 15:3570-3578.
- Meirovitch, E., D. Ignier, E. Ignier, G. Moro, and J. H. Freed. 1982. Electron spin relaxation and ordering in smectic and supercooled nematic liquid crystals. *J. Chem. Phys.* 77:3915-3938.
- Mouritsen, O. G., and M. Bloom. 1984. Mattress model of lipid protein interaction in membranes. *Biophys. J.* 46:141-153.
- Owicki, J. C., and H. M. McConnell. 1978. Theory of protein-lipid and protein-protein interactions in bilayer membranes. *Proc. Natl. Acad. Sci. USA*. 76:4750-4754.
- Parsegian, V. A., N. Fuller, and R. P. Rand. 1979. Measured work of deformation and repulsion of lecithin bilayers. *Proc. Natl. Acad. Sci. USA*. 76:2750-2754.
- Peschke, J., J. Riegler, and H. Moehald. 1987. Quantitative analysis of membrane distortions induced by mismatch of protein and lipid hydrophobic thickness. *Eur. Biophys. J.* 14:385-391.
- Prausnitz, J. M., R. N. Lichtenthaler, and E. G. Azevedo. 1986. *Molecular Thermodynamics of Fluid-Phase Equilibria*, 2nd. Ed. Prentice-Hall, Englewood Cliffs, NJ. Chap. 6.
- Rand, R. P., and V. A. Parsegian. 1989. Hydration forces between phospholipid bilayers. *Biochim. Biophys. Acta*. 988:351-376.
- Sackmann, E. 1983. Physical foundations of the molecular organization and dynamics of membranes. *Biophysics*. W. Hoppe, W. Lohmann, H. Markl, and H. Ziegler, editors. Springer-Verlag, New York. 425-457.
- Sackmann, E., R. Kotulla, and F.-J. Heiszler. 1984. On the role of lipid-bilayer elasticity for the lipid-protein interaction and the indirect protein-protein coupling. *Can. J. Biochem. Cell Biol.* 62:778-788.
- Schneider, D. J., and J. H. Freed. 1989. Calculating slow motional magnetic resonance spectra: a user's guide. In *Biological Magnetic Resonance*, 8, L. J. Berliner and J. Reuben, editors, Plenum Publishing Corp., New York. 1-76.
- Seelig, J., and A. Seelig. 1980. Lipid conformation in model membranes and biological membranes. *Quart. Rev. Biophys.* 13:19-61.
- Shankaram, M. B., and T. E. Thompson. 1991. Cholesterol induced fluid phase immiscibility in membranes. *Proc. Natl. Acad. Sci. USA*. 88:8686-8690.
- Shimshick, E. J., W. Kleemann, W. L. Hubbell, and H. M. McConnell. 1973. Lateral phase separations in membranes. *J. Supramolec. Struct.* 1:285-294.
- Shimshick, E. J., and H. M. McConnell. 1973. Lateral phase separation of binary mixtures of cholesterol and phospholipids. *Biochem. Biophys. Res. Commun.* 53:446-451.
- Shin, Y.-K. 1990. Thermodynamics and Dynamics of Phosphatidylcholine-Cholesterol Mixed Model Membranes: An ESR Study. Ph. D. thesis, Cornell University.
- Shin, Y.-K., and J. H. Freed. 1989a. Dynamic imaging of lateral diffusion by electron spin resonance and study of rotational dynamics in model membranes. Effect of cholesterol. *Biophys. J.* 55:537-550.
- Shin, Y.-K., and J. H. Freed. 1989b. Thermodynamics of phosphatidylcholine-cholesterol mixed model membranes in the liquid crystalline state studied by the orientational order parameter. *Biophys. J.* 56:1093-1100.
- Shin, Y.-K., J. K. Moscicki, and J. H. Freed. 1990. Dynamics of phosphatidylcholine/cholesterol mixed model membranes in the liquid crystalline state. *Biophys. J.* 57:445-459.
- Shipley, G. G. 1973. Recent x-ray diffraction studies of biological membrane components. *Biological Membranes*. D. Chapman and D. F. H. Wallach, editors. Academic Press, New York. Vol. 2, pp. 1-89.
- Simon, S. A., T. J. McIntosh, and R. Latorre. 1982. Influence of cholesterol on water penetration into bilayers. *Science*. 216:65-67.
- Tanaka, H., and J. H. Freed. 1984. Electron spin resonance studies on ordering and rotational diffusion in oriented phosphatidylcholine multilayers: evidence for a new chain ordering transition. *J. Phys. Chem.* 88:6633-6644.
- Thewalt, J. L., and M. Bloom. 1992. Phosphatidylcholine: cholesterol phase diagrams. *Biophys. J.* 63:1176-1181.
- Vist, M. R., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^2H nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry*. 29:451-464.