Dimorphism in Bile Salt/Lecithin Mixed Micelles†

W. J. Claffey and R. T. Holzbach*

ABSTRACT: We investigated the dependence of certain properties of bile salt/lecithin mixed micelles on the molar ratio of the two components. UV absorption suggested that the micellar system is fundamentally altered on passing through a bile salt:lecithin molar ratio of 1.8:1. Differential scanning calorimetry confirmed this transition ratio; in addition, it indicated a bilayer arrangement of lecithin, in support of the mixed-disk model of micellar structure, but only for micelles having a molar ratio less than 1.8:1. For micelles having larger ratios, which are those of physiological interest, calorimetry was inconsistent with the mixed-disk model. These observations support the X-ray structure analysis of Müller [Müller, K. (1981) *Biochemistry* (preceding paper in this issue)].

The two major components of bile salts and lecithin, which form mixed micelles. Cholesterol in bile is made soluble by incorporation into these micelles insofar as other components of bile, which amount to less than 20% of the total solids, may be assumed to play no role in cholesterol solubilization. The structure of bile salt/lecithin micelles should be important in determining their ability to incorporate cholesterol.

There have been two recent investigations of the structure of these micelles. Quasi-elastic light scattering (QLS) was used by Mazer and collaborators (Mazer et al., 1977, 1979, 1980; Mazer, 1978). X-ray small-angle scattering (XSAS) was used by Müller (1981) in work described in the preceding paper in this issue. The results of these two investigations agree only partially.

Temperature and counterion concentration influence the structure of bile salt/lecithin micelles (Mazer, 1978), but here we consider only the physiological conditions of 37 °C and 0.15 M NaCl. Under these conditions, the structure is primarily determined by the bile salt:lecithin molar ratio (BS:L for short). It has been known for about a decade that micelle size increases dramatically as BS:L decreases, according to NMR (Small et al., 1969) and Rayleigh scattering (Shankland, 1970). The micelle model (Small, 1967) which was believed to account for these data is now thought to be incorrect on the basis of the new QLS and XSAS data. Mazer et al. (1977) devised the "mixed-disk" model to explain their QLS data. In this model, lecithin forms a cylindrical piece of bilayer surrounded by a ring of bile salt molecules oriented so that their hydrophilic parts interact with aqueous solvent; in addition, bile salt molecules are inserted into the bilayer in pairs. This model is shown as Figure 10 (top) in Müller (1981). Mazer et al. (1977) found impressive agreement between the micelle radius predicted by this mixed-disk model and the Stokes radius measured by QLS for BS:L ranging from about 0.8:1 to about 2:1. The XSAS analysis of Müller (1981) supports the mixed-disk model for this range of BS:L.

The disagreement between QLS and XSAS arises over the question of micelle structure for BS:L ~2 or larger. This is an important issue because assays of human gallbladder specimens show BS:L ratios that are almost always in this range (e.g., Carey & Small, 1978).

For all BS:L larger than about 2:1, the mixed-disk model does not fit the Stokes radii measured by QLS. The exact transition ratio at which the fit begins to be lost depends slightly on the bile-salt species and on the total lipid concentration, but it is never far from 2:1 except at total lipid concentrations below 1%. However, a good fit to the QLS data at all BS:L was achieved by adopting the following model (Mazer et al., 1977; Mazer, 1978): The mixed micelles take the mixed-disk structure at all BS:L. For BS:L larger than about 2:1, simple bile salt micelles coexist with the mixed micelles. Throughout this coexistence range, the mixed micelles retain an internal molar ratio equal to the molar ratio at the transition. This internal BS:L is about 2:1, although, as mentioned above, it may vary somewhat according to the bile salt species and the total lipid concentration. The coexistence concept is supported by QLS measurements of the polydispersity, which rises to a maximum within the presumed coexistence range.

Müller (1981) disagrees that the mixed disk is the correct micelle model for all BS:L. His analysis has led him to a new micelle model for BS:L >2, a centrosymmetric and nearly spherical particle, shown in Figure 10 (bottom) of this paper. He also finds no evidence for the degree of polydispersity predicted by the coexistence of simple and mixed micelles.

Thus, it is still uncertain how cholesterol is solubilized in the range of BS:L that is physiologically relevant. According to QLS, it is incorporated into both simple bile salt micelles and mixed micelles with a BS:L of about 2:1. According to XSAS, cholesterol is incorporated into a single population of centrosymmetric mixed micelles.

In this paper, we again take up the question of how the structure of the bile salt/lecithin micelle depends on the molar ratio of its components. Of particular interest is the issue of whether a transformation between two fundamentally different micelle structures takes place at BS:L ~2. We have used two techniques not previously applied to these systems: ultraviolet (UV) absorption and differential scanning calorimetry (DSC).

Materials and Methods

Materials. The conjugated bile salts sodium glycochenodeoxycholate (SGCDC), sodium taurochenodeoxycholate (STCDC), and sodium taurodeoxycholate (STDC) were A grade from Calbiochem. They were stored as methanol stock solutions of about 100 mg/mL. The exact concentrations of these solutions were determined by enzymatic assay (Palmer, 1969). L-α-Lecithin from egg yolks was obtained from Nutritional Biochemicals. It was purified chromatographically (Marsh & Holzbach, 1973) and stored as a methanol stock solution. Synthetic L-α-dipalmitoyllecithin was obtained in benzene solution from Grand Island Biological Co. The purity of the synthetic lecithin was indicated by its DSC behavior

† From the GI Research Unit, the Cleveland Clinic Foundation, Cleveland, Ohio 44106. *Received September 19, 1980.* Supported by National Institutes of Health Grant AM-17562.

0006-2960/81/0420-0415$01.00/0

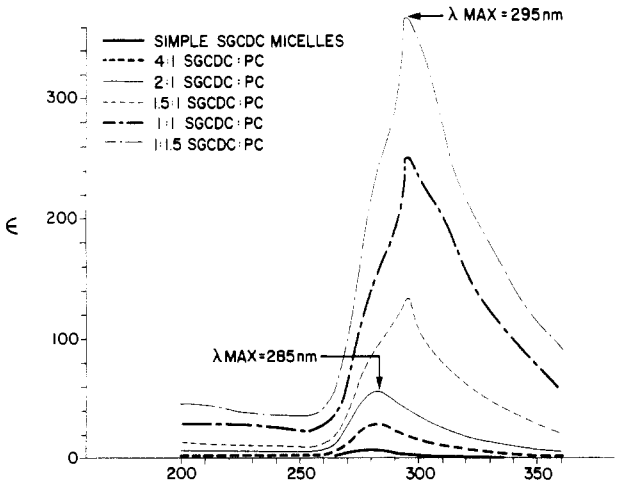

FIGURE 1: UV spectra at various sodium glychochenodeoxycholate: phosphatidylcholine (SGCDC:PC) molar ratios.

as will be further discussed below. Lecithin stock solutions were assayed by the method of Bartlett (1959).

Micellar Solutions. Mixtures of the stock solutions of bile salts and lecithin were first dried with a stream of nitrogen and then dried under vacuum over P_2O_5 for 72 h. A 0.15 M NaCl solution adjusted to pH 10 was then added to the dried mixture. Detailed descriptions of solution preparation have been previously given (Holzbach et al., 1973). The solutions for DSC were always made to contain 10% total lipids; but for UV the percent of total lipids was variable for reasons to be discussed below.

UV Spectroscopy. A Beckman Acta III double-beam instrument was generally used. A single-beam Gilford 240 instrument equipped for temperature control was used for a few experiments in which the temperature was varied.

Differential Scanning Calorimetry. This was done with a Perkin-Elmer DSC-2 instrument with a subambient cooling unit. The calorimeter was double calibrated against the melting transitions of water and of indium. About 10 μL of micellar solution was weighed into the sample pan, which was quickly sealed. An equal weight of water was sealed into a reference pan. The pans were placed in the calorimeter at 50 °C. Each sample could be cooled and heated several times, with reproducible results, if care was taken not to cool to below the freezing point of water. Heating and cooling rates of 10 °C/min were used.

Results

UV Spectroscopy. The spectra of SGCDC egg lecithin micelles for various BS:L are shown in Figure 1. The ordinate is the extinction coefficient per mole of SGCDC. As lecithin is added to the micelles, the absorption becomes successively greater and λ_{max} is shifted to longer wavelengths. There are two possible reasons for this. First, if lecithin is a stronger absorber than bile salt and has a larger λ_{max}, the spectra could merely represent the increasing enrichment of the solution by lecithin. Second, the changes in the spectra as lecithin is added could reflect modifications in the environment of the bile salt chromophores. The chromophores are probably the conjugating groups. One way of deciding between these two possibilities would be to obtain the spectrum of lecithin in 0.15 M saline and subtract it from the spectra of the mixed micelles. But here the difficulty arises that insoluble lecithin dispersions would be strong scatterers of radiation. A way around the problem is to record all the micelle spectra at constant lecithin concentration, varying the molar ratio in the micelles by

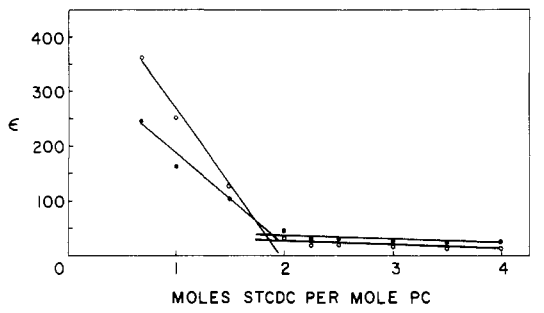

FIGURE 2: Extinction coefficient per mole of SGCDC as a function of molar ratio at 285 (closed circles) and 295 nm (open circles).

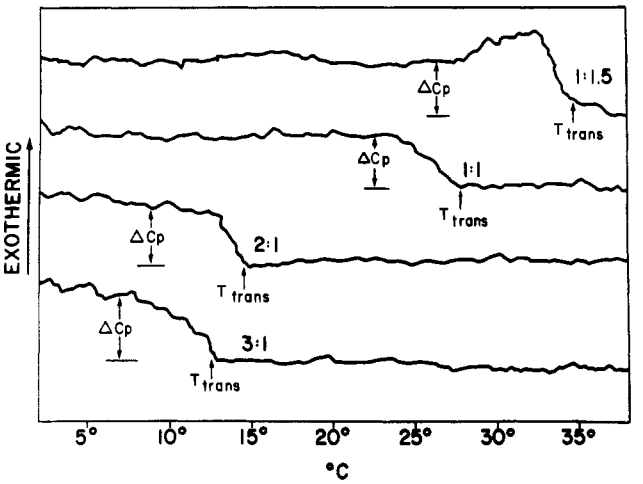

FIGURE 3: DSC scans obtained by cooling the selected sodium taurochenodeoxycholate:dipalmitoyllecithin (STCDC:DPL) molar ratios.

varying the concentration of the bile salt present. This had been done in recording the spectra of Figure 1, for which the lecithin concentration is 10 mg/mL in all cases and the bile salt concentration varies from 24 mg/mL for the 4:1 mixture to 4 mg/mL for the 1:1.5 mixture. Thus we can argue that differences in the spectra can only arise from differences in the environment of the bile salt chromophores.

The spectra of Figure 1 fall into two distinct groups. As lecithin is initially added to bile salt, the increase in absorption is fairly modest and λ_{max} = 285 nm. At lower BS:L ratios, the increase is far greater and λ_{max} = 295 nm. In Figure 2, we have plotted an extinction coefficient per mole of bile salt vs. composition for these two wavelengths. The data fall on straight lines intersecting at a molar ratio of 1.8:1.

Differential Scanning Calorimetry. Our initial DSC experiments were done with mixed micelles containing egg-yolk lecithin, but we failed to detect any thermotropic behavior over a range of 5 to 50 °C. This was not surprising since the transition between the gel and liquid-crystalline phases of egg-yolk lecithin is broad and close to 0 °C and can only be broadened and lowered when bile salts are mixed with lecithin.

We then studied mixtures of bile salts and synthetic L-α-dipalmitoyllecithin (DPL). We considered that the small acyl chain differences between DPL and egg-yolk lecithin would have little effect on the structure of the mixed micelles. Justification for this assumption is given below. DPL has a sharp, reversible gel to liquid-crystalline phase transition at 41–42 °C when the water content of the sample exceeds 20% by weight (Ladbrooke & Chapman, 1969; Cassel, 1973). We measured the transition temperature of our DPL samples as 42.1 °C, and this was taken to indicate a high degree of purity.

DSC scans for micellar solutions with 15 different STCDC:DPL molar ratios, all with 10% total lipids, were recorded,

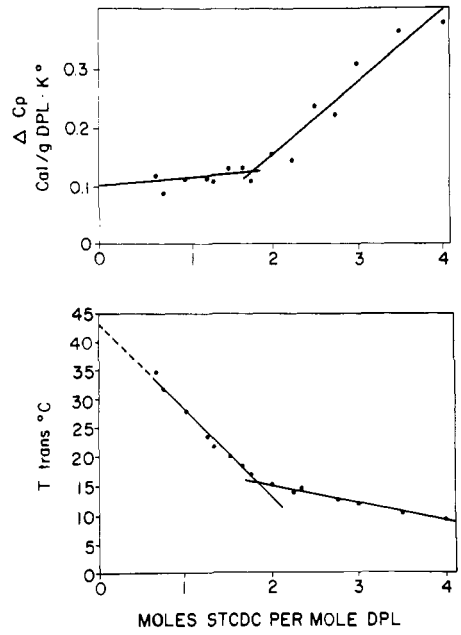

FIGURE 4: Gel to liquid-crystal transition temperatures (below) and heat capacity change per unit weight of DPL (above) as a function of STCDC:DPL.

and some of these are shown in Figure 3. Only exotherms obtained by cooling are presented, although we found that all transitions were reversible. In Figure 4 the transition temperature is plotted as a function of micelle composition. The change in heat capacity at the transition per unit mass of DPL is similarly plotted. Both graphs indicate that a transition between two different types of thermotropic behavior occurs at an STCDC:DPL of 1.8:1.

Discussion

UV Absorption. In obtaining the results shown in Figures 1 and 2, the lecithin concentration was held constant. Differences between the spectra at different BS:L must therefore be due to differing contributions from the bile salts. As bile salts are added to an initially lecithin-rich system, the absorption due to each bile salt molecule decreases sharply until a BS:L of 1.8:1 is reached. Both QLS and XSAS agree that this is the range over which the micelles take the mixed-disk form. In this structure, it appears that strong interactions between bile salt chromophores can take place, leading to hypochromism and increasing as the micelle size diminishes. At BS:L > 1.8:1, λ_{max} shifts from 295 to 285 nm, the absorption is weak throughout this BS:L range, and the addition of bile salts has only a moderate effect on the absorbance for each bile salt molecule.

Simple bile salt micelles are weak absorbers, as shown in Figure 1. Therefore the results for the mixed micelles are consistent with Mazer's view that at BS:L > 1.8, any added bile salts form weakly absorbing simple micelles which coexist with mixed disks having an internal BS:L of 1.8:1. But the results are also consistent with Müller's (1981) view that the micelles take a centrosymmetric structure for BS:L > 1.8:1, if the bile salts in this structure are weak absorbers with λ_{max} = 285 nm. Since Müller argues that his centrosymmetric structure represents only a small perturbation of the structure that he finds for simple micelles, it is reasonable that the bile salts should be weak absorbers in the centrosymmetric structure.

The UV analysis assumes that micelle structure type depends only on BS:L and is independent of total lipid concentration. This assumption is supported by Müller's work, which shows that lecithin-rich micelles take the mixed-disk form and bile-salt-rich micelles take the centrosymmetric form, for total lipid concentrations ranging from 10 to 100 mg/mL.

It was convenient to fit the extinction coefficient data in Figure 2 by straight lines. Actually, the data for lecithin-rich micelles may not be linear. But this does not change the essential point made by Figure 1, which is that the spectra for bile-salt-rich and lecithin-rich micelles are fundamentally different. Taking a linear approximation to the data in Figure 2 is merely a convenient way of estimating the transition ratio, which must certainly lie between 1.5:1 and 2:1 regardless of how the data is analyzed.

Thus the UV results agree with both QLS and XSAS in that the bile salt/lecithin system appears to be fundamentally altered on passing through a BS:L of about 1.8:1. But no definite choice can be made between the rather different views of micelle structure developed by QLS and XSAS.

Differential Scanning Calorimetry. The transition temperatures in Figure 4 fall linearly ($p < 0.001$) as the STCDC:DPL ratio increases to a value of 1.8:1. This line can be extrapolated to zero bile salt concentration; the intercept is 43.6 °C. This is quite close to the value measured for pure DPL, and we take this to indicate that up to a BS:L of 1.8:1 the lecithin in the micelle is arranged in the same bilayer form as in pure DPL (Levine et al., 1968). The phase transitions recorded for the mixed micelles are the same gel to liquid-crystal transitions seen in pure lecithin, but the transition temperatures are lowered in proportion to the amount of bile salt present. Added bile salt acts as an "impurity", lowering the transition temperature linearly. However, at BS:L > 1.8:1, the transition temperatures no longer fall on the same line, but on another line of different slope ($p < 0.005$). We conclude that for micelles in this range of compositions the lecithin is not in a bilayer but in some other arrangement.

Suppose that for BS:L > 1.8:1 the system contains mixed-disk micelles of internal BS:L = 1.8:1 coexisting with simple micelles. Then all of the transition temperatures in this range should be identical, and equal to the transition temperature for the 1.8:1 mixture. This is because the addition of bile salts as simple micelles can have no effect on the lecithin contained in the mixed micelles. But in Figure 4 the line for BS:L > 1.8:1 has a definite downward slope, which means that bile salts add to the system in a way that affects the transition temperature of the lecithin. Again with reference to Figure 4, if bile salt that is added beyond BS:L = 1.8:1 takes the form of simple micelles, this can have no effect on the heat capacity change at the transition per unit weight of lecithin. But, in fact, this quantity is strongly influenced by added bile salts for BS:L > 1.8:1.

In summary, the DSC data indicate a bilayer arrangement of lecithin for BS:L < 1.8:1, consistent with the mixed-disk model. But for micelles richer in bile salt, the DSC data indicate that the mixed-disk model cannot be correct. Lecithin assumes some structure other than a bilayer for BS:L > 1.8:1, and this structure must be one in which added bile salts can influence the thermotropic behavior of lecithin. This view is consistent with the centrosymmetric structure of Müller (1981).

Since the same transition ratio of 1.8:1 is obtained from both the UV and DSC data, substitution of DPL for egg lecithin or exchange of a taurine-conjugated for a glycine-conjugated bile salt seems not to affect the transition ratio.

Added in Proof

Since this work was completed and accepted, we have demonstrated by freeze–etch electron microscopy that a mi-

cellar structural dimorphism with sphere to disk transition compatible with the results of this and the preceding paper (Müller, 1981) is applicable to native human bile.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.
Carey, M. C., & Small, D. M. (1978) *J. Clin. Invest. 61*, 998.
Cassel, R. B. (1973) *Perkin-Elmer Technical Report.*
Holzbach, R. T., Marsh, M., Olszewski, M., & Holan, K. R. (1973) *J. Clin. Invest. 52*, 1467.
Ladbrooke, B. D., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 304.
Levine, Y. K., Bailey, A. I., & Wilkins, M. H. F. (1968) *Nature (London) 220*, 557.
Marsh, M. E., & Holzbach, R. T. (1973) *Clin. Chim. Acta 43*, 87.
Mazer, N. A. (1978) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.
Mazer, N. A., Kwasnick, R. F., Carey, M. C., & Benedek, G. B. (1977) *Micellization, Solubilization, Microemulsions, Proc. Int. Symp. 1*, 383.
Mazer, N. A., Carey, M. C., Kwasnick, R. F., & Benedek, G. B. (1979) in *Gallstones* (Fisher, M. M., Ed.) p 143, Plenum Press, New York.
Mazer, N. A., Benedek, G. B., & Carey, M. C. (1980) *Biochemistry 19*, 601-615.
Müller, K. (1981) *Biochemistry* (preceding paper in this issue).
Palmer, R. H. (1969) *Methods Enzymol. 15*, 280.
Shankland, W. (1970) *Chem. Phys. Lipids 4*, 109.
Small, D. M. (1967) *Gastroenterology 52*, 607.
Small, D. M., Penkett, S. A., & Chapman, D. (1969) *Biochim. Biophys. Acta 176*, 178.

Carbon-13 and Phosphorus-31 Nuclear Magnetic Resonance Studies on Interaction of Calcium with Phosphatidylserine†

Dennis L. Holwerda, Paul D. Ellis, and Roy E. Wuthier*

ABSTRACT: The interaction between Ca^{2+} and phosphatidylserine was studied by ^{13}C and ^{31}P NMR spectroscopy, by IR analysis, by binding constant measurements, and through use of space-filling molecular models. NMR measurements of various salt forms of the lipid were made in two types of organic solvents that allowed sufficient averaging of chemical shift anisotropy and dipolar couplings to yield high resolution spectra. ^{13}C resonances of the polar head-group carbons were broadened relative to those of the acyl chains. This was especially true in samples prepared at neutral pH where ionic interactions appeared to restrict molecular motion. In $CDCl_3$ the marked line broadening of the resonances of the polar head-group atoms in the Ca^{2+} form indicated the formation of large, slow tumbling micelles. In the amphipathic solvent the large reduction in line broadening indicated the presence of freely tumbling Ca-(phosphatidylserine)$_2$ dimeric complexes. The 2:1 binding stoichiometry and the low chemical activity of the Ca-phosphatidylserine complex support this view. Analysis of the chemical shifts of the various lipid atoms under the differing ionic environments indicates that Ca^{2+} enhanced the deprotonation of both the carboxyl and amino groups and stabilized the entire polar head group against the effects of changing pH. The marked upfield shift of the ^{31}P phosphate resonance in the Ca^{2+} form and its insensitivity to changing pH indicate strong coordination binding. IR data indicate direct involvement of the carboxyl group in Ca^{2+} binding, as evidenced by the appearance of a C=O stretching mode. Binding studies indicated that the phosphate group was the primary binding force but that the carboxyl group also contributes positively. The amino group appears to exert a repulsive effect, which is supported by the chemical shift data which indicate that Ca^{2+} enhances the deprotonation of the amino group. Molecular models indicate direct involvement of the carboxyl and phosphate oxygens and that the amino group must be deprotonated to participate.

Calcium has long been known to bind with phosphatidylserine (Nash & Tobias, 1964; Abramson et al., 1964; Hendrickson & Fullington, 1965), and such interaction is considered to be of physiological significance in a variety of biological processes. In addition to the well-known function of Ca^{2+} binding to phospholipids in blood clotting (Davie & Fujikawa, 1975) and in various soft-tissue membrane-associated processes, Ca^{2+} has been shown to be complexed with phosphatidylserine and other acidic phospholipids in mineralizing tissues [e.g., Wuthier (1968, 1971), Eisenberg et al. (1970), and Boskey & Posner (1976)]. Inorganic phosphate has been shown to enhance quantitatively the binding of Ca^{2+} to phosphatidylserine (Cotmore et al., 1971) leading to the formation of stable noncrystalline lipid-calcium-phosphate complexes. Subsequently, both Anghileri & Dermietzel (1973) and Boskey & Posner (1976) found significant amounts of such complexes in mineralizing tissues. Most recently, phosphatidylserine and complexes of it with calcium phosphate have been discovered in membrane-enclosed matrix vesicles associated with the initiation phase of mineral deposition in calcifying cartilage (Wuthier & Gore, 1977; Majeska et al., 1979). However, the characteristics of the affinity of phosphatidylserine for Ca^{2+} which permit concomitant binding of inorganic phosphate are not well understood.

While numerous studies have been conducted on the binding of Ca^{2+} with phosphatidylserine by using a wide variety of techniques, the details of the specific interactions between Ca^{2+} and the various atoms of the polar head group remain unclear. Recently, increased attention has been focused on the use of NMR spectroscopy in determining the nature of metal binding sites (Behr & Lehn, 1973; Hauser et al., 1976; Czarniecki & Thornton, 1977) and of solution structural features such as

† From the Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208. *Received June 25, 1980.* This research was supported by funds from U.S. Public Health Service Grant No. AM 18983, National Institute of Arthritis, Metabolism and Digestive Diseases.

0006-2960/81/0420-0418$01.00/0