brief communication

Effect of bile salts on monolayer curvature of a phosphatidylethanolamine/water model membrane system

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ABSTRACT A partial phase diagram of the ternary system dioleoylphosphatidylethanolamine (DOPE)/sodium cholate/water has been determined using ^{31}P Nuclear Magnetic Resonance (NMR) spectroscopy. In the absence of cholate, it is well known that the DOPE/water system forms a reversed hexagonal (H_{II}) phase. We have found that addition of even small amounts of cholate to the DOPE/water system leads to a transition to a *lamellar* (L_α) phase. At higher cholate concentrations, a *cubic* (*I*) phase (low water content) or a *micellar solution* (L_1) phase (high water content) is present. Thus, cholate molecules have a strong tendency to alter the lipid monolayer curvature. Increasing the concentration of cholate changes the curvature of DOPE from negative (H_{II} phase), through zero (L_α phase), and finally to a phase of positive curvature (micellar solution). This observation can be rationalized in terms of the molecular structure of cholate, which is amphipathic and has one hydrophobic and one hydrophilic side of the steroid ring system. The cholate molecules have a tendency to lie flat on the lipid aggregate surface, thereby increasing the effective interfacial area of the polar head groups, and altering the curvature free energy of the system.

INTRODUCTION

Bile salts are known to have a strong effect on the physicochemical properties of phospholipid aggregates. One of their major functions is to form micelles with lipids in the process of digestion and absorption of fats in the intestinal lumen (see Erlinger, 1987, for a recent review). Phospholipid/bile salt mixed micelles in turn can solubilize hydrophobic and amphiphilic molecules, and thereby increase the efficacy of the epithelial cells lining the small intestine to take up, for example, lysophospholipids, cholesterol, and fatty acids (Shiau, 1987). Recently, it was demonstrated (Shoemaker and Nichols, 1990) that bile salts and lysophospholipids form aggregates at concentrations that mimic the physiological conditions. It was proposed that these lysolipid/bile salt aggregates are involved in facilitation of lipid absorption in the intestine. Because solubilization of aggregates of different lipids may lead to changes in their size and structure, alterations are expected to occur due to changes in the phospholipid/bile salt ratio. Such variations in the relative concentrations of bile salts and phospholipids are known to occur in the gallbladder, and during dilution upon the ultimate emptying of the mixture into the duodenum. Obviously, a detailed knowledge of the structure of the aggregates formed by bile salts and lipids is desirable to understand the mechanisms behind fat digestion and absorption. Moreover, bile salts are also useful in various applications in biochemistry; for example, in extraction of membrane proteins (Helenius et al., 1979), or preparation of single-walled lipid vesicles (Schurtenberger et al., 1985). In medical treatments, naturally occurring bile acids such as ursodeoxycholic and chenodeoxycholic acids have been introduced as drugs to dissolve cholesterol gallstones (Danziger et al., 1972; Bachrach and Hoffmann, 1982; Roda et al., 1982), and bile salt/insulin mixtures have been utilized to facilitate the uptake of insulin through the skin.

Recently, a substantial amount of attention has focused on the polymorphism of assemblies of lipids formed by dispersal in water. This polymorphism has been described in terms of the average molecular shape (Israelachvili et al., 1976; Tanford, 1980) or curvature free energy of the lipid aggregates (Helfrich, 1973; Tate et al., 1991). Here we propose that the biological effect of cholate is associated with an effect on the monolayer curvature free energy of lipid aggregates. All previous studies have involved phosphatidylcholines which form primarily lamellar phases with zero net curvature in the absence of cholate. Therefore, to test the effect of bile salts on the curvature of lipid aggregates, we have studied aqueous mixtures of dioleoylphosphatidylethanolamine (DOPE), which forms nonlamellar phases, together with sodium cholate. The most striking observation from this investigation is that the addition of even very small amounts of sodium cholate to DOPE/water gives rise to a transition from a reversed hexagonal (H_{II}, *negative* curvature) phase to a lamellar phase (L_α, *zero*

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0006-3495/91/09/728/05 $2.00

curvature) and/or a micellar solution (L_1, *positive* curvature).

MATERIALS AND METHODS

Sodium cholate was purchased from Sigma Chemical Co. (St. Louis, MO) and dioleoylphosphatidylethanolamine (DOPE) from Avanti Polar Lipids (Alabaster, AL). The samples were prepared in small test tubes from lipids dried to constant weight in vacuum, water was added, and the tubes were immediately sealed. The samples were then mixed by centrifugation. Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra were acquired repeatedly from all samples during a period of one to two months. The experimental determination of phase diagrams using classical methods is usually very time consuming. However, use of NMR techniques for investigating phase equilibria has been shown to be very efficient (Ulmius et al., 1977). Convenient methods, based on ^{2}H and ^{31}P NMR spectroscopy, have been frequently utilized for such studies (Lindblom and Rilfors, 1989, and references therein), and in this work we have employed ^{31}P NMR. The samples were thermally equilibrated for about 1 h before the measurements were made. The ^{31}P NMR spectra were obtained with a General Electric (Fremont, CA) GN-500 spectrometer operating at 202 MHz. A phase-cycled Hahn echo sequence (Rance and Byrd, 1983) was used to acquire the data. Inverse gated, high-power proton decoupling was applied during the acquisition. The echo time was 40 μs, spectral width 40 kHz, pulse length 22 μs (90°), and relaxation delay 1 s. For a typical spectrum 500–2,000 scans were accumulated, and an exponential multiplication corresponding to 100 Hz line broadening in the frequency domain was applied before Fourier transformation.

RESULTS AND DISCUSSION

Although bile salts are surface active substances, their physicochemical properties differ appreciably from those of common amphiphiles, like soaps and lipids. Thus, bile salts aggregate in aqueous solution, but they do not form typical micelles with a well defined critical micelle concentration (Wennerström and Lindman, 1979; Mazer et al., 1979), nor have they been observed to form liquid-crystalline phases with water. The bile salt molecules consist of a rigid steroid fused ring system, which is roughly planar, in which one side is hydrophobic and the other one is hydrophilic. The latter property is due to the presence of up to three hydroxyl groups located on the same side of the ring system (as in sodium cholate). This unique structure of the molecule is the main reason for its atypical behavior.

The molecular organization of the lipid aggregates formed in the ternary system phosphatidylcholine/sodium cholate/water has been thoroughly studied in the liquid-crystalline phases (Ulmius et al., 1982), and in the micellar solution phase (Mazer et al., 1980; Lindblom et al., 1984; Schurtenberger et al., 1985; Müller, 1981; Hjelm et al., 1990; Nichols and Ozarowski, 1990). In this system, the lamellar phase (L_α) is present in the absence of sodium cholate. Addition of cholate leads to formation of the *normal* hexagonal (H_1), cubic (I), and micellar solution (L_1) phases (Ulmius et al., 1982). The normal hexagonal phase is formed by rod-like aggregates having a *continuous hydrocarbon core* with the lipid polar head groups at the surface (Ulmius et al., 1982), as found in the H_1 phases of soap/water systems (Ekwall, 1975). It has previously been shown using a variety of NMR techniques (^{1}H, ^{2}H, ^{31}P NMR spectroscopy, and pulsed magnetic field gradient NMR diffusion methods) that the cubic liquid-crystalline phase is composed of a bicontinuous structure (Lindblom et al., 1976; Lindblom and Rilfors, 1989). It was also shown (Ulmius et al., 1982) using ^{2}H NMR that for palmitoyloleoylphosphatidylcholine-d_{31} the acyl chain order profiles of the lamellar (L_α) or normal (H_1) hexagonal phases decrease substantially due to the presence of sodium cholate. In pure phospholipid bilayers, a characteristic plateau region in the order profile is generally found (Seelig and Seelig, 1974; Ulmius et al., 1982; Davis, 1983; Thurmond et al., 1990, 1991), and the order parameter is nearly constant for the first five to eight methylene carbons. It is clear that this order profile depends strongly on the average cross-sectional area available for the acyl chains (Ulmius et al., 1982; Salmon et al., 1987; Thurmond et al., 1991). From simple geometrical considerations, it is also obvious that the larger the cross-sectional area the more disordered the acyl chains. It was concluded (Ulmius et al., 1982) that a substantial fraction of the cholate molecules lies flat on the bilayer surface as a result of the two-sided, hydrophilic-hydrophobic nature of the cholate molecule. Moreover, the effect of cholate on the chain order parameters is opposite to that of cholesterol (another steroid), which is known to increase the order profile (Mantsch et al., 1977), with only minor influences on the average polar head group orientation (Brown and Seelig, 1978). Similar conclusions were drawn about the location of the cholate molecules on the rods in the normal hexagonal (H_1) phase (Ulmius et al., 1982), in agreement with the model suggested by Nichols and Ozarowski (1990).

Therefore, we reasoned that the major effect of sodium cholate is to alter the monolayer curvature of lipid aggregates. To test this hypothesis, we investigated the effect of cholate on the molecular organization of DOPE dispersions by the determination of phase equilibria. It is known from previous studies that DOPE in water forms the *reversed* hexagonal (H_{II}) phase over a wide range of concentrations (Rand et al., 1990), in which the monolayer curvature is negative. Addition of cholate should lead to formation of phases with increasingly positive monolayer curvature. Fig. 1 *a–d* shows some typical proton-decoupled ^{31}P NMR spectra for the various phases observed. The L_α phase gives rise to ^{31}P NMR spectra with a low-frequency peak and a high-

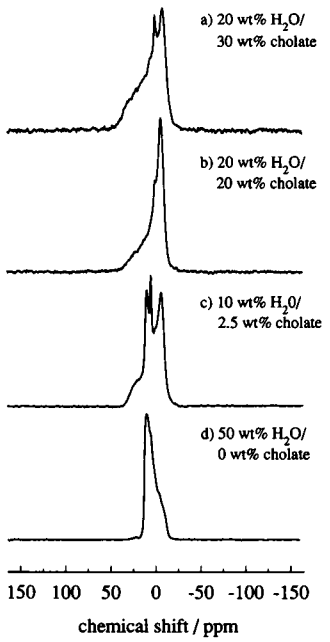

FIGURE 1 Proton-decoupled ^{31}P NMR spectra of dioleoylphosphatidylethanolamine (DOPE)/sodium cholate/water mixtures in the (*a*) $L_\alpha + L_1$; (*b*) L_α; (*c*) $L_\alpha + H_{II} + I$; and (*d*) H_{II} phases at 25°C. L_1 stands for the micellar solution; H_{II}, the reversed hexagonal phase; I, the cubic phase; and L_α, the lamellar phase, respectively. The results indicate that the presence of a rather small amount of cholate leads to a change in the phase formed by DOPE.

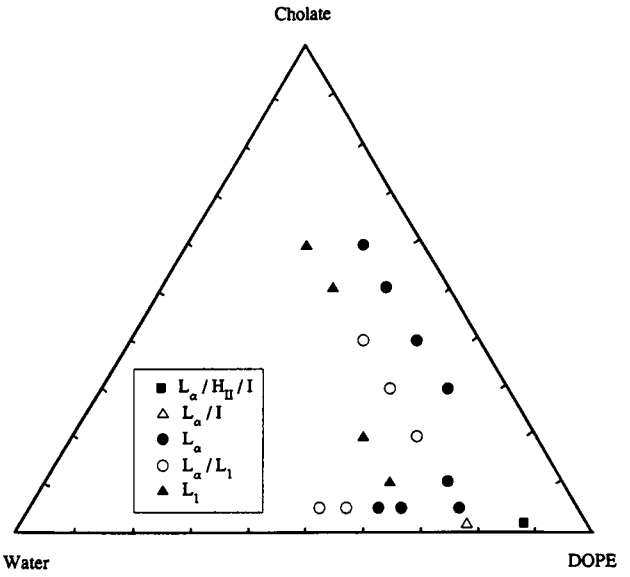

FIGURE 2 Partial phase diagram of the ternary system dioleoylphosphatidylethanolamine (DOPE)/sodium cholate/water at 25°C. The axes indicate wt% DOPE, water, and sodium cholate. The addition of only a small amount of cholate leads to a change in the phase formed by DOPE. The changes involve transitions from a reversed hexagonal, H_{II}, phase (negative curvature) to a lamellar, L_α, phase (zero curvature), and to a micellar solution, L_1 (positive curvature).

frequency shoulder, whereas micellar solutions (at high water contents) and the cubic liquid-crystalline phase (at low water contents) both show a narrow single peak (Lindblom and Rilfors, 1989). It seems improbable that a micellar solution would form at low water content between the L_α and H_{II} phases. Therefore, in analogy with the phase diagram obtained for the egg phosphatidylcholine/sodium cholate/water system, the mixture of phases present at low water content (Fig. 1 *c*) most probably includes a cubic liquid-crystalline phase (Ulmius et al., 1982). This suggestion is also strongly supported by the stiff texture and optically isotropic appearance of the sample. Thus, by following the ^{31}P NMR spectra as a function of concentration of the various components, it is possible to map out the phase diagram of the phospholipid system (cf Lindblom et al., 1986).

Fig. 2 shows a partial phase diagram for the ternary system DOPE/sodium cholate/water, determined from ^{31}P NMR spectra recorded at different concentrations of the three components. The ^{31}P NMR measurements were performed at 25°C. The water concentration was varied between 10 and 50 wt% and sodium cholate concentrations between 0 and 60 wt%. All the samples with DOPE and water (without cholate) gave ^{31}P NMR spectra exhibiting a high-frequency peak and a low-frequency shoulder, characteristic of hexagonal phase symmetry (cf McLaughlin et al., 1975; Seelig, 1978). This is in agreement with the well-known observation that DOPE forms an H_{II} phase with water (Gruner, 1985; Seddon, 1990). However, the addition of rather small amounts of sodium cholate (<2 wt%, cf Fig. 2) to a DOPE/water sample leads to the formation of an L_α phase as seen by ^{31}P NMR spectroscopy. Thus, the presence of even a small amount of sodium cholate leads to a drastic change in the phase behavior. It can be seen in Fig. 2 that the addition of sodium cholate to DOPE at a constant water content of 10 wt% gives rise to a transition from the H_{II} to the L_α phase. At 20 wt% water a cubic, I, phase, L_α phase, and finally a micellar solution, L_1, phase are observed with increasing wt% cholate. At high water and cholate concentrations only the micellar solution phase exists. It may perhaps be expected that a normal hexagonal phase, H_I, might also form in this system. We have, however, been unable to locate this phase even though an extensive number of samples were investigated for this purpose.

A qualitative understanding of the phase diagrams of lipid-water systems can be obtained by applying simple, current theoretical models for lipid aggregation. One of

these models is based on a consideration of the average molecular shape (Israelachvili et al., 1980; Tanford, 1980), i.e., the lipid molecules will adopt different geometrical average shapes depending on the lipid aggregate structure in which it is situated. Although such a simple approach is useful for qualitative considerations, it is difficult to use for quantitative calculations, mainly due to the complex behavior of the interfacial surface area of the lipid molecules in the aggregates. A model introduced by Helfrich (1973), therefore, has several advantages, where the lipid aggregate stability is governed by (*i*) the so called spontaneous curvature of the lipid monolayer and (*ii*) an elastic free energy of curvature (bending rigidity) (Helfrich, 1973; Gruner, 1985; Lindblom and Rilfors, 1990). On the other hand, the molecular details are not considered in such a thermodynamic model. Gruner and co-workers have shown that the free energy of curvature plays an important role in the formation of H_{II} phases, and that there also will be a nonzero energy for the packing of lipid molecules in the aggregates (Gruner, 1985, Tate et al., 1991). In particular, the acyl chains of the phospholipid molecules must stretch to fill the interstitial regions between the cylindrical aggregates in the H_{II} phase. The smaller the radius of the water cylinder, the smaller the hydrophobic interstices, making it possible for the lipid acyl chains to elongate to fill the volume (Gruner, 1985; Tate and Gruner, 1987; Tate et al., 1991; Lindblom and Rilfors, 1990, and references therein).

The phase behavior of the phospholipid/cholate mixtures investigated in this work can be explained conceptually in terms of these simple theories. Thus, DOPE with its comparatively small head group will adopt a wedgelike average shape, and forms an H_{II} phase with water. However, the uptake of water in this liquid crystalline phase is much less than the corresponding L_α phase of phosphatidylcholines, since larger lipid/water cylinders will create void volumes between the cylinders as discussed above. When cholate is added to the cylinders, the average size of the polar head groups at the interfacial surface of the aggregate increases, since the cholate molecules will tend to lie flat on the DOPE monolayer (Ulmius et al., 1982; Nichols and Ozarowski, 1990). As a result an L_α phase will form, and thus the effect of cholate is to increase the curvature of the DOPE monolayer. At even higher cholate concentrations, the curvature is further increased and eventually changes sign, leading to formation of a normal micellar solution phase. For the cubic phase structure, a subtle and critical balance of the free energy of the monolayer curvatures is prevailing (Tate et al., 1991). Generally the extension of the cubic phase is therefore limited to a narrow area in the phase diagram (Lindblom and Rilfors, 1989).

In summary, we have shown that cholate molecules have a very strong tendency to *increase* the curvature of membrane lipid aggregates. The effects of cholate may be due to an influence on the spontaneous curvature and/or the bending rigidity (curvature elastic modulus) of assemblies of membrane lipids. For DOPE, the curvature changes from *negative* (H_{II} phase), through *zero* (L_α phase), and finally, at high cholate concentrations, a phase of *positive* curvature (micellar solution) is obtained. This also explains the influences of cholate on phosphatidylcholines where the curvature goes from zero (L_α phase) to positive (normal hexagonal, H_I, and micellar solution). We propose that this intrinsic property of cholate could account for its high efficiency in solubilizing various membrane components.

This work was supported by grants GM41413, EY03754, and RR03529 from the United States National Institutes of Health and by the Swedish Natural Science Research Council.

Received for publication 7 March 1991 and in final form 22 May 1991.

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