Head Group and Chain Length Dependence of Phospholipid Self-Assembly Studied by Spin-Label Electron Spin Resonance†

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ABSTRACT: The critical micelle concentrations (cmc's) of a variety of spin-labeled phospholipids, 1-acyl-2-[4-(4,4-dimethyloxazolidine-*N*-oxyl)valeryl]-*sn*-glycero-3-phospho derivatives, have been determined by electron spin resonance (ESR) spectroscopy. The narrow, three-line ESR spectra of the rapidly tumbling monomers are clearly distinguished from the spin–spin broadened spectra of the micellar aggregates, allowing a direct determination of the concentrations of the two species. The influence of both the hydrocarbon chain length and the polar head group on the energetics of self-assembly has been studied. For phosphatidylcholine, ln [cmc] decreases linearly with the length of the *sn*-1 chain. The gradient of this linear dependence corresponds to a free energy of transfer of the monomer from the aqueous phase to the micelle of $\Delta G_{tr} = -1.1RT$ per CH_2 group. The cmc's of the 1-lauroyl derivatives of both phosphatidylcholine and phosphatidylglycerol have relatively shallow, biphasic temperature dependences with a minimum at approximately 20 °C. Both of these properties are characteristic of the hydrophobic effect, with the free energy of transfer being slightly less than that for the solubility of *n*-hydrocarbons in water, corresponding to the reduced configurational entropy of the lipid chains in the micellar state. The cmc's of the 1-lauroyl derivatives of the phospholipids in 0.15 M NaCl, for their various charge states, are as follows: phosphatidic acid(2−), 0.77 mM; phosphatidic acid(1−), 0.13 mM; phosphatidylserine(1−), 0.24 mM; phosphatidylglycerol(1−), 0.17 mM; phosphatidylcholine, 0.10 mM; phosphatidylethanolamine, 0.05 mM. For the negatively charged phospholipids, the cmc's decrease by 1–2 orders of magnitude on increasing the ionic strength from approximately 0 to 2.0 M NaCl, with a characteristic Debye–Hückel type of screening. For the zwitterionic lipids, the salt dependence is considerably less; the cmc's for phosphatidylcholine and phosphatidylethanolamine decrease by a factor of approximately 4 on going from 0 to 2 M NaCl. At a constant ionic strength of 0.1, the cmc of the 1-lauroyl derivative of phosphatidic acid increases by almost an order of magnitude on titration from pH 5 to pH 9, with a pK_a of 7.4 corresponding to the second deprotonation state. These results demonstrate that the polar head group can also have a very marked influence on the energetics of self-assembly and correspondingly on the kinetics of lipid transfer between vesicles. The range of the effects of the polar head group and salt on the free energy of transfer corresponds to those of changing the chain length by up to approximately four CH_2 groups. The rate of monomer–micelle collisions has been measured from the exchange broadening of the monomer signal as a function of the micelle concentration, for the 1-lauroyl derivatives of both phosphatidylcholine and phosphatidylglycerol in 0.15 M NaCl at pH 7. The collision rates follow an Arrhenius law temperature dependence with activation energies of 4.0 kcal·mol^{-1} and 5.3 kcal·mol^{-1} for phosphatidylcholine and phosphatidylglycerol, respectively. The interpolated second-order rate constants at 25 °C are $k^+/\bar{n} = 9.4 \times 10^7$ and 4.4×10^7 L·(mol of micelle)$^{-1}$·s^{-1} for phosphatidylcholine and phosphatidylglycerol, respectively. The differences in rates correspond to the effects of the electrostatic surface potential for phosphatidylglycerol and also to possible differences in the micelle size $\bar{n}$. The overall rates and activation energies are consistent with a diffusion-controlled process.

The self-assembly of phospholipid molecules into bilayers is one of the fundamental aspects of biological membrane stability. The thermodynamics of the self-assembly process can be studied by measurement of the critical micelle concentration (cmc),[1] which is determined by the free energy of transfer $\mu_{mic}° - \mu_w°$ of a lipid monomer from water to the micelle [see, e.g., Tanford (1973)]. Many of the factors governing this transfer, hydrophobic effects, electrostatics, and head group hydration, are those that are also operative at the polar–apolar interface in membranes (Israelachvili et al., 1980). Indeed, the free energy of transfer contains the free energy of the monomer within the micelle or bilayer as one of its components. In addition, the factors affecting micelle formation are also relevant to dynamic membrane processes such as lipid transfer, membrane fusion, and membrane biosynthesis.

In this work we have studied the effects of polar head group and acyl chain length on the cmc of spin-labeled phospholipid derivatives. Lipid monomers and micelles can be distinguished from the ESR spectrum, allowing a direct quantitation of the cmc. By varying the lipid head group at constant chain length or the chain length for a fixed head group, it is possible in principle to separate the hydrophobic contributions from the

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[1] Abbreviations: cmc, critical micelle concentration; ESR, electron spin resonance; V12PCSL, V12PASL, V12PGSL, V12PSSL, and V12PESL, 1-lauroyl-2-[4-(4,4-dimethyloxazolidine-*N*-oxyl)valeryl]-*sn*-glycero-3-phosphocholine, -phosphoric acid, -phosphoglycerol, -phosphoserine, and -phosphoethanolamine; Vn_cPCSL, 1-acyl-2-[4-(4,4-dimethyloxazolidine-*N*-oxyl)valeryl]-*sn*-glycero-3-phosphocholine, where n_c is the number of C atoms in the *sn*-1 acyl chain, as in V12PCSL; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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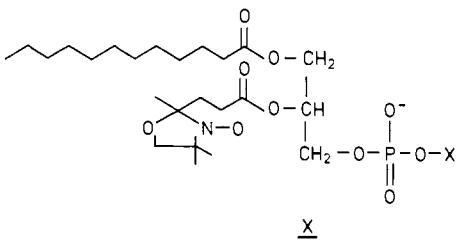

X

V12PASL : -H

V12PSSL : $-CH_2CH(COO^-)NH_3^+$

V12PGSL : $-CH_2CH(OH)CH_2OH$

V12PCSL : $-(CH_2)_2N(CH_3)_3^+$

V12PESL : $-(CH_2)_2NH_3^+$

FIGURE 1: Structures of the spin-labeled phospholipids. Valeric acid spin-labeled on the C4 atom is acylated at the *sn*-2 position and lauric acid is acylated at the *sn*-1 position. V12PASL, phosphatidic acid; V12PSSL, phosphatidylserine; V12PGSL, phosphatidylglycerol; V12PCSL, phosphatidylcholine; V12PESL, phosphatidylethanolamine. V14PCSL has myristic acid acylated at the *sn*-1 position.

polar and the steric contributions to the membrane stability. From the chain length dependence a value is derived for the interfacial hydrophobic free energy density and hence the effective lateral pressure in the bilayer, by use of the arguments of Evans and Waugh (1977). It is found that the phospholipid head group can have a pronounced effect on the micelle (and therefore bilayer)[2] stability, for negatively charged phospholipids responding to both ionic strength and pH titration.

Materials and Methods

Spin-Labels. Lauroyl- and myristoyl-L-α-lysophosphatidylcholine were obtained from the Sigma Chemical Co., St. Louis, MO.

The phosphatidylcholine spin-labels V12PCSL and V14PCSL (see Figure 1) were prepared by acylation, with the imidazole complex of spin-labeled valeric acid, at the *sn*-2 position of lauroyl- or myristoyllysophosphatidylcholine, respectively. The procedure has been described in detail by Marsh and Watts (1982). Valeric acid, with a nitroxide oxazolidine ring attached at the C4 position, was synthesized from 4-ketovaleric acid (ethyl ester) (Merck, Darmstadt, FRG) essentially according to the method given in Marsh and Watts (1982). The remaining spin-labeled phospholipids, V12PASL, V12PSSL, V12PGSL, and V12PESL (see Figure 1), were synthesized from the phosphatidylcholine spin-label V12PCSL by head group exchange in the presence of phospholipase D (Comfurius & Zwaal, 1977; Marsh & Watts, 1982). These materials were purified by column chromatography on Whatman CM-52 cellulose essentially as described by Comfurius and Zwaal (1977) except that the column was prewashed with methanolic acetic acid followed by an extensive methanol wash prior to equilibration in chloroform. The valeric acid phospholipid spin-labels are susceptible to hydrolysis and it was found that the acid prewash resulted in a significant reduction in this rate of degradation on the column.

Lipid dispersions were prepared in water, salt solution, or constant ionic strength buffer and adjusted to the appropriate pH. With the exception of phosphatidic acid, optically clear dispersions were obtained. Thus, all lipids formed small micelles, except phosphatidic acid which formed larger aggregates. Quasi-elastic light scattering measurements by Schmidt et al. (1981) for the phosphatidylcholine derivatives have indicated that the micellar size distribution is rather narrow and that the micelle size is practically independent of temperature and micellar concentration.

ESR Measurements. ESR spectra were recorded on a Varian 9-GHz ESR spectrometer equipped with a nitrogen-flow temperature regulation system. Lipid solutions were sealed in 100-μL capillaries, which were then placed in 3-mm quartz ESR tubes containing silicon oil for temperature stability. The sample temperature was monitored by a thermocouple placed at the top of the cavity within the silicon oil. cmc determinations were made from ESR recordings made with a modulation amplitude of 0.5 G and a nominal microwave power of 5 mW. A modulation amplitude of 0.05 G and a nominal microwave power of 1 mW were used for making line-width measurements.

cmc Determinations. cmc's were calculated with a simple graphical procedure in which the cmc is equated to the intercept of the monomer ESR signal intensity vs. concentration curves obtained above and below the cmc (cf. Figure 3). Below the cmc, a concentration range was chosen such that there was negligible spin–spin broadening of the monomer signal. Above the cmc, the spin–spin interactions between monomers, if not negligible, do not vary appreciably with total lipid concentration. The measurements above the cmc were restricted to a micellar concentration range such that the lifetime broadening of the monomer signal arising from monomer–micelle collisions was negligible. Phospholipid concentrations were measured by the Eibl and Lands (1969) phosphate assay.

Lipid Exchange Rates. Exchange rates were calculated from the concentration dependence of the peak-to-peak Lorentzian line width (ΔH_{pp}^L) of the monomer ESR signal. ΔH_{pp}^L was estimated from the measured peak-to-peak line width (ΔH_{pp}^O) with (Bales, 1982)

$$\Delta H_{pp}^L = [1 - (\Delta H_{pp}^G/\Delta H_{pp}^O)^2]\Delta H_{pp}^O \quad (1)$$

A Gaussian line-width contribution (ΔH_{pp}^G) of 0.76 G was used in the calculations. This figure was obtained from EPR measurements made on the valeric acid spin-label in aqueous glycerol solutions and with eq 9 and 4 of Bales (1982). The intrinsic Gaussian broadening was found by these measurements to be essentially independent of temperature.

The collision or exchange frequency τ_{coll}^{-1} between monomer and micelle is related to the dependence of the monomer Lorentzian peak-to-peak line width on micelle concentration c by [see, e.g., Marsh (1986)]

$$\tau_{coll}^{-1}/c = (\sqrt{3}\gamma/2)\cdot d(\Delta H_{pp}^L)/dc \quad (2)$$

where γ is the gyromagnetic ratio of the electron. Equation 2 is essentially a lifetime broadening of the monomer spectrum, arising from the Heisenberg spin-exchange interaction occurring on association between the monomer and the exchange-narrowed micelle. Because of the rapid rates of translational diffusion in low-viscosity media, dipole–dipole interactions between monomer and micelles will be averaged to zero and therefore will not contribute to the monomer line width. The second-order rate constant for monomer–micelle

[2] Here the term micelle is used quite generally to include all multimolecular aggregated phospholipid systems, including bilayers. The thermodynamic parameters governing self-assembly will be similar for all types of aggregates. This applies particularly to the hydrophobic effect (which is the principal driving force), as evidenced by the single continuous linear dependence of the logarithm of the cmc on chain length for both micelle- and bilayer-forming diacylphosphatidylcholines [see, e.g., Cevc and Marsh (1987)]. Quantitative differences will occur, depending on the differences in area per polar head group in the different aggregate states, but these are likely to be secondary perturbations compared with the primary energetic contributions.

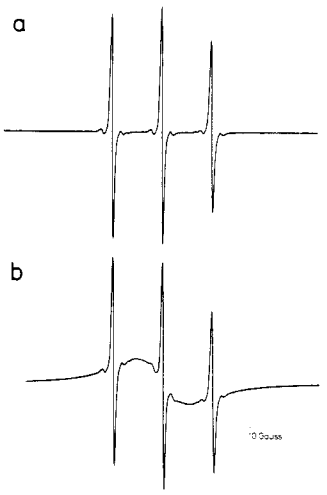

FIGURE 2: ESR spectra at 20 °C of spin-labeled phosphatidylcholine, V12PCSL, in 0.5 M NaCl, pH 7: (a) at a concentration of 0.08 mM; (b) at a concentration of 40 mM.

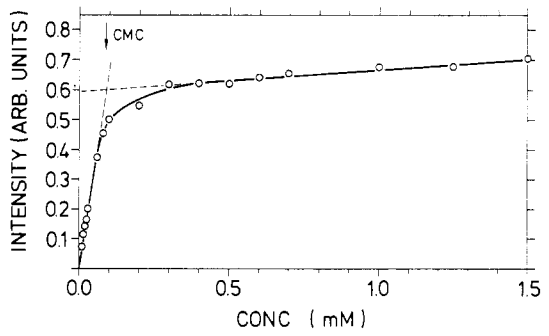

FIGURE 3: Low-field line height at 20 °C in the monomer ESR spectrum of V12PCSL in 0.5 M NaCl, pH 7, as a function of total spin-label concentration.

association is then given simply by the gradient of the monomer Lorentzian line width with respect to concentration, according to

$$k^+ = d(\tau_{coll}^{-1})/dc \qquad (3)$$

RESULTS

Measurement of the cmc. The ESR spectra at 20 °C of the V12PCSL phosphatidylcholine spin-label at low and high concentrations in 0.5 M NaCl, pH 7, are given in spectra a and b of Figure 2, respectively. At low concentration the spectrum consists of three sharp lines (with ^{13}C satellites), corresponding to the rapidly tumbling spin-label monomer. At high concentration the spectrum consists of a superposition of the spectrum from the spin-labeled monomers and an underlying spin–spin-broadened spectrum from the spin-label micelles. The resolution of the two signals is extremely good, and thus, the amount of monomer in the mixture can be quantitated directly from the ESR spectrum. The dependence of the peak-to-peak monomer signal line height on the total spin-label concentration is given in Figure 3. In the low-concentration regime, the monomer signal intensity increases linearly with concentration, indicating that only spin-label monomers are present in solution. Departures from linearity are seen at higher concentrations, concomitant with the appearance of a micelle signal in the ESR spectrum. The monomer intensity then rapidly levels off to a value that increases only very slowly with further increase in total spin-label concentration. A critical micelle concentration, cmc, can be defined by the intersection of the two linear regions of the

Table I: Critical Micelle Concentrations (cmc) and Free Energies of Monomer Transfer ($\Delta G_{tr}/RT$)[a] at 20 °C of Spin-Labeled Phosphatidylcholines, Vn_cPCSL, as a Function of the *sn*-1 Chain Length, n_c

n_c	cmc (M)	$\Delta G_{tr}/RT$	ref
8	6.5×10^{-3}	−9.0	1[b]
10	9.0×10^{-4}	−11.0	1
12	1.0×10^{-4}	−13.2	2[c]
14	8.4×10^{-6}	−15.7	2

[a] $\Delta G_{tr}/RT$ is the natural logarithm of the cmc in mole fraction units. [b] Schmidt et al. (1981). [c] This work.

Table II: Critical Micelle Concentrations (cmc) and Free Energies of Monomer Transfer ($\Delta G_{tr}/RT$) at 20 °C for the Different Spin-Labeled Phospholipids with C12 *sn*-1 Chain (V12PXSL) in 0.15 M NaCl at pH 7, except for PA^{2-} and PAH^- Which Represent Phosphatidic Acid at pH 8 and pH 5, Respectively

	PA^{2-}	PAH^-	PS^-	PG^-	PC	PE
cmc (mM)	0.77	0.13	0.24	0.17	0.10	0.05
$\Delta G_{tr}/RT$	−11.2	−13.0	−12.4	−12.7	−13.2	−13.9

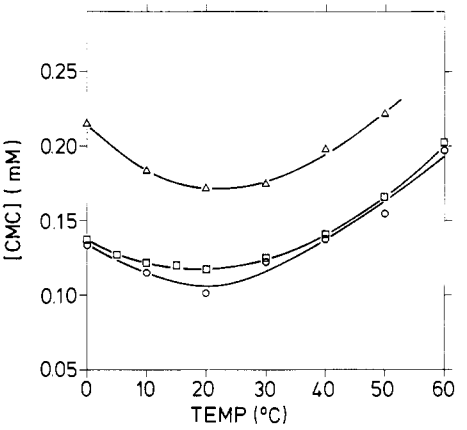

FIGURE 4: Temperature dependence of the cmc's of spin-labeled phosphatidylcholine, V12PCSL, and spin-labeled phosphatidylglycerol, V12PGSL: (□) V12PCSL in water at pH 7; (○) V12PCSL in 0.15 M NaCl, pH 7; (△) V12PGSL in 0.15 M NaCl, pH 7.

concentration dependence, as indicated in Figure 3.

Chain Length and Head Group Dependence of the cmc. The chain length dependence of the cmc's of the Vn_cPCSL spin-labels at 20 °C is given in Table I. The values for the 1-myristoyl and 1-lauroyl derivatives are from this work, and those for the 1-decanoyl and 1-octanoyl derivatives are taken from Schmidt et al. (1981). It is seen that the logarithm of the cmc decreases approximately linearly with the number of methylene groups, n_c, in the *sn*-1 acyl chain of the phosphatidylcholine. As will be seen later, this behavior is characteristic of the hydrophobic effect, which is the primary factor governing the lipid self-assembly. The gradient of the chain length dependence of the monomer free energy of transfer is in the region of $-1.1RT$ per CH_2 group, corresponding to the approximately 10-fold decrease in cmc for every two methylene groups added to the *sn*-1 chain. The temperature dependences of the cmc's of the spin-labeled phosphatidylcholine, V12PCSL, and phosphatidylglycerol, V12PGSL, are given in Figure 4.[3] The cmc's go through a minimum at 20 °C, which is again characteristic of the hydrophobic effect, as exemplified for instance by the temperature dependence of hydrocarbon solubility in water [see, e.g., Tanford (1973)].

[3] The dispersions were adjusted to pH 7 at 20 °C. No attempt was made to control the variation of pH with temperature, since the lipid pK_a's lie well outside this range.

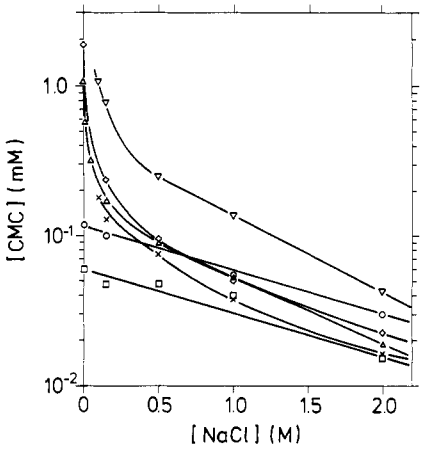

FIGURE 5: cmc's of spin-labeled phospholipids with different head groups as a function of NaCl concentration: (○) V12PCSL; (△): V12PGSL$^-$; (□) V12PESL; (◇) V12PSSL$^-$; (×) V12PASL$^-$; (▽) V12PASL^{2-}. All measurements were made at 20 °C and at pH 7, except for V12PASL$^-$ and V12PASL^{2-} which were measured at pH 5 and 8, respectively.

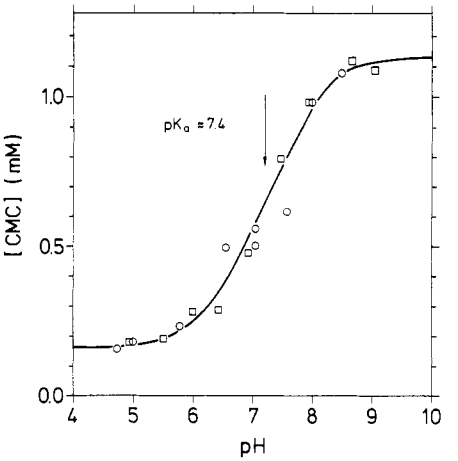

FIGURE 6: pH dependence of the cmc and monomer solubility of spin-labeled phosphatidic acid, V12PASL. (○) cmc measurements made in constant ionic strength buffers (I = 0.1, acetate, phosphate, or Tris, as appropriate) at 20 °C. (□) Low-field line height of the monomer ESR signal of V12PASL in 0.1 M unbuffered NaCl solution as a function of pH at 20 °C. The monomer line-height data are scaled to fit the cmc data at pH 5 and 8.

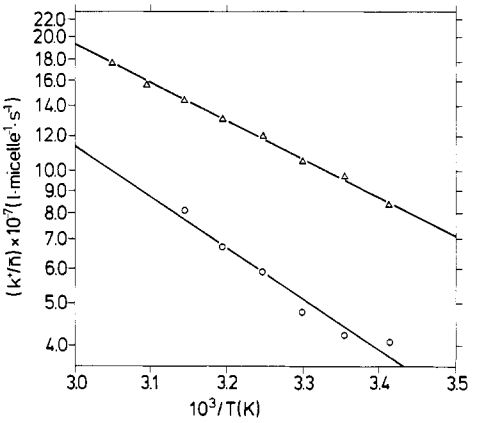

FIGURE 7: Arrhenius plot of the temperature dependence of the monomer–micelle exchange rate, $k^+/\bar{n}$, where k^+ is the monomer on-rate constant and $\bar{n}$ is the micelle size: (△) Phosphatidylcholine, V12PCSL, in 0.15 M NaCl, pH 7; (○) phosphatidylglycerol, V12PGSL, in 0.15 M NaCl, pH 7. The activation energies obtained by linear regression are E_a = 4.0 and 5.3 kcal·mol^{-1} for V12PCSL and V12PGSL, respectively.

The dependence of the cmc's on the phosholipid head group has been studied for the 1-lauroyl derivatives, V12PXSL (cf. Figure 1). The cmc's of the different spin-labeled phospholipids at 20 °C in 0.15 M NaCl are given in Table II. In this case only the phospholipid polar group is varied; all other parts of the molecule, including the spin-label group, are kept constant. For phosphatidic acid, the head group charge is also varied by changing the pH. It is seen from Table II that there is a considerable variation in the cmc with polar head group, the values ranging over more than a factor of 10, equivalent to a change in chain length by more than two methylene groups.

Salt and pH Dependence of the cmc. The cmc's in Table II were measured in the presence of salt, and thus, electrostatic effects may be partly suppressed. The salt dependence of the cmc's of the various spin-labeled phospholipids at 20 °C is given in Figure 5. For the zwitterionic phospholipids V12PCSL and V12PESL, the salt dependence is not very steep: the cmc decreases by approximately a factor of 4 on going from 0 to 2.0 M NaCl. For the negatively charged phospholipids the salt dependence is much greater. The cmc's decrease very steeply with increasing salt, particularly at the lower concentrations, corresponding to the shielding of the electrostatic interactions. For phosphatidylserine, V12PSSL, the cmc decreases by almost a factor of 100 on going from 0 to 2.0 M NaCl, and for phosphatidylglycerol, V12PGSL, the decrease is not much smaller. For phosphatidic acid the salt effects are similar, but to ensure good buffering, the lowest salt concentration measured was 0.05 M. Over the range 0.05–2.0 M NaCl, the cmc changes by a factor of 10–20, depending on the charge state of phosphatidic acid.

The effect of the head group charge of phosphatidic acid has been studied by pH titration of the V12PASL spin-label. The pH dependence of the cmc in buffers of constant ionic strength, I = 0.1, is given in Figure 6. Because of the chemical instability of the lipid at high pH, the cmc determinations have been checked by also measuring the monomer solubility. The latter method simply involves single measurements of the monomer ESR signal line height at the different pHs and is therefore much more rapid and far less susceptible to chemical degradation. Both methods yield titration curves of identical form (see Figure 6), with an apparent $pK_a \approx 7.4$ for the spin-labeled phosphatidic acid micelle formation. The cmc increases by a factor of approximately 7 on going from the singly to the doubly negatively charged state (at I = 0.1).

Kinetics of Monomer–Micelle Interaction. The on-rate constant for the association of the lipid monomer with the micelles has been measured for V12PCSL and V12PGSL from the lifetime broadening of the monomer ESR signal as a function of micelle concentration. The measurements for the V12PCSL lipid have been reported previously in a different context (King & Marsh, 1986). In 0.15 M NaCl the critical micelle concentrations are sufficiently low (see Table II) that the spin–spin interactions between monomers are negligible, and therefore, the line broadening of the monomer ESR signal arises solely from monomer–micelle collisions. The temperature dependences of the on-rate constants, k^+, for the monomer–micelle interaction are given as an Arrhenius plot for the V12PCSL and V12PGSL spin-labeled phospholipids in Figure 7. The rate constants are normalized with respect to the micelle size, n, which is required to calculate the micelle concentrations from those of the monomer. The logarithm of the rate constant decreases linearly with $1/T$ for both systems, clearly demonstrating the absence of any contribution to the line broadening from dipole–dipole interactions, which would have the opposite temperature dependence. An acti-

vation energy for the monomer–micelle association of 4.0 kcal·mol^{-1} for V12PCSL, and of 5.3 kcal·mol^{-1} for V12PGSL, is obtained from the Arrhenius plot. The value for V12PCSL is very close to the effective activation energy of 3.7 kcal·mol^{-1} associated with the viscosity of water (Weast, 1980), and the higher value for V12PGSL reflects the electrostatic repulsion associated with the micelle surface charge.

Discussion

Systematic studies have been made of the dependence of the phospholipid cmc's on hydrocarbon chain length, polar head group structure and charge, and composition of the aqueous phase, using ESR spectroscopy. In all the measurements, the spin-label group has been maintained constant on the *sn*-2 chain, hence allowing a direct intercomparison between the various phospholipids. A short-chain fatty acid (spin-labeled valeric acid) has been used on the *sn*-2 position to keep the cmc's in a range that is easily accessible experimentally. In this way it is hoped to use the results to extrapolate the self-assembly properties to those of longer chain diacyl phospholipids, such as are commonly found in biological membranes (cf. footnote 2).

Thermodynamics of Self-Assembly. The energetics of phospholipid self-assembly can be characterized by the free energy of transfer, ΔG_{tr}, of a lipid monomer from water to the micelle. At equilibrium the free energy of transfer to a micelle of size n is given by (Tanford, 1973)

$$\Delta G_{tr} = \mu^{\circ}_{mic,n} - \mu^{\circ}_{W} = RT \ln X_W - (RT/n) \ln (X_m/n) \quad (4)$$

where $\mu^{\circ}_{mic,n}$ and μ°_{W} are the chemical potentials of a monomer in the micelle and in water, respectively, in the standard state, and X_m and X_W are the mole fractions of monomer in the micelle and in water, respectively. It is assumed that the monomer activity coefficient in water is unity since the concentration is very low and that the micelle activity coefficient is also unity since the micelles are independent entities. For very large micelles, the micellar number, n, can be taken equal to infinity ($n \rightarrow \infty$), and the pseudophase approximation is obtained:

$$\Delta G_{tr} = RT \ln X_W \quad (5)$$

This approximation corresponds to a true phase separation, and according to the Gibbs phase rule, X_W is the single monomer concentration that can exist in equilibrium with the micelles. This is the thermodynamic definition of the critical micelle concentration, cmc:

$$\Delta G_{tr} = RT \ln [\text{cmc}] \quad (6)$$

where [cmc] is the cmc expressed in mole fraction units. The finite micelle size will cause the free energy of transfer deduced from the experimentally defined cmc (cf. Figure 3) according to eq 6 to be somewhat in error. However, to a first approximation this will be a systematic error, if the micelle size does not vary excessively, and therefore will not affect intercomparisons of the energetics of self-assembly between the different systems very greatly.

Chain Length Dependence of the cmc and Bilayer Properties. Keeping the *sn*-2 chain constant, the variation in the free energy of transfer of the Vn_cPCSL phosphatidylcholines with length of the *sn*-1 chain is found by linear regression analysis ($r^2 = 0.998$) of the data in Table I to be given by

$$\Delta G_{tr}/RT = -1.2 - 1.1(n_c - 1) \quad (7)$$

where $n_c - 1$ is the number of aliphatic carbon atoms in the *sn*-1 acyl chain. This is very similar to the chain length dependence of the free energy of transfer for monoacyl(lyso)-phosphatidylcholines, which is given by [see, e.g., Cevc and Marsh (1987)]

$$\Delta G_{tr}/RT = 0.2 - 1.1(n_c - 1) \quad (8)$$

The similarity arises because both series of lipids have a short (or no) chain on the *sn*-2 position. The incremental free energy of transfer is the same in both cases, and the larger negative chain length independent contribution for the Vn_cPCSL phospholipids corresponds to the hydrophobic contribution from the short *sn*-2 chain. For comparison, the chain length dependence of the free energy of transfer of hydrocarbons from water to the hydrocarbon liquid is given by [see Tanford (1973)]

$$\Delta G_{tr}/RT = -4.11 - 1.49n_c \quad (9)$$

The increment per CH_2 group is somewhat larger than, but nonetheless of a similar magnitude to, that deduced from the phospholipid micellization. Together with the linear chain length dependence and the characteristic biphasic temperature dependence (Figure 4) of the cmc's, this indicates that the hydrophobic effect is the driving force for self-assembly of the phospholipid molecules. The reduced incremental free energy of transfer for the phosphatidylcholines can be attributed to the ordering of the chains in the micelle, relative to a hydrocarbon liquid. In support of this, it is found that the incremental entropy of chain melting is approximately $0.3R$ per CH_2 greater for liquid hydrocarbons than for phospholipid bilayers (Phillips et al., 1969).

Allowing for the ordering of the chains in the micelle, a more appropriate estimate of the hydrophobic component of the free energy of transfer from the phospholipid micelle is obtained by adding the chain entropy contribution of $0.3RT$, giving a total of $1.4RT$ per CH_2. Using the measurements of Reynolds et al. (1974) for the hydrocarbon molecular surface area accessible to water this corresponds to a hydrophobic free energy of transfer of 23 cal·mol^{-1}·Å^{-2}. For phospholipid bilayer self-assembly, we are more interested in the surface density of the hydrophobic free energy at a planar interface. If the molecular surface is approximated by a hemisphere, the interfacial area is half of that of the molecular surface area and the interfacial hydrophobic free energy density is $\gamma \approx 3.3 \times 10^{-20}$ J·nm^{-2} ≡ 33 dyn·cm^{-1}.

The interfacial hydrophobic free energy density plays a major role in bilayer stability. According to the "principle of opposing forces" (Tanford, 1973; Israelachvili et al., 1980), the cohesive hydrophobic tension, γ, is balanced by an effective internal lateral pressure Π arising from the repulsive interactions between the lipid head groups and the hard-core repulsive interactions between the chains. The condition for mechanical equilibrium leads to the following expression for the net tension $\bar{T}$ in each bilayer half (Evans & Waugh, 1977):

$$\bar{T} = \gamma - \Pi \quad (10)$$

Thus for a normal tension-free bilayer ($\bar{T} = 0$), the equilibrium surface pressure in each bilayer half is simply equal to the interfacial hydrophobic free energy density: $\Pi_0 = \gamma = 33$ dyn·cm^{-1}. This value is in good agreement with the equilibrium spreading pressure of 32 dyn·cm^{-1} for a monolayer derived from a bilayer vesicle suspension (Schindler, 1979), which strongly supports the above analysis. Therefore, bilayer properties should correspond to those of a lipid monolayer with a surface pressure of approximately 33 dyn·cm^{-1}.

For dioleoylphosphatidylcholine monolayers, a surface pressure of 33 dyn·cm^{-1} corresponds to an area per molecule of approximately 70 Å^2 (Yue et al., 1976), as expected from X-ray diffraction results from bilayers in the fluid L_α phase [see, e.g., Shipley (1973)]. In addition, phospholipid mono-

layers at a surface pressure of approximately 30 dyn·cm^{-1} are found to have similar thermotropic phase-transition properties to those of the corresponding lipid bilayers (Blume, 1979). The elastic properties may also be derived from monolayer data. The isothermal area compressibility modulus K_A is given from eq 10 to be

$$K_A = A(d\bar{T}/dA)_T = -A(d\Pi/dA)_T \quad (11)$$

where A can be taken as the area per molecule in this case. Using the monolayer data at a surface pressure of $\Pi = 33$ dyn·cm^{-1} (Yue et al., 1976) yields a value of 70 dyn·cm^{-1} per bilayer half, i.e., a value of $K_A = 140$ dyn·cm^{-1} for the whole bilayer. This is in very good agreement with the experimental measurements for phosphatidylcholine bilayers in the fluid phase of $K_A = 130 - 140$ dyn·cm^{-1} (Kwok & Evans, 1981; Evans & Kwok, 1982).

Head Group Dependence of the cmc. Keeping the apolar part of the molecule constant, the dependence of the cmc on polar headgroup for phospholipids in 0.15 M NaCl, with a lauroyl chain in the *sn*-1 position, is given in Table II. The cmc's vary by a factor of 15 for the different head groups. The largest differences are found between the negatively charged and zwitterionic phospholipids. However, the cmc's of phosphatidylcholine and phosphatidylethanolamine differ by a factor of 2, presumably because of differences in hydration as observed for phosphatidylcholine and phosphatidylethanolamine bilayers (Cevc et al., 1986). This smaller difference also corresponds to the more limited salt dependence of the cmc's for the zwitterionic phospholipids (Figure 5).

The steep salt dependence of the cmc's for the negatively charged phospholipids arises from electrostatic screening of the surface charges on the micelle (Figure 5). This can be understood semiquantitatively in terms of the Debye–Hückel theory of electrolytes. For a negatively charged micelle of radius r_0 the electrostatic contribution to the free energy per monomer in the micelle is (Tanford, 1973)

$$\Delta G_{el} = [N_A z^2 e^2 \bar{n}/2\epsilon][1/r_0 - \kappa/(1 + \kappa r_0 + \kappa a_i)] \quad (12)$$

where ϵ is the effective dielectric constant at the micelle–water interface, a_i is the counterion radius, $\bar{n}$ is the average micellar number, z is the charge on the lipid monomer, e is the electronic charge, k is Boltzmann's constant, and $\kappa = (8\pi N_A e^2 I/1000\epsilon kT)^{1/2}$ is the reciprocal Debye–Hückel screening length in a 1:1 electrolyte of ionic strength I, N_A being Avogadro's number. The electrostatic contribution is additive to the free energy of transfer in eq 6, and hence, its effect on the cmc can be calculated. The first term in eq 12 is not dependent on ionic strength and therefore may be absorbed into the value for the cmc in the absence of salt in eq 6. In Figure 8, the values of ln [cmc] for the PG$^-$, PS$^-$, and PA$^-$ spin-labels from Figure 5 have been normalized to zero at 0.5 M NaCl, at which salt concentration the screening becomes less steep. The values then all lie approximately on the same smooth curve, suggesting a common mode of screening for the three singly negatively charged phospholipids. Values for the electrostatic free energy of transfer, $\Delta G_{el}/RT$, have been calculated according to eq 12, and these are also (arbitrarily) normalized to zero at 0.5 M NaCl. Values of $\epsilon = 80$ and $a_i = 2.5$ Å have been used in this calculation. The micelle sizes are unfortunately not known, but values of $\bar{n} = 63$ and $r_0 = 28.5$ Å measured for V12PCSL (Schmidt et al., 1981) have been taken for the sake of comparison. In spite of the uncertainties involved, the simple Debye–Hückel theory offers a qualitative description of the observed screening behavior. At zero ionic strength, the cmc's do not increase as rapidly as predicted by the theory, due at least in part to the residual counterions. At high ionic strength, the cmc's continue to decrease slowly to an extent that closely parallels that of the zwitterionic phospholipids. This is presumably a nonelectrostatic effect, possibly arising from a decrease in hydration of the lipid head groups with increasing salt concentration.

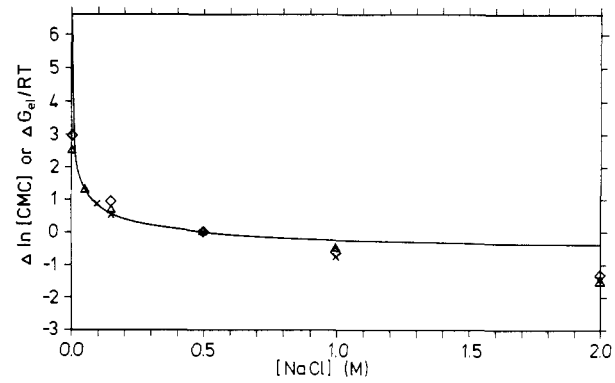

FIGURE 8: Ionic strength dependence of ln [cmc] for the V12PGSL$^-$ (△), V12PSSL$^-$ (◇), and V12PASL$^-$ (×) negatively charged phospholipids, obtained from the data of Figure 5. The curves are shifted to coincide at 0.5 M NaCl, at which point ln [cmc] is arbitrarily set to zero. The full line is the calculated micellar electrostatic free energy per monomer, ΔG_{el}, deduced from Debye–Hückel theory, according to eq 12. This is also set equal to zero at 0.5 M NaCl. Values of $\epsilon = 80$, $a_i = 2.5$ Å, $r_0 = 28.5$ Å, $\bar{n} = 63$, and $T = 293$ K, were used in the calculation.

In principle, the change in cmc on titration of V12PASL (Figure 6) can be calculated with Debye–Hückel theory. For this, the full eq 12 is required, since the lipid head group charge, z, is changing from 2– to 1– on going from high pH to low pH. The calculated change in ΔG_{el} is $7.32RT$ for $I = 0.1$, whereas the cmc only changes by a factor of 10, corresponding to a change in ΔG_{tr} calculated from eq 6 of $1.9RT$. One possible reason for the discrepancy could be a change in micelle size on pH titration. It is observed that the samples become visibly cloudy on titration to low pH, and an increase in the micelle size by a factor 3–4 would be enough to account for the difference. Other reasons lie in the shortcomings of the simple Debye–Hückel theory, as reviewed by Tanford (1973), in particular the counterion condensation at the very high charge densities arising in the doubly negatively charged state.

The apparent pK_a associated with the cmc of V12PASL is p$K_a = 7.4$, which lies between that of glycerol 1-phosphoric acid with p$K_a = 6.65$ (Datta & Grzybowski, 1958) and that of egg phosphatidic acid in 0.1 M NaCl with p$K_a = 8.0$ (Abramson et al., 1964). The value for glycerol 1-phosphoric acid can be taken as representative of the V12PASL lipid monomer. The value for egg phosphatidic acid, on the other hand, contains contributions from surface electrostatics and the reduced polarity at the lipid–water interface (Fernandez & Fromherz, 1977) and can be taken as representative of the aggregated form of V12PASL. An intermediate value is to be expected for the self-assembly process.

Monomer–Micelle Association Kinetics. The temperature dependences of the on-rate constants, k^+, for the monomer–micelle interaction are associated with activation energies of 4.0 kcal·mol^{-1} for V12PCSL and 5.3 kcal·mol^{-1} for V12PGSL. The value for V12PCSL is very close to the effective activation energy of 3.7 kcal·mol^{-1} associated with the viscosity of water (Weast, 1980), suggesting a diffusion-controlled process. With a value of $\bar{n} = 63$ for the micelle size of V12PCSL (Schmidt et al., 1981), the on-rate constant at 20 °C is $k^+ = 5.1 \times 10^9$ M^{-1} s^{-1}. This is to be compared with a diffusion-controlled

rate of $k_2^{diff} = 2RT/(3000\eta)\cdot(r_B/r_A) = 6.5 \times 10^9$ M^{-1} s^{-1} at 20 °C, where the approximation $r_B/r_A = \bar{n}^{1/3}$ has been used in determining the ratio of micelle to monomer radii. Again, this strongly suggests that for V12PCSL the monomer–micelle on-rate corresponds to a diffusion-controlled process. Since the spin-label group in V12PCSL is located close to the polar–apolar interface of the micelle, it is not entirely clear whether the on-rate represents only monomer–micelle collision or true incorporation of the monomer into the micelle. If the latter is the case, then the off-rate constant k^- can also be determined, since from detailed balance

$$k^- = k^+[\text{cmc}] \quad (13)$$

At 20 °C this yields an off-rate constant of $k^- = 5.1 \times 10^5$ s^{-1} for V12PCSL. These results for k^+ and k^- differ somewhat from those reported previously by Schmidt et al. (1981), because in the latter work no correction was made for the inhomogeneous broadening of the monomer ESR signal (cf. eq 1).

The higher value of the activation energy for V12PGSL$^-$ compared with V12PCSL (see Figure 7) arises from the electrostatic repulsion between monomer and micelle for the charged lipid. In principle, the size of this effect can be estimated from an extension of Debye–Hückel theory. The additional electrostatic contribution to the activation energy is given by (Debye, 1943)

$$\Delta E_a = \frac{N_A \bar{n} e^2}{2\epsilon} \frac{e^{-\kappa(r_0+a_i)}}{r_0 + a_i}\left(\frac{e^{\kappa r_0}}{1+\kappa r_0} + \frac{e^{\kappa a_i}}{1+\kappa a_i}\right) \quad (14)$$

As previously noted, the micelle size for V12PGSL is not known. Therefore, the values of $\bar{n} = 63$ and $r_0 = 28.5$ Å, appropriate to V12PCSL (Schmidt et al., 1981), have been used for the purpose of this estimate. Using the values for the remaining parameters given in the legend to Figure 8 then gives a value of $\Delta E_a = 740$ cal·mol^{-1} for the electrostatic contribution to the activation energy. This is somewhat smaller than the difference between the activation energies for V12PGSL and V12PCSL, but in view of the uncertainties in the values for the micelle size and the interfacial dielectric constant, it gives satisfactory order of magnitude agreement. Again assuming the same micelle size as for V12PCSL, the on-rate constant for V12PGSL at 20 °C is $k^+ = 2.4 \times 10^9$ M^{-1} s^{-1}, and the off-rate constant assuming that eq 13 can be applied is $k^- = 4.1 \times 10^5$ s^{-1}.

Extrapolated cmc's for Diacyl Phospholipids. Finally, with the additivity of the contributions to the free energy of transfer, the present data on the phospholipid head group dependence can be used to predict the cmc's of the longer chain diacyl phospholipids such as are found in biological membranes. The cmc's have been measured for saturated diacylphosphatidylcholines and indicate a linear dependence of the free energy of transfer on chain length, up to the bilayer-forming dipalmitoyl derivative [see, e.g., Reynolds et al. (1977)]. Linear regression yields the following expression for the phosphatidylcholines:

$$\ln [\text{cmc}] = -0.4 - 1.7(n_c - 1) \quad (15)$$

where $(n_c - 1)$ is the number of aliphatic carbon atoms in either acyl chain and the cmc is in mole fraction units. The corresponding values for other phospholipids can then be predicted from eq 15 by adding the difference in the value of $\Delta G_{tr}/RT$ in Table II from that for phosphatidylcholine. For example, the cmc's of the dipalmitoyl derivatives of phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine, in 0.15 M NaCl, pH 7, are predicted to be approximately 1.5 $\times 10^{-10}$, 5.6 $\times 10^{-10}$, and 7.6 $\times 10^{-10}$ M, respectively, relative to a predicted cmc of 3.3 $\times 10^{-10}$ M for dipalmitoylphosphatidylcholine. The salt dependence may also be predicted by adding the increments from Figure 8 to eq 15, or simply adding the electrostatic contributions estimated with eq 12. These corrections will not be exact because of the difference in area per polar head group between micelles and bilayers but can be expected to give a good first approximation. The method may be extended to mixed-chain lipids by using a combination of eq 7 and 15 and may even be applied to unsaturated lipids by taking data from the relative solubilities of saturated and unsaturated hydrocarbons [see, e.g., Tanford (1973)]. Further details may be found in Marsh and King (1987).

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Calcium Binding to Mixed Phosphatidylglycerol–Phosphatidylcholine Bilayers As Studied by Deuterium Nuclear Magnetic Resonance†

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ABSTRACT: The binding of calcium to bilayer membranes composed of mixtures, in various proportions, of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) plus 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) was investigated by using atomic absorption spectroscopy and deuterium nuclear magnetic resonance. The number of bound calcium ions, X_2, was determined in the low calcium concentration range (up to 100 mM) via atomic absorption spectroscopy. Simultaneous measurements of the deuterium magnetic resonance spectra of POPC, specifically deuteriated at the α-methylene segment of the choline head group, revealed a linear relationship between the quadrupole splitting, $\Delta\nu_Q$, and X_2 for each particular proportion of POPC–POPG. The amount of bound calcium was then determined at much greater calcium concentrations, where the atomic absorption spectroscopy measurements were unreliable, using deuterium magnetic resonance. At low Ca^{2+} concentrations, the amount of bound Ca^{2+} increased linearly with increasing proportion of POPG, demonstrating an electrostatic contribution to Ca^{2+} binding. At high Ca^{2+} concentrations, the calcium binding isotherms exhibited saturation behavior with a maximum binding capacity of 0.5 Ca^{2+} and 1.0 Ca^{2+} per phospholipid for pure POPC and mixtures of POPC–POPG, respectively. Simultaneous deuteriation of POPG and POPC showed that both lipids remained in a fluidlike lipid bilayer at all Ca^{2+} concentrations tested. Any phase separation of quasi-crystalline Ca^{2+}–POPG clusters could be excluded. The residence time of Ca^{2+} at an individual head group binding site was shorter than 10^{-6}–10^{-5} s. Thus, Ca^{2+} ions accumulate near the negatively charged POPG–POPC membrane surface but move freely in a "trough" of the electrical potential. The effective surface charge density, σ, could be determined from the measured amount of bound Ca^{2+}. Subsequently, the surface potential, ψ_0, and the concentration of free Ca^{2+} ions at the plane of ion binding could be calculated by employing the Gouy–Chapman theory. The availability of these parameters allowed a rigorous evaluation of various models for the chemical contribution to Ca^{2+} binding. For mixed POPC–POPG bilayers, a simple Langmuir adsorption model yielded the best fit to the experimental data, and the binding constants were 19.5 and 18.8 M^{-1} for POPG contents of 20 and 50 mol %, respectively. Sodium binding was comparatively weak with a binding constant of 0.6–0.85 M^{-1}. The values of the calcium association constants indicate that the increased binding of calcium with increasing proportion of POPG is predominantly an electrostatic effect, rather than the result of an intrinsically greater affinity of POPG for calcium. Calcium was able to reduce the surface potential by binding and neutralizing negative surface charges in addition to having a screening effect.

Negatively charged lipids are common components of biological membranes and can be expected to attract and to bind calcium and other ions (Eisenberg et al., 1979; Lau et al., 1981). The binding of cations may alter the conformation and physical properties of membrane lipids [e.g., see Fleming & Keough (1983)], as well as the electrical and functional properties of the membrane proper (McLaughlin, 1977), and can have important physiological consequences (McLaughlin, 1977; Scarpa & Carafoli, 1979).

Previously, the various techniques employed to study the binding of ions to membrane surfaces have not measured directly the amount of bound ion but rather have inferred this quantity indirectly from measurements of such parameters as the electrophoretic mobility (Eisenberg et al., 1979; Lau et al., 1981; McLaughlin et al., 1983), the electrostatic interbilayer repulsive force (Lis et al., 1981a,b; Oshima et al., 1982), or the competition with lanthanide shift reagents (Grasdalen et al., 1977). A direct measurement of the amount of bound ion can be obtained, however, over a limited concentration range, by using atomic absorption spectroscopy. The available range of concentrations can then be greatly extended by using deuterium nuclear magnetic resonance (^2H NMR).[1] The change in the quadrupolar splitting in spectra of specifically deuteriated lipid polar head groups, which occurs

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[1] Abbreviations: NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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