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Effect of tryptophan derivatives on the phase properties of bilayers

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Binding of several tryptophan derivatives and tryptophan-containing peptides to bilayers is examined by monitoring fluorescence enhancement as a function of lipid concentration. The thermodynamic and spectral parameters of the solutes in the bilayers of vesicles and liposomes do not exhibit any anomalous dependence upon the gel or the liquid-crystalline phase state of the bilayer. Effects of these solutes on the phase-transition profiles of the bilayers of liposomes and vesicles are examined, and the lowering of the phase-transition temperature is correlated with the mole fraction of the solute in the bilayer. The partition coefficients do not change at the main phase-transition temperature. These observations contradict the thermodynamic explanation of the solute-induced lowering of the phase-transition temperature which is based on the Van't Hoff relationship for distribution of the solute in the two coexisting phases at the phase-transition temperature. It is postulated that solute molecules bound to defect sites in bilayers modulate the phase properties of bilayers. These defect sites are induced in the gel phase of bilayers of liposomes above the subtransition temperature.

Introduction

Incorporation of a solute in phospholipid bilayers perturbs the phase equilibrium [1,2], which in turn modulates the system properties and molecular properties of a bilayer, including the phase-transition temperature [1,3], permeability [4] and protein functions [5]. Such solute-induced changes in the phase properties of bilayer are responsible not only for inhibition of phospholipase A_2 action [6] and fusion of vesicles [6], but also for modulation of the blood-brain barrier [7] and for anesthesia [8].

The physical processes underlying solute-induced changes in bilayers are not well understood. In this paper we formulate some of the questions underlying the distribution of a solute between water and bilayer, as well as between the various coexisting phases and regions in the bilayer. We have measured the distribution of a series of structurally related solutes, viz. tryptophan derivatives and tryptophan-containing peptides, in bilayers of vesicles and liposomes as a function of the phase properties, temperature and concentration. These results show that the microenvironment of tryptophan in a bilayer is sensitive to the structure of the solute.

Materials and Methods

Phosphatidylcholines were from Avanti or Calbiochem, tryptophan esters from Sigma, and all the other compounds were the highest grade available. Dipalmitoylcyclopentanophosphatidylcholine was a gift from Dr. A. Hancock. Peptides containing tryptophan as a terminal residue were synthesized by established procedures. Peptide couplings were accomplished by the mixed

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

anhydride method [9] using isobutyl chloroformate and *N*-methylmorpholine. In some cases the N-terminus of the peptides was protected by *N-t*-butyloxycarbonyl (Boc) groups, and in all cases the carboxyl end was protected as a methyl ester. IUB nomenclature of amino acids is used: L = leucine, A = alanine, W = tryptophan. Liposomes were prepared by dispersing solid phospholipids in aqueous buffer at 10–15°C above their phase-transition temperatures. The vesicles were prepared by sonic disruption of the liposomes in a bath-type sonicator (Sonicor) till clear (typically about 15 min). For inducing subtransition phase the vesicles and liposomes were annealed by incubating them for 1 h at 10°C above the phase-transition temperature, and then allowed to cool slowly (about 2 h) to room temperature and then at 4°C for 4 days. The unannealed vesicles or liposomes were obtained by freezing and then thawing below the main phase-transition temperature. The annealed liposomes exhibit the subtransition, whereas the unannealed liposomes do not. Vesicles do not exhibit the subgel transition in either annealed or unannealed forms.

All fluorescence measurements were done with an SLM4800S spectrofluorimeter. Unless stated otherwise, excitation was set at 300 nm for quenching runs, and at 295 nm for the emission spectra, which were scanned from 300 to 400 nm. Slit widths were typically set at 4 nm for both the excitation and emission. The probe (2.5 ml of 8 μM solution in 10 mM Hepes, 100 mM KCl at pH 8.0) was titrated with the lipid dispersions containing 50 mg lipid in 1 ml of the stock solution. Appropriate corrections for volume changes were made. The contribution of light scattering to fluorescence intensity change was less than 3% with up to 3 mM phospholipid as vesicles. However, significant corrections were necessary at more than 0.4 mM phospholipid as liposome. Binding of solutes to liposomes was therefore studied only under the conditions where about 75% binding could be achieved at 0.4 mM phospholipid.

Calorimetric studies were performed on a Mettler 2000B differential thermal analyzer. Typically 5 μmol phospholipid in 30 μl buffer (100 mM Hepes, 100 mM KCl at pH 8.0) containing appropriate amounts of the probe were scanned at 1 or 2°C per minute. The transition temperatures reported here were obtained to an accuracy of ± 0.05°C, and the enthalpies are within $\pm 5\%$. Other details are as given elsewhere [10].

Results

Distribution of tryptophan-containing solutes between bilayer and water

The fluorescence properties of the indole ring of tryptophan make it a useful probe for monitoring its distribution between bilayer and water. When excited at its absorption maximum (290 nm), the fluorescence emission in the 320–370 nm region exhibits a maximum that depends upon the microenvironment in which the probe is localized. For example, the alkyl esters of tryptophan exhibit an emission maximum at 354 nm in an aqueous medium. However, the fluorescence intensity increases and the emission maximum shifts towards 338 nm in the presence of phospholipid vesicles. The spectral contribution from the probe localized in the bilayer was obtained by subtracting the aqueous-phase spectrum from the spectrum in the presence of lipid vesicles. Except for a difference in the peak intensity, the difference spectra are

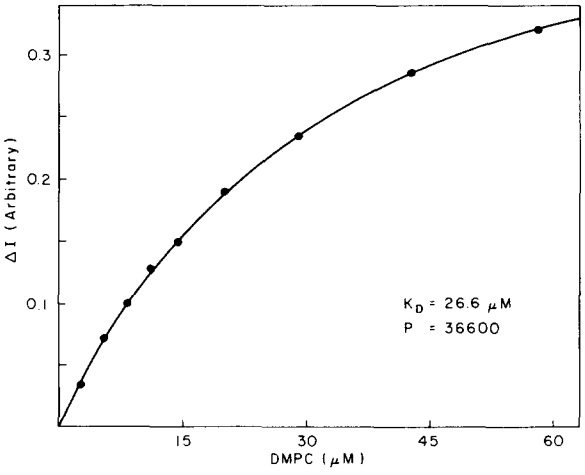

Fig. 1. The change in the fluorescence intensity of octyltryptophan at 333 nm vs. the dimyristoylphosphatidylcholine (DMPC) concentration added in the form of sonicated vesicles. The reaction mixture contained 10 mM Hepes, 100 mM KCl at pH 8.0 and 31°C. Excitation at 295 nm. Independent experiments showed that all the aqueous and lipid compartments are at equilibrium under these conditions.

TABLE I

PROPERTIES OF TRYPTOPHAN DERIVATIVES IN DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES (AT 30°C, pH 8.0)

I_m/I_o is the ratio of the change in the fluorescence intensity to the intensity in the aqueous phase; K_d is dissociation constant of the complex containing one probe molecule and n lipid molecules; γ_{ss} is steady-state anisotropy; EtTRP is ethyl tryptophan; BuTRP is butyltryptophan; octTRP is octyltryptophan.

	$\frac{I_m}{I_o}$	$K_d(\mu M)$		n		γ_{ss}	
		19°C	30°C	19°C	30°C	30°C	19°C
LW	1.97	600	710	< 5	< 5	0.156	0.147
BocLW	1.65	6.4	7.1	22	24	0.127	0.139
LALW	1.02	41	38	16	15	0.136	0.152
BocLALW	1.78	10	6	27	21	0.149	0.148
$(LA)_2LW$	1.42	8.3	7.2	28	39	0.139	0.148
$Boc(LA)_2LW$	2.0	12	5	29	32	0.132	0.160
EtTRP	2.24			< 5	< 5		
BuTRP	2.80			< 5	< 5		
OctTRP	4.47			< 5	< 5		

identical at the various lipid-to-probe ratios. Thus, the change in the emission intensity (ΔI) at the peak of the difference spectrum is directly proportional to the fraction of the probe in the bilayer. As shown in Fig. 1, the change in the fluorescence intensity (ΔI) increases with the lipid concentration in the medium, and it approaches a maximum value (I_m) at high lipid concentrations when all the fluorophore is localized in the bilayer. I_m/I_o ratios for the various probes are given in Table I, where I_o is the intensity in the aqueous phase.

The hyperbolic isotherm of the type shown in Fig. 1 results from the equilibrium distribution of the probe between water and bilayer:

$$P + nL \rightleftharpoons PL_n$$

that is, one probe molecule interacts with n lipid molecules to form a complex, or binding of each probe molecule makes n lipid molecules unavailable for binding of other probe molecules. As shown elsewhere [11,12], the binding isotherm for this is described by the dissociation constant

$$K_d = \frac{[\text{free probe}][\text{free lipid}]}{[\text{complex}]}$$

$$\frac{I_m}{\Delta I} = 1 + \frac{nK_d}{[L] - n[PL]} \quad (1)$$

[L] is the total lipid concentration, and [PL] is the concentration of the bound probe. By nonlinear regression analysis of [L] vs. ΔI plot (cf. Fig. 1) we have obtained values of K_d, n and I_m. The apparent dissociation constant of the complex, nK_d, has been used to calculate the apparent association constant ($K_a = 1/nK_d$) or the partition coefficient which is generally defined as

$$P = \frac{\text{mol probe/g lipid}}{\text{mol probe/g water}}$$

Association constants expressed in mole fraction terms were used for calculation of unitary thermodynamic parameters.

Goodness of fit by nonlinear regression analysis was evaluated as standard deviation and correlation coefficient. Covariance of n, K_d and I_m was evaluated to ascertain interdependence of these three parameters. Poor covariance shows that the parameter values are independent. Overall, the values of apparent dissociation constant obtained by nonlinear regression are much more reliable than the corresponding values obtained by the double reciprocal or Scatchard plot of [L] vs. ΔI data. For weakly associating solutes both of the methods give good fit, except that in the nonlinear regression analysis a low value of n is obtained

and generally there is significant covariance between n and K_d. Under these conditions the concentration of free lipid is approximately equal to the concentration of total lipid, and therefore K_d may be determined from the $1/[L]$ vs. $1/\Delta I$ or the corresponding Scatchard plots. For strongly associating solutes the linearized plots showed significant departure at low lipid concentration. This is probably because incorporation of a probe molecule makes n lipid molecules unavailable for incorporation of other probe molecules. Thus for probes with low dissociation constants, the bilayer/water distribution would be similar to binding on a site. On the other hand, for probes with high dissociation constants, the distribution would approach partitioning between bulk solvents. In order to eliminate problems arising from covariance of n and K_d, only the apparent dissociation constants (nK_d) are reported in this paper.

The unitary association constant K_u, where the concentrations are expressed as mole fraction, for several tryptophan derivatives are summarized in Table II. In general, K_u increases with the hydrophobicity of the alkyl esters or for the Boc-derivatives of the peptides. The hydrophobic contribution of the leucine-alanine sequence is rather small and the incremental free energy per methylene group is about 400 cal. The number of lipid molecules per binding site for the probe also increases with the number of peptide residues.

Microenvironment of tryptophan in the bilayer

The fluorescence intensity of the various tryptophan-containing solutes changes on mixing with vesicles. With larger solutes (tetra- and hexapeptides) at low lipid concentrations ($< K_d$) about 65% of the total change in intensity (ΔI) is seen almost instantaneously, and the rest of the increase takes a first-order time course with half-time of several minutes. We think that the first increase is due to binding of the solute to the outer monolayer of the vesicles, and the second phase is due

TABLE II

UNITARY THERMODYNAMIC PARAMETERS FOR BINDING OF TRYPTOPHAN DERIVATIVES TO DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES

	Temp. (°C)	nK_d (μM)	K_u (M^{-1})	ΔG (kcal)	ΔS (e.u.)	ΔH (kcal)
LW	19	600	91887	−6.61	13.6	−2.64
	30	710	77731	−6.76		
BocLW	19	142	380136	−7.43	17.3	−2.38
	30	170.4	325704	−7.62		
LALW	19	800	69030	−6.44	29.1	2.06
	30	710	77731	−6.76		
BocLALW	19	228	239224	−7.16	31.8	2.12
	30	200	272059	−7.51		
$(LA)_2LW$	19	510	107977	−6.70	50.0	7.9
	30	310	176752	−7.25		
$Boc(LA)_2LW$	19	228	239224	−7.16	43.6	5.57
	30	160	338414	−7.64		
EtTRP	19	1695	32745	−6.05	22.7	0.58
	30	1538	36075	−6.30		
BuTRP	19	541	102675	−6.67	28.2	1.56
	30	495	112110	−6.98		
OctTRP	19	28.4	1712963	−8.30	30.9	+0.72
	30	27.2	1780335	−8.64		

to a slow transfer of the solute to the inner monolayer. The total change in intensity in these two phases is found to be identical to the total change observed with the vesicles sonicated in the presence of the solute. The half-time for transmembrane movement increases with increasing peptide length, and the rate of transfer of hydrophobic Boc-peptides is somewhat slower. These data are not discussed further in this paper. However, such considerations were taken into account when measuring the equilibrium binding isotherms, i.e., the binding had reached equilibrium with all the available lipid on both sides of the bilayer.

The motional freedom of a fluorophore is reflected in the values of steady-state anisotropy (γ_{ss}). In the aqueous phase γ_{ss} values are less than 0.02 for all the solutes, and these values increase to 0.12–0.15 when these solutes are incorporated into vesicles (Table I). This suggests that the tryptophan derivative is immobilized in the bilayer [13]. Since these values are not significantly dependent upon the gross structure of the solute or the bulk phase properties of the bilayer, it would mean that the microenvironment of the probe remains the same and that these solutes are indeed immobilized in a large particle whose tumbling motion is considerably slower than the fluorescence life-time [13]. This conclusion is also consistent with the observation that I_m for the peptide probes (directly proportional to their quantum yield) is the same, and they do not exhibit an anomalous change in I_m at the phase-transition temperature.

Further evidence for the localization of these solutes in the bilayer was obtained by quenching experiments. As shown in Fig. 2, bromo-substituted fatty acids in bilayers of ternary codispersions quench the fluorescence of the peptides. The quenching efficiency for the 2-bromo derivative appears to be better than that for the bromo-substituent in 6-, 9- or 11-positions. However, the hexapeptide, $Boc(LA)_2LW$, is better quenched by the 6-bromo-derivative. Similarly, the extent of quenching increases in the order of increasing length of peptide: LW < LALW < $(LA)_2LW$. This probably means that the average residence time of the fluorophore at the level of 2-bromo substituent is also in the same order. As summarized in Table III, this conclusion is further substantiated by the second-order quenching constants (k_q) for a

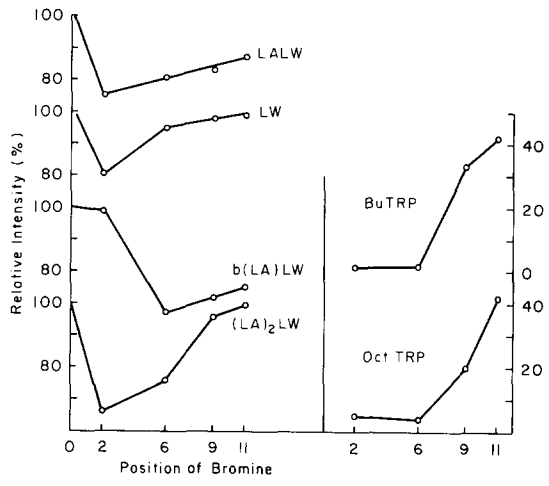

Fig. 2. Relative fluorescence intensity of the various solutes in the presence of ternary co-dispersions containing dimyristoylphosphatidylcholine + lysophosphatidylcholine + stearic acid. Stearic acid was brominated in 2-, 6,7-, 9,10- or 11,12-positions.

charged polar quencher 1-methyl nicotinamide. The k_q values for collisional quenching decrease when the accessibility of the fluorophore for the quencher is hindered. The k_q values for these

TABLE III

1-METHYLNICOTINAMIDE MEDIATED QUENCHING CONSTANTS (25°C, pH 8.0) FOR TRYPTOPHAN-CONTAINING PEPTIDES IN AQUEOUS PHASE AND IN DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES

τ_p^{18} is phase lifetime at 18 MHz; K_d is Stern-Volmer quenching constant; k_q is bimolecular quenching constant.

		Lipid K_d (M^{-1})	τ_p^{18} (ns)	k_q ($\times 10^{-9}$) ($M^{-1} \cdot s^{-1}$)
LW	–	15	1.35	11.1
	+	13.3	2.73	4.87
BocLW	–	21.3	1.60	13.3
	+	11.1	2.73	4.0
LALW	–	18	1.54	11.7
	+	10.6	2.45	4.3
BocLALW	–	13.5	1.58	8.5
	+	10.2	3.04	3.4
$(LA)_2LW$	–	14	1.8	7.7
	+	8.3	2.95	2.8
$Boc(LA)_2LW$	–	< 2	1.58	< 1.2
	+	5.9	3.04	1.9

peptides in bilayers are less than half of those observed in the aqueous phase. Also k_q decreases for the longer peptides (tetra- and hexapeptides), suggesting that they are somewhat better shielded in the bilayer. The fluorescence quantum yield and k_q for $Boc(LA)_2LW$ in the aqueous phase shows anomalous behavior, as if the fluorophore is shielded and/or internally quenched. Since the absorbance of its aqueous solution is comparable to that of other peptides one can essentially rule out aggregation resulting from the formation of microcrystals.

Dependence of association constant upon temperature

The lipid titration method was used to study the temperature dependence of binding parameters. As shown in Fig. 3, the partition coefficient increases with temperature; however, little or no anomalous change in the partition coefficient or I_m (data not shown) was observed at the phase-transition temperature of dimyristoylphosphatidylcholine vesicles. The unitary enthalpy, entropy and free energy values calculated from the temperature dependence of the unitary association constant K_u are given in Table II. The data show that the binding process is essentially entropy-driven,

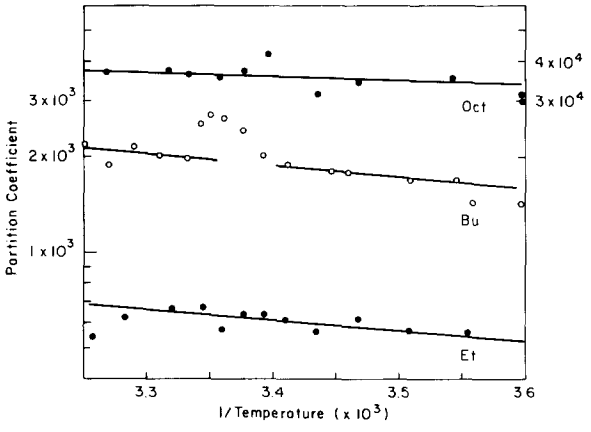

Fig. 3. Van't Hoff plots of partition coefficients of ethyl-, butyl- and octyltryptophan in dimyristoylphosphatidylcholine vesicles. Partition coefficients are expressed as moles of solute per gram of lipid divided by moles of solute per gram of water. Left ordinate is for ethyltryptophan (Et) and butyltryptophan (Bu), and the right ordinate is for octyltryptophan (Oct). The enthalpy and entropy values calculated from these data are given in Table I.

and the enthalpy of the process becomes more positive as the solute becomes larger. Insight into the solvent nature of vesicles can be obtained from the Barclay-Butler plots of ΔS vs. ΔH (Fig. 4). The slope of this plot, $b = 0.0034$, is more than twice as large as it is for bulk nonpolar solvents (< 0.0015) and water (0.00212). The high b values suggest that a molecule suffers a much greater loss of mobility when incorporated into bilayer than if dissolved in a bulk solvent [14]. This is consistant with an increased steady-state anisotropy (Table I) and a decreased half-height width of the emission spectral peak for the tryptophan-containing solutes in bilayers (data not shown).

Effect of solutes on the phase-transition temperature of bilayer

Incorporation of solutes at low mole fractions has been shown to alter the phase-transition temperature of bilayers [1,3,10,15–17]. As summarized in Table IV, between 0.03 and 0.09 mole fraction in vesicles and liposomes, these solutes lower the phase transition temperature by 1°C, and under these conditions the enthalpy of transition remains unchanged. The solute-induced lowering of phase-transition temperature (ΔT) is given by [1,3,15,16]:

$$\Delta T = \frac{RT_m^2}{\Delta H}(X_g + X_l) \qquad (2)$$

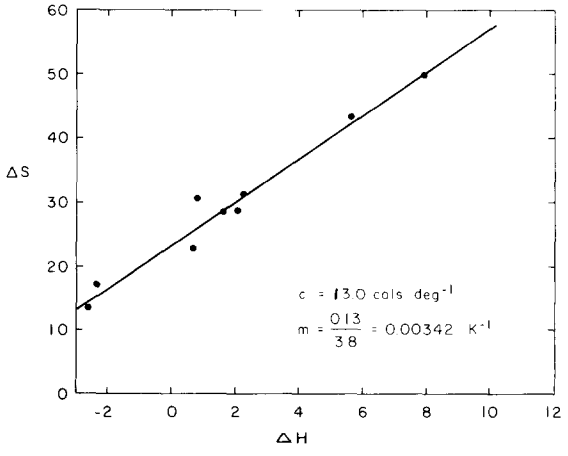

Fig. 4. Barclay-Butler plot for unitary enthalpy (ΔH) and entropy (ΔS) of partitioning of the several tryptophan derivatives in dimyristoylphosphatidylcholine vesicles (see Table II for the data). Intercept 13.0 cal·deg^{-1}; slope 0.00342 K^{-1}.

TABLE IV

MOLE FRACTIONS OF SOLUTES REQUIRED TO LOWER THE PHASE TRANSITION TEMPERATURE BY 1°C

DMPC is dimyristoylphosphatidylcholine, DPPC is dipalmitoylphosphatidylcholine. Partition coefficients given in Table II were used for the calculation of the mole fractions of solutes in bilayers, and the phase-transition temperature was obtained from the peak position of the phase transition profile. The theoretical values were calculated as $\Delta H/RT_m^2$ from the experimental ΔH and T_m values by assuming that the solute partitions only in the liquid-crystalline phase.

Solute	X_s for 1°C decrease in		
	DMPC vesicles	DMPC liposomes	DPPC liposomes
EtTRP	0.033	–	–
ButTRP	0.035	0.059	0.071
OctTRP	0.030	0.028	0.030
LW	0.034	0.045	–
BocLW	0.042	0.038	–
LALW	0.028	0.057	–
BocLALW	0.022	0.055	–
$(LA)_2LW$	0.046	0.052	0.030
$Boc(LA)_2LW$	0.021	0.055	–
Theoretical	0.031	0.037	0.044

where R is the gas constant, T_m is the midpoint of the transition, ΔH is the enthalpy of transition, and X_g and X_l are the mole fractions of the solute in the gel and the liquid-crystalline phases, respectively. If all the solute partitioned into the liquid-phase bilayer (that is $X_g = 0$), X_l would be 0.03–0.06, which is the range that we have observed (Table IV). This would imply that the association constant of these solutes for a bilayer in the gel phase would be very small. However, as shown in Fig. 3 and Table II, this is not the case. In all the cases we have examined K_a is found to be the same in the gel and in the liquid-crystalline phases for vesicles and liposomes. In fact, as shown in Fig. 5, the association constant in liposomes does not decrease significantly until the bilayer temperature falls below the subtransition temperature in annealed dipalmitoylphosphatidylcholine [18,19] or in dipalmitoylcyclopentanophosphatidylcholine [20], which remains in the crystalline phase up to the main transition temperature [21]. Vesicles do not exhibit the subtransition, and they do not exhibit an anomalous change in the associ-

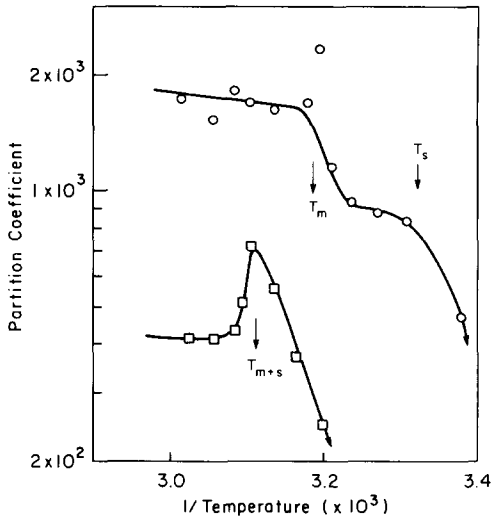

Fig. 5. Van't Hoff plots of partition coefficients (P) of butyltryptophan in annealed liposomes of dipalmitoylphosphatidylcholine (open circles) and of dipalmitoylcyclopentanophosphatidylcholine (squares). The corresponding data for octyltryptophan exhibit a similar but less steep increase in the partition coefficient just above the subtransition temperature. The values of P below the phase transition temperature have a much higher scatter (±20%), and only the lower estimates are given. The main (T_m) and sub (T_s) transition temperatures for the two types of liposomes are indicated by the arrows. The single transition for cyclopentanophosphatidylcholine (T_{s+m}) is from L_C to the liquid crystalline phase. The ordinate for cyclopentanophosphatidylcholine is moved downwards for clarity. See Table II for actual data.

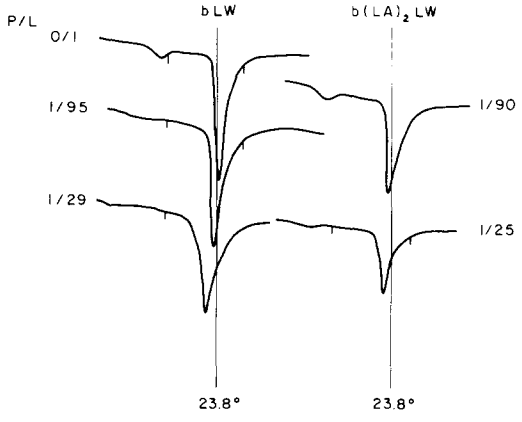

Fig. 6. Endothermic phase-transition profiles of dimyristoylphosphatidylcholine liposomes in the presence of BocLW (left) and $Boc(LA)_2LW$ (right) at mole ratios indicated with the curves. The major effect of the solutes is on the enthalpy of transition. The vertical lines indicate the transition temperature of the liposomes without additives.

TABLE V

PARTITION COEFFICIENT AND SOLUTE CONCENTRATION REQUIRED TO LOWER THE PHASE-TRANSITION TEMPERATURE OF DIMYRISTOYLPHOSPHATIDYLCHOLINE LIPOSOMES BY 1°C

Solute	Partition coeff. [a]		X_S(obs)	X_S (calcd)
	< 25°C	> 25°C		
Butyramide	0.409	0.507	0.17	0.154
Ethyl acetate	2.39	2.52	0.1625	0.685
Acetone	0.953	1.05	0.425	0.37

[a] Data from Katz and Diamond [14].

ation constant over the temperature range we examined (Fig. 3).

Effect of solutes on phase transition profile

As shown in Fig. 6, at high mole fractions ($X_s > 0.05$) most of the solutes we have examined not only somewhat lower T_m, but also significantly lower the enthalpy of transition. These results show that the solute induces formation of a phase which apparently phase separates and exhibits lower cooperativity.

Katz and Diamond [14] have reported that butyramide, ethyl acetate and acetone exhibit an anomalous increase (< 1.25-fold) in the partition coefficient in dimyristoylphosphatidylcholine liposomes. The phase-transition profile in the presence of these solutes does not broaden nor does the enthalpy change significantly. As summarized in Table V, the mole fractions of these solutes in the bilayer required to lower T_m by 1°C are significantly higher. For butyramide and acetone there appears to be a correlation between the relative partition coefficient below and above the phase transition and the mole fraction of the solute required to lower T_m by 1°C.

Discussion

The observations summarized in the preceding section show that a variety of solutes containing the same fluorophore can provide significant information about their microenvironment in the bilayer. The lipid binding isotherm can be described by a single dissociation constant, suggesting that the binding is to one class of noninteracting sites. The association constant as well as several other properties (anisotropy, quenching constant and I_m) do not show any anomalous change in binding or the quantum yield as a function of temperature or with the phase state of the bilayer vesicles and liposomes. This strongly suggests that the microenvironment of the solute in the bilayer is not significantly affected even though the bulk phase properties of the bilayer change at the gel to liquid-crystalline phase-transition temperature. However, as discussed later, there is a significant change in association constant at the subtransition temperature (cf. Fig. 5).

The region of localization of the solutes appears to be near the surface of the bilayer. Experiments with bilayer-localized as well as the aqueous-phase quenchers suggest that this region is somewhere in the vicinity of the 2-position of the acyl chain. However, the precise location changes somewhat with the nature of the solute. Larger hydrophobic solutes (particularly the hexapeptide) are quenched appreciably even by 6- and 9-bromo fatty acids. While this could mean that these solutes penetrate that far into the bilayer, it is also possible that the acyl chains at the solute binding site are reorganized and distorted away from the normal to the plane of the bilayer, in order to accomodate the solute.

Accomodation of a solute in the bilayer would perturb organization of the acyl chain to create a putative binding site. Binding of the solutes to the bilayer is entropy driven, that is, the association of the solute to membrane results in a disordering of the bilayer. Perturbation of the bilayer by a solute is indicated by modification of the phase-transition profiles. It is often assumed [3,15,16] that incorporation of a low mole fraction of solutes in the bilayer approximates ideal miscibility. Supporting evidence of this is sought in the phase-transition profiles, where T_m shifts in the presence of solutes. However, such an experimental validation of the ideal mixing behavior of a solute in the bilayer is not tenable because a lack of change in the shape of the transition profile at low mole fraction of the solute does not necessarily rule out localized membrane perturbation.

The shift of 1°C in T_m induced by the various solutes is at 0.03–0.09 mole fractions (Table IV).

According to the Van't Hoff equation this implies that the partition coefficient of the solute in the gel phase of both vesicles and liposomes is small. Experimentally, the partition coefficients are essentially identical below and above T_m. We believe that the reason for this discrepancy is in the model to which Eqn. 2 is applied, rather than in the applicability of the underlying thermodynamic principles. The behavior of solutes in a bilayer suggests that these solutes are localized at 'sites' that are intrinsically present in the bilayer or are formed in the presence of a solute. In either case the solute is present in an environment in which the surrounding lipid molecules have undergone a change in the state of the acyl chains, and such a solute-induced local isothermal change is not unlike the thermotropic change of state. Thus the solute bound to the bilayer in the gel phase occupies a microscopic region resembling the liquid crystal in organization. We have not extensively investigated, but it appears that at least at $X_s < 0.05$ these solutes do not cause a gross disruption of the vesicles or liposomes. This would imply that the loss of enthalpy is not due to formation of lipid microaggregates. This possibility is being pursued further.

The observations reported in this paper bear strongly on the mechanism underlying the phase change in bilayers. Elsewhere [2] we have suggested that the phase transition in bilayers occurs along defect sites. The lipid molecules at such sites will be isoenergetic, and their energy will be lower than that of the other molecules away from the defects. Of the various types of defects, line defects appear to be most attractive in the context of the phase transition in a bilayer. If the transition occurs in a linear array along the line defects, the cooperative unit would be made up of the isoenergetic molecules along a line defect. This model can be qualitatively extended to account for the solute-induced perturbation of the phase properties of bilayers. Solutes in a bilayer can create new defects, and they can change the number or energy of the isoenergetic molecules at the line defects. The experimental consequences of these would be reflected in the parameters that define the phase-transition profile: T_m, ΔH and the cooperativity as is experimentally observed [10,16,17].

The origin of defects in the gel phase of a bilayer is an intriguing problem. As shown in Fig. 5, the partition coefficients of butyl- and octyltryptophan decrease markedly only below the subtransition temperature in annealed dipalmitoylphosphatidylcholine liposomes. The decrease is not seen in unannealed liposomes nor in annealed vesicles, both of which do not show the subtransition. Similarly, below the main phase-transition temperature (T_{s+m}) in dipalmitoylcyclopentanophosphatidylcholine liposomes the partition coefficient decreases sharply (Fig. 5). The state of organization in these two bilayers (annealed diacylphosphatidylcholines and in dipalmitoylcyclopentanophosphatidylcholine) is similar [21] and is considerably different from that in the gel phase. Packing of lipid molecules in the phase that exists below the subtransition temperature is pseudo-orthorhombic, and the lipids have little rotational freedom. By contrast, in the gel phase the molecules are packed in a hexagonal lattice and have significant rotational freedom. This difference between these two states of packing in a bilayer offers a possible origin of the defects that act as the solute binding sites in the gel phase of diacylphosphatidylcholine liposomes and that appear only above the subtransition temperature. The formation of these defects may be analogous to the formation of cracks in an ice sheet before melting in order to relieve strain [2]. In a bilayer, the life-time of defects in the presence and absence of solutes may be an important consideration for understanding the equilibrium distribution of a solute and the resulting change in the properties of bilayers.

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