Molecular Organization in the Liquid-Crystalline Phases of Lecithin–Sodium Cholate–Water Systems Studied by Nuclear Magnetic Resonance†

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ABSTRACT: The molecular organization in the hexagonal and lamellar phases of the ternary systems lecithin–sodium cholate–water has been investigated by using a variety of nuclear magnetic resonance techniques. The main findings and conclusions are the following: (i) When calculated on a mole fraction basis, the phase equilibria are insensitive to changes in the alkyl chains of the lecithin. (ii) When incorporated into a lecithin bilayer, cholate exerts a strong perturbation on the lecithin alkyl chain order, giving a large decrease of the order parameters. (iii) This decrease of the order occurs since the average cross-sectional area per alkyl chain increases probably as a result of cholate placing itself flat on the bilayer surface. (iv) The diffusion of lecithin molecules is approximately equally rapid in the lamellar and hexagonal phases. (v) The hexagonal phase is formed by rodlike aggregates with the polar groups at the surface of the rods and with a continuous hydrocarbon core. The rods are *not* formed by stacking disklike mixed micelles. (vi) With the interpretations of the molecular packing and the phase structures, the observed phase equilibria are in good agreement with current theories of the factors that govern phase behavior in amphiphile–water systems.

Sodium cholate and other bile salts have a strong effect on the physicochemical properties of phospholipid bilayers. This is, for example, of significance in the intestinal absorption of fat [see review by Borgström (1977)]. Lipolysis seems to be dependent on a combined action of bile salts and phospholipids, mainly lecithins, on the emulsified triacylglycerol substrate (Lairon et al., 1978, 1980). In the formation of bile, phospholipid membrane fragments are dissolved by the bile salts, and the phospholipid molecules reach the gallbladder in mixed micelles. Similarly in the use of phospholipid liposomes for the oral administration of drugs, the stability of these liposomes in the small intestine depends strongly on the bile salt–phospholipid interaction (Rowland & Woodley, 1980).

In vitro cholate can be used in the extraction of integral membrane proteins (Helenius et al., 1979) and also in the preparation of unilamellar vesicles [see, for example, Brunner et al. (1976)]. One part of the understanding of these processes in molecular terms consists in characterizing the nature of the interaction between bile salts and phospholipids. In the present work we try to reveal the main features of this interaction by studying the model system sodium cholate–lecithin–water using a variety of nuclear magnetic resonance techniques.

The physicochemical properties of bile salts in various binary, ternary, and quaternary systems have been extensively studied by Small and co-workers (Small, 1971). The bile salts are surface active compounds but have several properties that differ from those of typical amphiphiles, as, for example, soaps. Thus the aggregation is of low cooperativity (Carey & Small, 1972; Fontell, 1972; Lindman et al., 1979; Wennerström & Lindman, 1979; Mazer et al., 1979). Furthermore, mixtures of bile salts and water have not been observed to form liquid-crystalline phases. This behavior of the bile salts can be understood as due to the stiffness of the fused ring system giving the molecules few internal degrees of freedom that can be excited when going from a solid to a liquidlike state. As was pointed out by Small (1971), the bile salts are roughly planar, with one hydrophobic and one hydrophilic side. This seems to be a key for the understanding of the atypical properties of the bile salts as surfactants.

Lecithins (phosphatidylcholines), on the other hand, aggregate so strongly in an aqueous system that a lamellar liquid-crystalline phase is formed over a wide temperature range (Luzzati & Tardieu, 1974). In the binary system lecithin–water, the concentration of free lecithin is minute so that the redistribution of lecithin molecules between different aggregates is a slow process. Since the stability of an emulsion is related to the stability of a lamellar phase, the lecithins act as emulsifiers (Friberg & Larsson, 1976).

In the ternary system sodium cholate–lecithin–water, interesting aggregation effects result from the balance between the very strong association tendency of lecithin and the rather weak association in cholate. This is illustrated by the ternary phase diagram in Figure 1 determined by Small and co-workers (Small et al., 1966; Small & Bourges, 1966). There are four one-phase regions: an isotropic solution of higher water contents, where the amphiphilic molecules are aggregated to mixed micelles, and three liquid-crystalline phases. The mixed micellar solution has been thoroughly studied by Mazer et al. (1980). They used quasi-elastic light scattering and were able to characterize the aggregate size and shape. In the mixed micelles, the lecithins retain their preference for a bilayer-like structure, and disklike micelles are formed.

Among the three liquid-crystalline phases, the structure of the lamellar phase is well established for the binary lecithin–water system, and the gross structure does not seem to change on addition of cholate. The structures of the cubic and hexagonal phases have, on the other hand, not been clearly established. We have previously (Lindblom et al., 1976c) shown that the cubic phase contains a bicontinuous structure, but the more precise nature of the three-dimensional structure is not known. For the hexagonal phase, Small and co-workers (Small, 1971) have suggested that infinite rods are formed by

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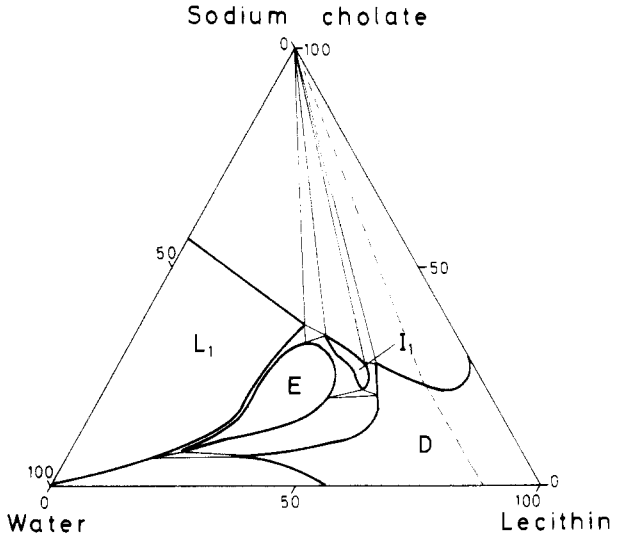

FIGURE 1: Phase diagram for the ternary system egg yolk lecithin–sodium cholate–water at 22 °C [redrawn after Small et al. (1966)]. L_1 is the isotropic aqueous liquid, E the hexagonal, D the lamellar, and I the cubic liquid-crystalline phases.

stacking disklike micelles on top of each other and that these rods pack in a two-dimensional hexagonal lattice. This structure of a hexagonal phase has not been found in other amphiphile–water systems, and one of the aims of the present work is to test this suggestion on the basis of the NMR[1] measurements. Due to the long-range order in the liquid-crystalline phases, one can obtain direct information on the molecular structure of these phases. Particularly nuclear magnetic resonance techniques have provided a wealth of information on molecular order in liquid-crystalline and model membrane systems (Seelig, 1977; Seelig & Seelig, 1980; Mantsch et al., 1977; Lindblom et al., 1976a–c). In this article we report a series of NMR studies of the lamellar and hexagonal liquid-crystalline phases in ternary systems of different phosphatidylcholines–sodium cholate–water, using ^1H, ^2H, and ^{31}P NMR in order to reveal the molecular organization and to understand the relation between molecular interactions and phase behavior.

Materials and Methods

Egg yolk lecithin was prepared from freshly extracted egg yolk lipids by column chromatography on aluminum oxide followed by chromatography on silicic acid. Established procedures were adopted for the preparation of synthetic phosphatidylcholines (Gupta et al., 1977) and for manufacturing the fatty acid anhydrides (Brockerhoff & Yurkowski, 1965) and the *sn*-glycero-3-phosphocholines (Selinger & Lapidot, 1966) used in the synthesis. The synthetic lecithins were purified by column chromatography on silicic acid. All lecithin preparations were subjected to analysis by thin-layer and gas chromatography for the assessment of purity. Serial dilutions of each sample over a 100-fold concentration range were applied to silica gel thin-layer plates which run in different solvent systems. After development, the plates were sprayed with dichlorofluorescein and viewed under UV light, followed by spraying with ninhydrin and finally with a phospholipid-specific spray reagent. Only lecithins with a purity of 99% were used. Perdeuterated palmitic acid (hexadecanoic-d_{31} acid) with a chemical purity exceeding 99% and an enrichment of deuterium exceeding 95% was obtained from Larodan Fine Chemicals, Malmö, Sweden. The fatty acids were characterized by their melting points and mass spectra. Cholic acid was obtained from BDH, Poole, England, and the sodium salt was prepared by neutralizing an alcoholic solution of cholic acid with an equimolar amount of sodium methoxide and then boiling off the alcohol until precipitation started. The sodium cholate was dried under vacuum. The water was double distilled in an all-quartz apparatus. Heavy water purchased from Ciba-Geigy, Switzerland, was used.

Samples were prepared in two different ways. In one method lecithin and sodium cholate were weighed into an ampule, and the mixture was dissolved in methanol. Then the solvent was evaporated under vacuum. A given quantity of water was added with a microsyringe, and the contents of the ampule were mixed. The ampule was then sealed and the mixture allowed to equilibrate for several days. In the other method, sodium cholate was dissolved in water, and known amounts of this solution and lecithin were mixed. No differences between these two methods of sample preparation could be observed by X-ray diffraction or NMR spectroscopy. Macroscopically aligned phases were prepared by pressing a sample between microscope cover glasses.

^1H NMR measurements were performed at 100.0 MHz with a Varian XL-100-15 NMR spectrometer operating in the continuous wave mode with a sweep width of 31 kHz. The same spectrometer was used to record ^2H FT NMR spectra at 15.4 MHz and ^{31}P at 40.5 MHz. For the [$^2H_{31}$]-palmitoyloleoylphosphatidylcholine samples, the ^2H spectra were measured at 39 MHz by using a home-built spectrometer with a superconducting magnet. In addition some of the ^2H measurements were made at 13.8 MHz by using the quadrupolar echo technique (Davis et al., 1976) on a modified Bruker 322 spectrometer. This spectrometer was also used for the diffusion measurements with the pulsed field gradient NMR technique as described previously (Lindblom & Wennerström, 1977). The phase structure of the samples was also studied qualitatively by using a polarizing microscope and by low-angle X-ray diffraction. In the calculations of order parameters, the deuteron quadrupole coupling constant was assumed to be 170 kHz.

Results

Phase Behavior for Different Phosphatidylcholines. In addition to the egg yolk lecithin studied by Small and coworkers, we have performed studies of systems with pure phosphatidylcholines as dilauroyl-, dimyristoyl-, dioleoyl-, and palmitoyloleoylphosphatidylcholines. In each case the phase structure was checked in a polarizing microscope and also by X-ray diffraction. It was a general finding that if compositions were calculated on a mole fraction basis the phase boundaries are very similar to those of Figure 1 (effective molecular weight of EYL was chosen as 767; all the "pure" lecithins contain 1 mol of H_2O per mol of lecithin). An implication of this insensitivity of the phase equilibria to changes of the acyl chains is that the most important interactions determining the phase structure occur in the polar head region. At room temperature all the investigated phosphatidylcholines give liquid-crystalline structures, and the hydrocarbon chains have a liquidlike character. Thus there are no strong specific effects due to hydrocarbon–hydrocarbon interactions that can have a strong influence on the phase equilibria. This agrees well with the present understanding of phase equilibria in soap–water systems (Jönsson & Wennerström, 1981).

Proton Magnetic Resonance Spectra of the Amphiphiles. From an experimental point of view, the chain packing is most easily studied by using proton NMR. In a nonoriented liq-

[1] Abbreviations: NMR, nuclear magnetic resonance; EYL, egg yolk lecithin; POL, 1-palmitoyl-2-oleoyllecithin; DOL, 1,2-dioleoyllecithin.

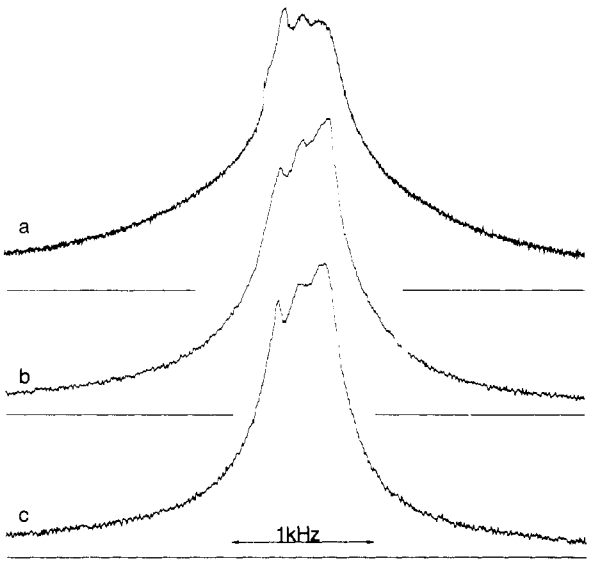

FIGURE 2: ^{1}H NMR spectra at 100 MHz of the lipids in lamellar (a and b) and hexagonal (c) phases of the system egg yolk lecithin–sodium cholate–heavy water. The cholate:EYL molar ratios are 0.14, 0.75, and 0.75 in (a), (b), and (c), respectively. The corresponding heavy water contents are [in % (w/w)] 24.2, 21.2, and 46.4. Temperature 28 °C. Total sweep width 31 kHz.

uid-crystalline sample the ^{1}H spectrum exhibits a characteristic shape ("super-Lorentzian") having a rather narrow central peak with unusually broad wings (Lawson & Flautt, 1968; Wennerström & Lindblom, 1977; Ulmius et al., 1975). This shape is caused by the nonvanishing dipole–dipole couplings in the anisotropic phase (Wennerström, 1973). Both lecithin and cholate contain a multitude of protons, and therefore the ^{1}H NMR experiment only provides information about the average molecular order. The width of the spectrum gives a measure of the molecular packing of the chains at the different compositions (the broader the peak the larger the order parameters). Figure 2 shows ^{1}H spectra of two lamellar and one hexagonal sample. A comparison between the lamellar samples 2a and 2b shows that the order decreases markedly with increasing cholate. For the samples in b and c of Figure 2 the lecithin/cholate ratio is approximately constant, and it is clear that the average order is approximately equal in the two phases under these circumstances. This finding is in sharp contrast with what is usually found in soap systems where the width changes by a factor of 2 (Charvolin & Rigny, 1973).

It should also be noted that we have not been able to reproduce the high resolution peak previously reported (Small, 1971) for the hexagonal phase. Theoretically such an observation is not to be expected either, so we have to conclude that this high resolution peak is due to a contamination from an optically isotropic phase, either a cubic liquid crystal or a micellar solution (see Figure 1).

Deuterium Magnetic Resonance of the Acyl Chains. The deuterium nucleus has a quadrupole moment giving rise to quadrupole splittings in the ^{2}H NMR spectra for the anisotropic lamellar and hexagonal phases. Studies of deuterated acyl chains of lecithins provide information about the average orientation of the chain segments through the order parameter S (Seelig, 1977). So that a detailed picture of the effect of cholate on the lecithin chain order could be obtained, deuterium spectra were measured for the perdeuterated palmitin chain in POL at a number of compositions. Figure 3 shows typical spectra of the lamellar and hexagonal phases, and one can see that a majority of the peaks can be resolved. The measured magnitude of the order parameters for 10 different compositions is summarized in Table I. The assignments are

Table I: Order Parameters at Different Positions in the Perdeuterated Palmitoyl Chain of Palmitoyloleoylphosphatidylcholine for Different Compositions in the POL–Sodium Cholate–Water System (t = 24 °C)

composition (cholate/POL molar ratio)	H_2O [% (w/w)]	phase	order parameter[a] at position													
			C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}
0.084[b]	22.5	lamellar	———	———	———	0.21 ———	———	———	0.20	0.18	0.164	0.144	0.130	0.111	0.093	0.069	0.020
0.25	21.6	lamellar	0.205	———	0.19 ———	———	0.18	0.158	0.147	0.131	0.121	0.104	0.094	0.080	0.068	0.052	0.015
0.52	19.6	lamellar	0.216	———	———	———	———	———	0.100	0.085	0.077	0.064	0.058	0.049	0.043	0.033	0.010
0.55	20.0	lamellar	0.206	——0.15——	———	——0.13——	———	0.110	0.086	0.071	0.064	0.053	0.047	0.040	0.035	0.026	0.0078
0.78	19.0	lamellar	0.186	——0.13——	———	0.108	0.100	0.081	0.071	0.057	0.049	0.038	0.033	0.027	0.024	0.018	0.0054
0.76	42.5	hexagonal[c]	0.19	———	———	0.16 ———	———	———	———	0.144 ———	———	0.105	0.096	0.084	0.070	0.052	0.015
0.82	45	hexagonal	0.18	———	———	0.15 ———	———	———	0.122	———	0.102 ———	0.086	0.078	0.066	0.056	0.044	0.012
0.96	41.0	hexagonal	0.19	———	———	0.15 ———	———	———	0.115	0.099	0.094	0.078	0.072	0.060	0.052	0.040	0.011
1.15	35	hexagonal	0.19	0.134	0.128	0.118	0.112	0.092	0.086	0.070	0.064	0.052	0.046	0.038	0.034	0.026	0.0076
1.28	36.6	hexagonal	0.19	——0.136——	———	——0.114——	———	0.092	0.086	0.068	0.062	0.050	0.044	0.036	0.032	0.024	0.0068

[a] Only the absolute value is given. [b] The S values at this low cholate content are in good agreement with those determined for the pure POL–H_2O system (Seelig & Seelig, 1977). [c] Experimentally determined order parameters are multiplied by 2.

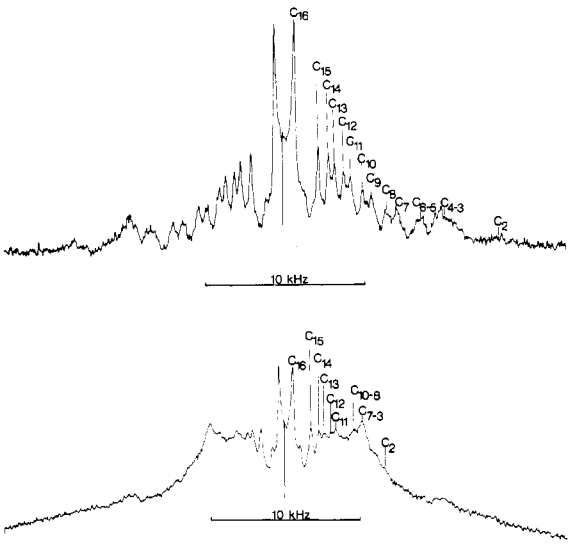

FIGURE 3: Typical ^2H NMR spectra at 39 MHz of the perdeuterated palmitoyl chain in a lamellar phase (top trace), cholate:POL molar ratio 0.52, and a hexagonal phase (bottom trace), cholate:POL molar ratio 0.76. The assignment of the signals from the deuterons on the C_2 carbon was performed with the aid of specifically labeled POL. Temperature 24 °C.

based on the assumption that the order parameters decrease along the chain. In some cases the assignment of the number of deuterons contributing to a certain peak is equivocal. In these cases the choice is made to be most consistent with the trend found when the composition is varied. For facilitation of the assignments of the signals from the deuterons on the C_2 carbon, samples with specifically labeled POL (1-[$^2H_2$]-palmitoyl-2-oleoyllecithin) were prepared. For several of these samples two slightly different splittings were observed (Seelig, 1977). Since the 2 position in the glycerol backbone is asymmetric, two deuterons on the same carbon are not symmetry equivalent, and this is probably the source of the two peaks. In reporting the order parameters for the hexagonal phase, we have chosen to multiply the observed S by a factor of 2 (Charvolin & Rigny, 1973) in order to obtain values that can be directly related to those observed in the lamellar phase as demonstrated by the similarity in the order parameters for the α position. It should be noted that it is only for certain models of the hexagonal phase that it is appropriate to adopt this procedure. For the model of the hexagonal phase suggested by Small, there is no reason to assume this difference of a factor of (minus) 2 in the order parameters in the hexagonal and lamellar phases.

The most notable features of the results summarized in Table I are the following: (i) For both the lamellar and hexagonal phases an increase in cholate content has the effect of decreasing S in general but also to decrease the order more at the chain end than near the polar group. (ii) After correction for the factor of 2 in the order parameter that arises from the difference in aggregate structure, the order parameter in the hexagonal phase is similar to those of the lamellar phase at much lower cholate contents. Thus at a molar ratio for cholate/lecithin of 0.25 in the lamellar phase, the order parameters are approximately the same as the ones found in the hexagonal phase (corrected by a factor of 2) at a molar ratio of 0.76. The same relation exists between the lamellar phase at a molar ratio of 0.55 and hexagonal phase at a molar ratio of 1.15. (iii) The clearly least affected position is the C_2 one, indicating that the conformation of the lecithin at the polar–apolar interface is relatively insensitive to the presence of cholate.

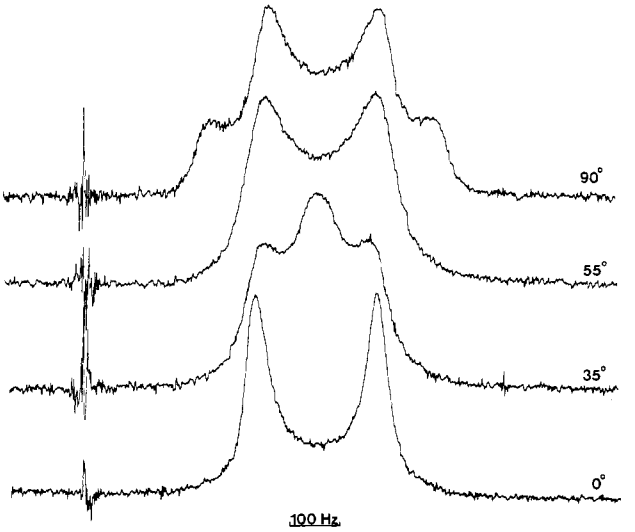

FIGURE 4: ^2H NMR spectra (at 15.4 MHz) of heavy water for a hexagonal phase macroscopically aligned between glass plates at different angles between the magnetic field and the normal to the glass plates. Temperature 28 °C.

Heavy Water Quadrupolar Splittings. Previous studies from this laboratory (Persson et al., 1974; Lindblom et al., 1976a) have shown that the water orientation decreases with increasing cholate content and furthermore that there is no jump in the quadrupole splitting of the water at the change from lamellar to hexagonal structure (see Figure 3; Persson et al., 1974). These results have here been confirmed by using a high resolution spectrometer. Due to the uncertainty of the structure of the hexagonal phase, the water deuteron splitting was measured for a sample oriented between two glass plates (Lindblom, 1972). As is clear from Figure 4, a good macroscopic orientation is obtained. The angular dependence of the signal follows what is predicted for a cylindrical distribution of directors parallel to the glass plates and with the asymmetry parameter zero (Wennerström et al., 1975). This latter finding implies that on the NMR time scale the water molecules experience an anisotropic environment with at least a 3-fold symmetry axis. The orientation behavior of the phase is thus consistent with that usually found for a hexagonal phase formed by rods.

Phosphorus NMR. In the ^{31}P NMR spectra of phospholipids in liquid-crystalline systems, the chemical shift anisotropy gives the dominating contributions (McLaughlin et al., 1975; Cullis & deKruijff, 1979). In contrast to the quadrupole splittings the spectrum depends on the sign of the order parameter, which in the present case provides a crucial extra piece of information. As seen from the spectra in Figure 5, the order parameters have different signs in the lamellar and hexagonal phases. In the two-phase region where the hexagonal and lamellar phases coexist, the order parameters in the two phases have similar magnitude, but due to the difference in the sign, the spectrum of Figure 5C clearly reveals the presence of the two phases.

Diffusion. So that additional information on the molecular structure of the different phases could be obtained, the dynamic properties of the lecithin molecules were studied through the translational diffusion. The translational diffusion coefficients have previously been reported for the cubic (Lindblom et al., 1976c, 1979) and the lamellar phases (Lindblom & Wennerström, 1977; Lindblom et al., 1981). Here these pulsed field gradient pulsed ^1H NMR studies have been extended, and the diffusion coefficients have been measured in the cubic, lamellar, and hexagonal phases for EYL, DOL, and POL. For

Table II: Diffusion Coefficients for Different Lecithins in Some Liquid-Crystalline Phases of the Lecithin-Sodium Cholate-Water Systems[a]

sample composition [% (w/w)]	phase	temp (°C)	diffusion coefficients (m^2/s)		
			$D \times 10^{12}$	$D_L \times 10^{12}$	$D_\parallel \times 10^{12}$
EYL-cholate-water					
80:0:20	lamellar	35	3	5[b]	
55.4:23.4:21.2	lamellar	23	6	9	
	lamellar	26	7	11	
	lamellar	39	11	17	
66.6:9.2:24.2	lamellar	23	5	8	
POL-cholate-water					
80:0:20	lamellar	35	4	6[b]	
50:26:24	cubic	35	1		4
35.5:25.5:39	hexagonal[c]	35	2		7
DOL-cholate-water					
80:0:20	lamellar	35	3	5[b]	
70:10:20	lamellar	35	3	5	
45:30:25	cubic	35	1		3
36.2:25.2:38.6	hexagonal[c]	35	3		8

[a] D is the experimentally determined effective diffusion coefficient. D_L represents the lateral diffusion coefficient in a lipid bilayer, obtained by correcting D for the magic angle. $D_\parallel$ is an estimated diffusion coefficient for translational motion along the surface of the amphiphile rodlike aggregate for the cubic and hexagonal phases. [b] From Lindblom et al. (1981). [c] Nonoriented.

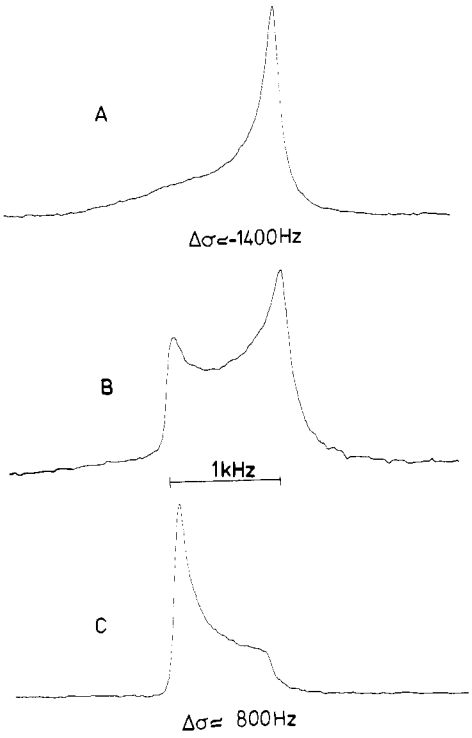

FIGURE 5: ^{31}P NMR spectra at 40.5 MHz for (A) a lamellar phase with a cholate:POL molar ratio of 0.40, 18% water, (B) a two-phase sample with a total composition cholate:POL molar ratio of 0.48, 30% water, and (C) a hexagonal phase with a cholate:POL molar ratio of 0.88, 46% water. Temperature 28 °C. $\Delta\sigma$ denotes the effective chemical shift anisotropy.

cubic phases the diffusion coefficient can be conveniently measured with the standard techniques (Charvolin & Rigny, 1973; Lindblom & Wennerström, 1977), but for anisotropic phases like the lamellar and hexagonal ones this method cannot in general be used directly (Lindblom & Wennerström, 1977). However, the amphiphile diffusion coefficient can be determined for a lamellar phase provided the samples are macroscopically aligned and oriented at the so-called magic angle in the magnetic field (Lindblom & Wennerström, 1977; Lindblom et al., 1979; Wieslander et al., 1981). For the hexagonal phase, alignment with glass plates does not give sufficient narrowing (cf. Figure 4) to make the measurement feasible on such samples. However, at high cholate contents, the unoriented samples give a sufficiently narrow signal to make the effective T_2 sufficiently long to allow for NMR diffusion measurements. The results are summarized in Table II. It has been shown previously that from a comparison between the diffusion coefficients obtained in different phases, information about amphiphile aggregate structure can be extracted [for a detailed description, see Lindblom & Wennerström (1977) and Lindblom et al. (1979)]. Thus, for example, the diffusion coefficient of a cubic phase composed of spherical micelles is about 1 order of magnitude smaller than that for a cubic phase with a rod network structure (Bull & Lindman, 1974; Lindblom & Wennerström, 1977). The local diffusion coefficient (lateral diffusion) is found to be independent of aggregate geometry (Lindblom & Wennerström, 1977; Lindblom et al., 1979). These local diffusion coefficients in the lamellar phase (D_L) and along the rodlike aggregates in the hexagonal and cubic phases ($D_\parallel$) have also been calculated and are shown in Table II. For the hexagonal phase the dominating contribution to the measured spin echo comes from aggregates, where the director is at the "magic angle" (Lindblom et al., 1977). In that case the diffusion along the director ($D_\parallel$) is a factor $(\cos^2 54.7)^{-1} = 3$ faster than the measured diffusion relative to the external field, assuming motions within the aggregates only. Thus in the rodlike aggregates of the hexagonal phase the diffusion coefficient of lecithins is of the same magnitude as in lamellar aggregates. Similarly for the cubic phase the lateral diffusion coefficient calculated (Lindblom & Wennerström, 1977) from the measured D, assuming a rod network structure (Fontell, 1973), agrees well with the measured lateral diffusion coefficient for the lamellar phase.

The similarity (within a factor of 2) between the diffusion coefficients strongly indicates that the local molecular environment is not affected by the gross phase structure. Moreover, in all three liquid-crystalline phases the hydrocarbon region must be continuous in at least one dimension. These findings agree with the general observation that local molecular properties are rather insensitive to changes in phase structure in amphiphile–water systems (Wennerström & Lindman, 1979; Tiddy, 1980; Ekwall et al., 1972).

Discussion

Molecular Order in the Lamellar Phase. Changes in the order parameters of molecules and/or molecular segments

when a second component is added to a phospholipid bilayer provide detailed information on how the solubilizate affects the bilayer structure. When, for example, cholesterol is added, there is a marked increase in the acyl chain order, particularly near the polar head (Rothman & Engelman, 1972; Stockton & Smith, 1976). Cholesterol is solubilized with the hydroxyl group at the bilayer surface, and the steroid skeleton seems to orient the acyl chain, possibly by reducing the number of kinks or by reducing the tilt of the chains. Integral membrane proteins appear to affect the acyl chain order to a remarkably small extent even at high weight fractions (Rice et al., 1979; Bloom, 1979; Davis et al., 1980). Although this problem is not yet fully explored, it is clear that these proteins do not mediate an extensive perturbation of the bilayer structure.

The effect of cholate on the bilayer contrasts in an interesting way to both the effect of cholesterol and the effect of proteins. As shown by the results of Figures 2 and 3 and Table I, there is a dramatic decrease of the order in the lamellar phase on addition of cholate. Indeed Rice et al. (1979), in a study of the cytochrome oxidase–phospholipid interaction, found that the presence of cholate, added in the bilayer reconstitution process, could give rise to artifacts dominating over the effect of the protein itself on the chain order.

To arrive at an interpretation of the molecular cause of the disordering induced by cholate, one can note not only that there is a general decrease in the order but also as seen from Table I that there is also a differential effect along the acyl chain in the deuterated POL. The tail of the alkyl chain is more affected than the positions close to the polar group. This has the effect that the order profile is changed and the plateau region disappears on addition of cholate.

In pure phospholipid bilayers one finds a characteristic plateau region, where the order parameter S is nearly constant for the first five to eight positions in the order parameter profile (Seelig, 1977). The same behavior is shown by lamellar soap–alcohol–water systems (Klason & Henriksson, 1981) where the cross-sectional area by alkyl chain is approximately 25 Å^2. In binary soap–water systems, where the area per chain is larger (30–40 Å^2) in the lamellar phase (Gallot & Skoulios, 1966), the order parameter is found to decrease monotonically along the chain (Davis & Jeffrey, 1977). Although the interpretation of order parameter changes along the alkyl chain in terms of molecular conformation can be accomplished in several different ways (Israelachvili et al., 1980), it is clear that one crucial parameter is the average cross-sectional area, a_c, available for the chains. Simple geometrical considerations show that the larger a_c the more disordered is the chain, with the largest effect at the tail.

The strong effect of cholate on the lecithin chain order can most directly be interpreted as due to an increase in the average cross-sectional area available to the alkyl chains in the lamellae. There are several conceivable molecular mechanisms that could lead to this effect. The most likely mechanism, in our opinion, is that a substantial fraction of the cholate molecules is placed flat on the bilayer surface. This is the simplest way of accounting for the two-sided hydrophilic–hydrophobic nature of the cholate molecule. A solubilized cholate dimer, which has been suggested to be the most important cholate species within a lamellar structure (Small, 1971), could hardly account for the observed effects on the lecithin chain order, particularly considering the well-documented effect of cholesterol and proteins on the chain order. A further consequence of a cholate molecule solubilized flat on the bilayer surface is that a local curvature will be induced. This could be the source of the reduced order in the polar head region observed by Rice et al. (1979) through ^{31}P NMR, by Persson et al. (1974) through ^{2}H NMR on the water, and by Lindblom et al. (1976a–c) through counterion NMR.

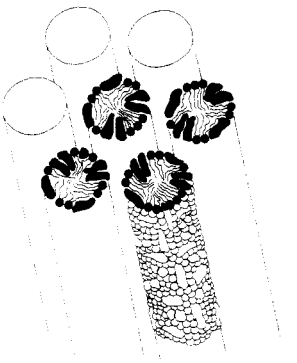

FIGURE 6: Suggested structure of the hexagonal phase in the lecithin–sodium cholate–water system, showing the continuous hydrocarbon regions of the rodlike aggregates.

Structure of the Hexagonal Phase. According to the original suggestion by Small et al. (1966), supported by Shankland (1977), the hexagonal phase consists of rodlike aggregates formed by stacking disklike micelles, and these (infinite) rods pack in a hexagonal fashion. This implies that there is a profound difference between the normal hexagonal phases found in binary (ionic or nonionic) amphiphile–water systems (Ekwall, 1975; Tiddy, 1980) and the one in the lecithin–cholate system. However, several of our experimental results are hard to reconcile with this suggested nonconventional structure of the hexagonal phase, while they are more consistent with the structure established for the soap–water systems, in which all the polar groups are at the surface of the rods and where the rods have a continuous hydrocarbon core (Figure 6). The observations that favor this conclusion are the following:

(i) The translational diffusion is equally rapid in the hexagonal and the lamellar phases (see Table II). If the rods in the hexagonal phase were formed by stacking disklike micelles, the translational motion of lecithin molecules would involve "flip-flop" and/or interaggregate exchange processes, which should give a considerably slower diffusion than in the lamellar phase. Again it should be noted that for liquid-crystalline phases in which the amphiphile forms closed aggregates, the amphiphile diffusion is at least 1 order of magnitude slower than that in corresponding phases with continuous amphiphile regions (Bull & Lindman, 1974; Lindblom & Wennerström, 1977).

(ii) The director in the hexagonal phase is along the rod axis (cf. Figure 4), and yet the sign of the order parameter is different in the hexagonal and lamellar phases (Figure 5). This is consistent with the conventional structure of the hexagonal phase (Cullis & deKruijff, 1979), while with a stacking of disklike micelles the phospholipid head groups have the same orientation relative to the director as in the lamellar phase.

(iii) The acyl chain order parameters in the hexagonal phase are considerably (at least a factor of 2) smaller than the order parameters in the pure phospholipid bilayer (cf. Table I; note that the order parameters for the hexagonal phase have been multiplied by 2). This is again consistent with what is found in soap systems (Klason & Henriksson, 1981). In the stacked micelle model, the chains have a similar orientation relative to the symmetry axis as in the pure bilayer, and the order parameters should be of the same magnitude.

Molecular Order in the Hexagonal Phase. Although the main features of the hexagonal phase are the same as those found in normal hexagonal phases of binary amphiphile–water

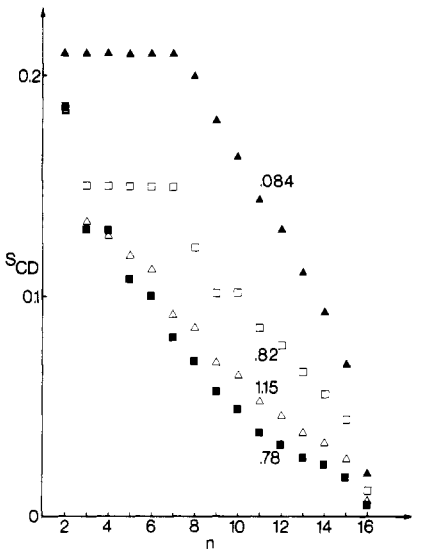

FIGURE 7: Deuterium order parameter profile for [$^2H_{31}$]palmitoyloleoyllecithin for two lamellar samples (filled symbols) with cholate:POL ratios of 0.084 and 0.78 and for two hexagonal samples (open symbols) with cholate:POL ratios of 0.82 and 1.15 (cf. Table I). Temperature 24 °C.

systems, there are clear differences. The most conspicuous one is that the average order is similar in the hexagonal phase and in the lamellar phase with the same cholate/lecithin ratio (cf. Figure 2b,c). In addition, the chain order decreases less rapidly along the chain in the hexagonal phase than in the corresponding lamellar phase. This is illustrated in Figure 7, which shows the order parameter profile for four different compositions. In carboxylate–alcohol–water systems, the trend is the reverse in that the transition to a lamellar phase on addition of alcohol leads to a decrease in the order gradient (Klason & Henriksson, 1981).

To account for these observations we propose that the infinite rods present in the hexagonal phase have a biaxial, elliptical, or rectangular cross section rather than a circular (or hexagonal) one, as in the usual picture of a normal hexagonal phase. The phospholipids are then able to partially adopt a bilayer-like structure, as indicated by the order parameter profiles, while the cholate molecules preferentially cover the shorter sides. The rods can be viewed as originating from an edge on association of the disklike mixed micelles minimizing the edge, rather than polar group–polar group association assumed in Small's model.

Biaxial structures have been observed in such soap–alcohol–water systems that tend to form nematic lyotropic phases (Charvolin et al., 1979; Yu & Saupe, 1980; Forrest & Reeves, 1981). The biaxiality in the hexagonal phase is, however, of a local nature, and on the NMR time scale the system is uniaxial due to the rapid rotation of the local symmetry axes. The deuterium NMR line shapes show that the asymmetry parameter is zero (within the experimental accuracy), which implies that the system has at least a 3-fold symmetry axes on the relevant time scale. There is thus no long-range correlation of the biaxial orientation. It seems necessary to invoke this rather complex idea to account simultaneously for the order parameter profile, the sign of the order parameter, the unaxiality, and the relation between the order in the lamellar and the hexagonal phases. The local biaxiality may also explain why the small-angle X-ray diffraction shows more diffuse reflections than found in the soap systems, and at some compositions two reflections are observed that are not, in any simple way, consistent with a hexagonal symmetry.

Relation between Molecular Interactions and Phase Behavior. On the basis of the rather detailed picture of the molecular organization developed in the previous sections, it is possible to obtain a qualitative understanding of the phase equilibria. In the binary lecithin–water system the lecithin bilayers are in equilibrium with a very dilute aqueous phase, and the amount of water incorporated into the lamellar phase is determined by an exponential "hydration" force (LeNeveu et al., 1977). The stability of the infinite lamellar structure toward dissolution is due to its two dimensionality (Israelachvili et al., 1976; Wennerström, 1979), and the stability relative to other liquid-crystalline phases is connected with the balance between head group cross-sectional area and alkyl chain volume (Tanford, 1973; Israelachvili et al., 1980). When cholate is added to the bilayer, the average size of the polar groups increases, and electrostatic interactions begin to operate between and within the bilayers. As a consequence the hexagonal phase becomes relatively more stable, and the interlamellar electrostatic repulsions lead to an incorporation of more water in the lamellar phase. The relative stability of the hexagonal and lamellar phases depends also on the water content, making the lamellar phase more favorable at low water contents. This effect is also found in binary carboxylic soap–water systems (Gallot & Skoulios, 1966), where it can be explained quantitatively in terms of the electrostatic interactions (Parsegian, 1966; Jönsson & Wennerström, 1981). The instability of the hexagonal phase toward dilution can be explained on the basis of rather general principles. For an aggregate that grows in one dimension, like a rod, the finite size is always the preferred one if aggregate–aggregate interactions can be neglected (Israelachvili et al., 1976; Wennerström, 1979). It is thus the unidimensionality of the basic structural unit that explains why the hexagonal phase decomposes into an isotropic micellar solution on addition of water.

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