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Coexistence of Simple and Mixed Bile Salt–Lecithin Micelles: An NMR Self-Diffusion Study†

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ABSTRACT: The aggregation behavior of bile salt and lecithin in aqueous solutions at 20 °C was studied from bile salt, lecithin, and aggregate self-diffusion coefficients obtained by means of a Fourier-transform NMR pulsed-gradient spin-echo technique. The results strongly support the coexistence of simple bile salt micelles and mixed bile salt–lecithin micelles under physiologic conditions.

In recent years, extensive investigations of the physical chemistry of model and native biliary lipid systems have been performed. In particular, the formation, size, and structure of bile salt (BS)[1]–lecithin (L) mixed micelles have been studied systematically by using various techniques such as quasi-elastic light scattering (QLS) (Mazer et al., 1980, 1984; Schurtenberger et al., 1984a,b), small-angle X-ray scattering (Müller, 1981), calorimetry (Claffey & Holzbach, 1981; Spink et al., 1982), or NMR (Stark & Roberts, 1984; Lindblom et al., 1984). Whereas the model for the structure of mixed micelles at high lecithin to bile salt molar ratios (L/BS) proposed by Mazer et al. (1980) has been widely accepted, a present controversy exists as to the precise nature of the aggregates formed at physiologic L/BS ratios (0.2–0.4) and high total lipid concentrations (C_{tot}). The model proposed by Mazer et al. (1980) suggests that simple bile salt micelles and small bile salt–lecithin mixed micelles of fixed composition coexist under these conditions. This coexistence of simple and mixed micelles was already proposed by Carey & Small (1978) and is consistent with recent cholesterol dissolution studies (Higuchi et al., 1981) and equilibrium dialysis data (Duane, 1977; Higuchi et al., 1984). However, on the basis of small-angle X-ray experiments, Müller (1981) proposed that only mixed micelles exist at physiologic L/BS ratios and that these aggregates have a structure different from those formed at higher L/BS ratios. This model was further supported by Claffey & Holzbach (1981) and Spink et al. (1982) on the basis of their calorimetry

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[1] Abbreviations: BS, bile salt; L, lecithin; QLS, quasi-elastic light scattering; L/BS, lecithin to bile salt molar ratio; C_{tot}, total lipid concentration; C_{BS}, bile salt concentration; C_L, lecithin concentration; FT-PGSE, Fourier-transform pulsed-gradient spin-echo; D_s, self-diffusion coefficient; D_c, collective diffusion coefficient; D_s^{BS}, self-diffusion coefficient of bile salt; D_s^L, self-diffusion coefficient of lecithin; $D_s^{Me_4Si}$, self-diffusion coefficient of Me_4Si; Me_4Si, tetramethylsilane; HMS, hexamethyldisiloxane; TC, taurocholate; TDC, taurodeoxycholate; C_{tot}^0, total lipid concentration of micellar stock solution; cmc, critical micelle concentration.

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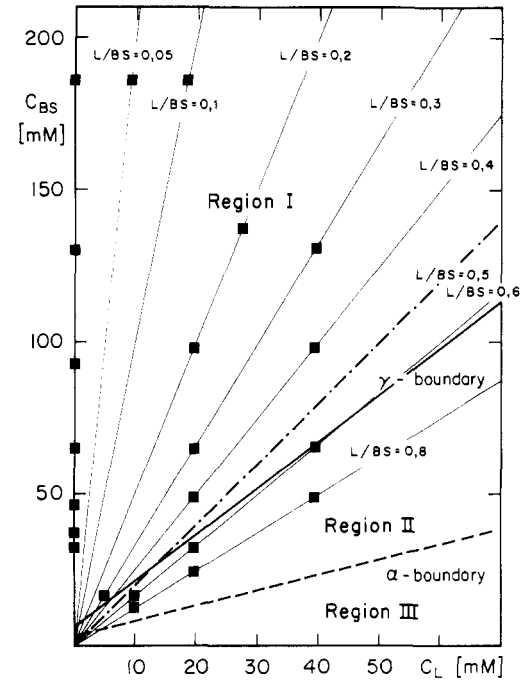

FIGURE 1: Phase diagram for the taurocholate–lecithin system in 0.15 M NaCl at 20 °C (Mazer et al., 1980) and investigated concentrations (■) of lecithin (C_L) and taurocholate (C_{BS}). Region I, coexistence of simple and mixed micelles; region II, mixed micelles only; region III, vesicles. Also shown are the coexistence boundary [γ boundary (—)] and the mixed micellar phase limit [α boundary (---)] separating region I from region II and region II from region III, respectively. Critical mixing ratio (–·–) for the transition "bile salt rich" mixed micelle–mixed disk micelle according to Müller (1981).

data. In view of the physiologic importance of understanding these model bile systems, we performed multicomponent self-diffusion studies by means of the Fourier-transform ^{1}H NMR pulsed-gradient spin-echo (FT-PGSE) technique in order to test the two current hypotheses. The FT-PGSE experiment (Stilbs et al., 1980; Stilbs & Moseley, 1980) has been shown to be a very powerful method for obtaining self-association and structural data in microemulsion or micellar systems (Lindman et al., 1981, 1984; Lindman, 1984) or for solubilization studies (Stilbs, 1982). With this technique, it is possible to simultaneously determine the self-diffusion coefficients (D_s) of all constituent molecules of complex multicomponent solutions. The NMR experiment permits one to distinguish between the two hypotheses for bile salt–lecithin solutions, since the values for the self-diffusion coefficients of lecithin (D_s^L) and bile salt (D_s^{BS}) should indicate whether there is only one micellar species or whether one molecular species (lecithin) is present in mixed micelles only, whereas the other (bile salt) is partitioned between coexisting simple and mixed micelles. Therefore, we have measured D_s^L, D_s^{BS}, and the self-diffusion coefficient of a hydrophobic marker molecule at various L/BS ratios and total concentrations (see Figure 1).

Materials and Methods

Materials. Egg yolk lecithin was obtained from Lipid Products [South Nutfield, Surrey, U.K. (grade 1)] and the sodium salt of taurocholic acid (TC) from Calbiochem. The bile salt was dissolved in ethanol, filtered, and recrystallized. D_2O (99.8%) was obtained from Norsk Hydro. Tetramethylsilane (Me_4Si, Merck, 99.7%) and hexamethyldisiloxane (HMS, Ega-Chemie, West Germany, 98%) were used as hydrophobic marker substances in order to measure a mean micellar self-diffusion coefficient (Stilbs, 1982; Carlfors & Stilbs, 1984). All other reagents used were of analytical grade. Solutions with different L/BS ratios were prepared by coprecipitation. After an appropriate amount of each lipid was dissolved in ethanol, the mixture was dried in vacuo until the dry weight was constant. D_2O containing 0.15 M NaCl was then added to obtain the desired final total lipid concentration, C_{tot}^0, of the stock solution. Solutions with lower total lipid concentration, C_{tot}, were then prepared from the stock solution by diltuion with D_2O (0.15 M NaCl). Each sample was flushed with N_2, sealed, and equilibrated at room temperature for 24 h. The mixtures studied are shown in Figure 1.

Methods. The diffusion studies were performed on two different spectrometers. The Fourier-transform ^{1}H PGSE measurements were made on a Jeol FX-60 spectrometer at a temperature of 20 ± 1.0 °C; the spectrometer modification was kindly made by Professor Peter Stilbs according to published principles (Stilbs & Moseley, 1980; Stilbs, 1982). The basic PGSE NMR experiment generates a spin-echo by a 90°–180° pulse sequence, with a magnetic field gradient G applied during a time δ in the form of two pulses separated by a time Δ, one before and one after the 180° pulse. Fourier transformation of the digitized second half of the echo separates the contributions to the echo from the different signals in the spectra. Individual signal amplitudes follow relations of the form (Stilbs, 1982)

$$A_i = \text{const}_i \exp[-\gamma^2 D_{s,i} G^2 \delta^2 (\Delta - \delta/3)] \quad (1)$$

where γ is the gyromagnetic ratio and $D_{s,i}$ is the self-diffusion coefficient of the molecule to which nuclei i belong. The constant in (const) eq 1 is proportional to $\exp(-2\Delta/T_{2,i})$, where $T_{2,i}$ is the transverse relaxation time for nuclei i. In a typical FT-PGSE experiment, signal amplitudes were determined at constant Δ and G values for a series of 10 δ values in the region between 3 and 99 ms. The measured $A_i(\delta)$ values were then fitted with a nonlinear least-squares fitting program to eq 1. At low concentrations and for pure bile salt solutions (due to their low T_2 values), signal averaging up to 100 transients per δ value was necessary. To obtain absolute values for the self-diffusion coefficients, the field gradient was calibrated from measurements on a reference D_2O sample. Except for the lower field applied here, the experimental details and the data analysis procedure were essentially as described by Stilbs (1982). Part of the samples were also measured with a Bruker 322-s pulsed NMR spectrometer using ^{1}H NMR at 60 MHz. The δ values varied between 0.4 and 4 ms, and the temperature was 20.0 ± 0.5 °C. In this PGSE experiment, proton contamination of the heavy water interferes with the measurements of lipid diffusion.[2] However, the water diffusion is fast and gives only a negligible contribution to the signal at longer field gradient pulses (larger δ values). To obtain absolute values of D_s, the field gradient was determined from measurements on a reference glycerol sample. The experimental details for this spectrometer have been described previously [Lindblom et al., 1981; cf. also Nilsson et al. (1983)]. The results from measurements with the two different experimental approaches on identical samples agreed within experimental error, thus confirming both experiment and data analysis.

Error limits for the diffusion coefficients were calculated by using a Monte Carlo simulated error analysis described by Stilbs & Moseley (1978) and correspond to 80% confidence intervals. The resulting error limits for a single measurement

[2] Since the spin echo is not Fourier transformed, the analyzed signal decay results from the superposition of the contribution from the different molecules.

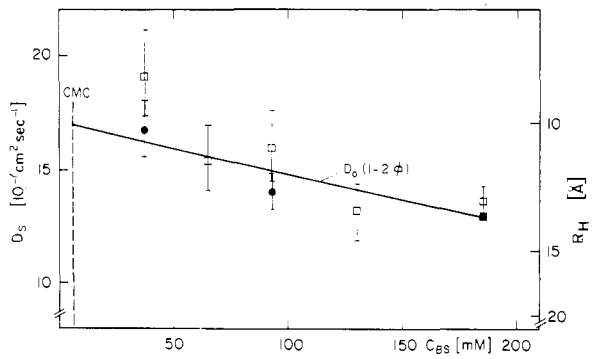

FIGURE 2: Self-diffusion coefficient (and corresponding hydrodynamic radius) of taurocholate, D_s^{BS} (□), and Me_4Si, $D_s^{Me_4Si}$ (●), as a function of the taurocholate concentration, C_{BS}. Also shown is the result of a measurement with the Bruker spectrometer (■) and the theoretical dependence of D_s upon the concentration (—).

were less than 5% for D_s^L and $D_s^{Me_4Si}$ and less than 10% for D_s^{BS}. The results shown in Figures 2–5 are average values from at least three different measurements on different samples, and the error limits given in these figures correspond to the standard deviation.

Results

Simple Bile Salt Systems. Self-diffusion coefficients of bile salt molecules at different concentrations in D_2O (0.15 M NaCl) at 20 °C are shown in Figure 2. Since the monomers contribute substantially to the measured total D_s at low concentrations (close to the cmc), a hydrophobic marker molecule has been used to measure the micellar diffusion coefficient. To ascertain that the addition of Me_4Si or HMS did not change the aggregation behavior, D_s^{BS} (and in case of mixed micellar systems, D_s^L) was measured before and after the addition of the marker substance. No measurable changes were observed. The results for $D_s^{Me_4Si}$ are also included in Figure 2. The difference between D_s^{BS} and $D_s^{Me_4Si}$ permits a deduction of the monomer concentration, C_{BS}^{mon}, by using eq 2 (Lindman et al., 1982; Lindman, 1984) where $D_s^{BS,mon}$ and

$$C_{BS}D_s^{BS} = C_{BS}^{mon}D_s^{BS,mon} + (C_{BS} - C_{BS}^{mon})D_s^{BS,mic} \quad (2)$$

$D_s^{BS,mic}$ are the self-diffusion coefficients of bile salt monomers and micelles, respectively. This leads to a value for C_{BS}^{mon} of 4.7 mM at C_{BS} = 37.2 mM, which is in good agreement with the cmc values[3] from the literature (Small, 1971; Roda et al., 1983).

The micellar self-diffusion coefficient decreases with increasing concentration (Figure 2). As will be shown later, this is due to the influence of interparticle interactions on the self-diffusion coefficient and is in quantitative agreement with recent theoretical work (Lekkerkerker & Dhont, 1984; van Megen & Snook, 1984). An extrapolation of $D_s^{BS,mic}$ to the cmc yields a value of 17.0×10^{-7} cm^2/s. The calculation of the hydrodynamic radius, R_H, using the Stokes–Einstein relation yields R_H = 10 Å, in close agreement with previous light-scattering results (Mazer et al., 1979).

Mixed Bile Salt–Lecithin Systems. The results from the measurements of D_s^{BS} and D_s^L at various L/BS ratios and total concentrations (see Figure 1) are summarized in Figure 3. It

[3] It is noted that bile salt self-association is gradual and of low cooperativity and the assignment of a cmc has been criticized (Mukerjee & Cardinal, 1976). Self-diffusion studies demonstrate a much lower cooperativity than for typical surfactants [see Lindman (1984) and references cited therein].

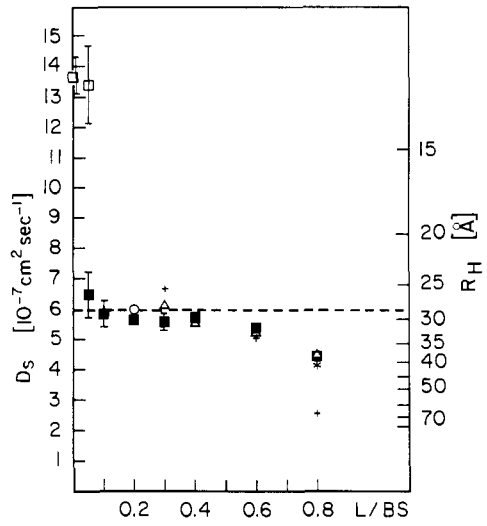

FIGURE 3: Self-diffusion coefficient and corresponding hydrodynamic radius of taurocholate and lecithin as a function of the L/BS ratio for different concentrations. D_s^{BS}: (□) dilution 1. D_s^L: (■) dilution 1; (○) dilution 1.4; (△) dilution 2; (*) dilution 4; (+) dilution 8. A dilution of 1 corresponds to the highest concentration, C_{tot}^0, indicated in Figure 1 for the respective L/BS ratio (L/BS = 0.05, C_{tot}^0 = 107 mg/mL; L/BS = 0.1, C_{tot}^0 = 113.6 mg/mL; L/BS = 0.2, C_{tot}^0 = 94.6 mg/mL; L/BS = 0.3, C_{tot}^0 = 100 mg/mL; L/BS = 0.4, C_{tot}^0 = 82.3 mg/mL; L/BS = 0.5, C_{tot}^0 = 71.7 mg/mL; L/BS = 0.6, C_{tot}^0 = 64.7 mg/mL; L/BS = 0.8, C_{tot}^0 = 55.9 mg/mL); a higher dilution corresponds to a concentration $C_{tot} = C_{tot}^0$/dilution.

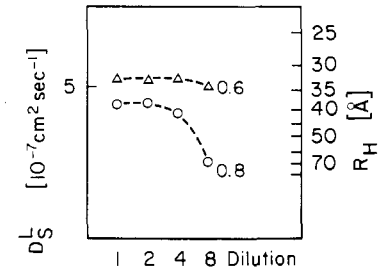

FIGURE 4: Lecithin self-diffusion coefficient, D_s^L (and corresponding hydrodynamic radius), as a function of dilution for L/BS = 0.6 [(△) C_{tot}^0 = 64.7 mg/mL] and L/BS = 0.8 [(○) C_{tot}^0 = 55.9 mg/mL].

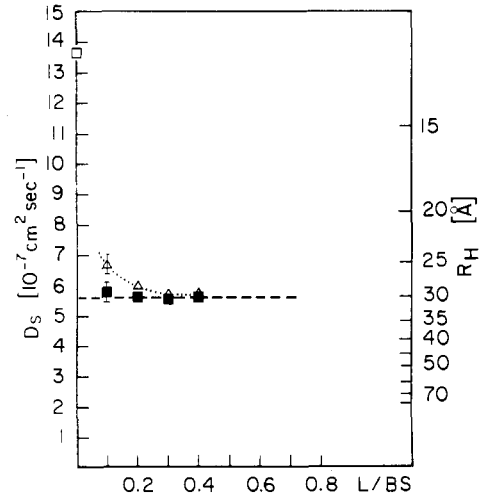

FIGURE 5: Self-diffusion coefficient (and corresponding hydrodynamic radius) for lecithin (■), Me_4Si (△), and TC (□) as a function of the L/BS ratio.

was only possible to measure D_s^{BS} at L/BS = 0.05 and in pure bile salt solutions due to the very low amplitude of the bile salt signals.[4] Whereas D_s^{BS} at L/BS = 0.05 (C_{tot} = 107

mg/mL) has a value of 13.4×10^{-7} cm^2/s, close to D_s^{BS} in pure bile salt systems at comparable C_{BS} values, D_s^L is substantially smaller. Moreover, D_s^L is constant within experimental error at all concentrations and mixing ratios in the controversial region of the phase diagram. The measurements yield a mean value for D_s^L of $(6 \pm 0.3) \times 10^{-7}$ cm^2/s or a hydrodynamic radius of 28 Å for the mixed micelles. Only at high L/BS ratios, close to the mixed micellar phase limit (Figure 4), we observe a concentration dependence of D_s^L, in agreement with previous light-scattering results (Schurtenberger et al., 1984a,b). The measurements with Me_4Si as a marker molecule are shown in Figure 5. Whereas at high L/BS ratios $D_s^{Me_4Si}$ is equal to D_s^L, the Me_4Si self-diffusion coefficient increases with decreasing L/BS ratio. The value for $D_s^{Me_4Si} - D_s^L$ increases from $(0.05 \pm 0.05) \times 10^{-7}$ cm^2/s at L/BS = 0.4 to $(0.88 \pm 0.08) \times 10^{-7}$ cm^2/s at L/BS = 0.1.

Discussion

Simple Bile Salt Systems. A strong argument for the existence of a different type of mixed micelles in solutions at low L/BS ratios emerged from the results of the small-angle X-ray scattering experiment, which showed only a small and almost linear increase of the micellar radius with increasing L/BS ratio from 28 Å for simple bile salt micelles to 32 Å for mixed micelles at L/BS = 0.5 (Müller, 1981). These results were interpreted in terms of a gradual uptake of lecithin in spherical bile salt micelles up to a critical mixing ratio, where disk-shaped mixed micelles form. However, a radius of 28 Å for simple bile salt micelles is in clear disagreement with previous publications which reported values of 10–12 Å for TC and 15–20 Å for TDC micelles on the basis of a number of different techniques [for QLS studies, see Mazer et al. (1979) and Schurtenberger et al. (1983); for ultracentrifugation techniques, see Small (1968) and Vitello (1971); for tracer diffusion, see Lindheimer et al. (1981)]. The QLS results in particular have been questioned because of the influence of intermicellar interaction effects and the resulting difficulties in the data analysis and interpretation. However, as far as the first-order concentration dependence of the collective diffusion coefficient D_c (measured in the QLS experiment) is concerned, there is now agreement in the expressions describing this effect (Batchelor, 1976; Felderhof, 1978; Kops-Werkhoven & Fijnaut, 1981). Nevertheless, the NMR PGSE experiment permits a further independent determination of micellar size in pure bile salt solutions. The results shown in Figure 2 strongly support a hydrodynamic radius of 10 Å for simple TC micelles in 0.15 M NaCl and are in clear disagreement with the X-ray results (Müller, 1981). It is interesting to compare the concentration dependence of the measured D_s with recent theoretical calculations of D_s of interacting particles (Lekkerkerker & Dhont, 1984; van Megen & Snook, 1984; Snook et al., 1983). In the case of hard spheres with no hydrodynamic interactions, the calculations predict the following relation for the self-diffusion coefficient (Lekkerkerker & Dhont, 1984):

$$D_s = D_0(1 - 2\phi) \qquad (3)$$

[4] The bile salt peaks have an extremely low amplitude due to the small T_2 values of the bile salt protons. The only echo signal with reasonable strength for accurate measurements comes from the CH_2 group at position 26 [for a ^{1}H spectrum with peak assignment, see, for example, Stark et al. (1984)]. However, this peak is close to the strong $(CH_3)_3$ peak of the lecithin molecule, and it was thus impossible to deduce D_s^{BS} with reasonable accuracy at high L/BS ratios, since the lecithin peak completely dominates.

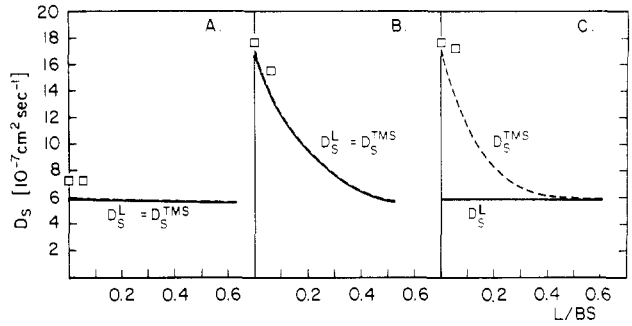

FIGURE 6: Theoretical dependence of the self-diffusion coefficients of bile salt (□), lecithin (—), and Me_4Si (TMS in figure) (---) upon the L/BS ratio for the three different models discussed in the text. (A) No coexistence, R = 28 Å for simple micelles; (B) no coexistence, R = 10 Å for simple micelles; (C) coexistence model (Mazer et al., 1980).

where D_0 is the diffusion coefficient in the absence of interactions and ϕ the volume fraction of colloidal particles. Brownian dynamics simulations which included hydrodynamic and electrostatic interactions have supported this result for charged spheres at volume fractions below 10% and moderate ionic strength (van Megen & Snook, 1984). As shown in Figure 2, the results from the FT-PGSE experiment are in quantitative agreement with the theory. Equation 3 should be compared with the concentration dependence of the collective diffusion coefficient of hard spheres (Batchelor, 1976):

$$D_c = D_0(1 + 1.45\phi) \qquad (4)$$

From eq 3 and 4, we see that for hard spheres QLS provides an *upper limit*, whereas FT-PGSE yields a *lower limit* of D_0, a prediction which can be verified by comparing the results of Mazer et al. (1979) with Figure 2. Therefore one cannot attribute the discrepancy between the X-ray results and the other measurements to intermicellar interactions.

Mixed Bile Salt–Lecithin. We try now to interpret the results from the FT-PGSE experiment on the basis of three different models summarized in Figure 6. If we assume that there is no coexistence between simple and mixed micelles and that the micellar size decreases with decreasing L/BS ratio from 32 Å at the critical mixing ratio to 28 Å at L/BS = 0.0 (Müller, 1981), we expect to see an almost constant D_s^L (Figure 6A). D_s^{BS} should be slightly larger due to the monomer contribution. Since there is only one micellar species acting as a carrier for Me_4Si, $D_s^{Me_4Si}$ should be equal to D_s^L. However, such a model is in clear disagreement with Figures 2, 3, and 5. In Figure 6B, we present the expected values for D_s^L and $D_s^{Me_4Si}$ based on the assumption of a radius of 10 Å for the simple TC micelles, a continuous uptake of lecithin with increasing L/BS, no coexistence of simple and mixed micelles, and a consequent micellar growth from 10 Å to R = 32 Å at the critical mixing ratio. This model can be ruled out from Figures 3 and 5. Finally, Figure 6C shows the expected changes of D_s^L and $D_s^{Me_4Si}$ with changing L/BS on the basis of the coexistence model of Mazer et al. (1980). At concentrations above the coexistence limit (γ line, Figure 1), simple TC micelles (R_H = 10 Å) are supposed to coexist with minimum-size mixed micelles (R_H = 30 Å) of constant composition. The hydrophobic marker Me_4Si should be partitioned between simple and mixed micelles. The population of simple micelles is expected to decrease with increasing L/BS at constant C_L. $D_s^{Me_4Si}$ should thus decrease from the value in pure bile salt systems to the value of D_s^L at the γ line, whereas D_s^L should be constant over the entire composition range. As can be seen from Figures 2, 3, and 5, the results of the NMR diffusion experiment are consistent with the coexistence model

but in clear disagreement with the idea of a structural dimorphism. Part of the controversy is probably due to the fact that some of the authors were not aware of the importance of phase diagrams. Claffey & Holzbach (1981) and Spink et al. (1982) interpreted their calorimetry results only in terms of the L/BS ratio. However, the aggregation behavior is determined not only by the mixing ratio but also by the actual values of C_L and C_{BS}. It has been shown that even at low L/BS ratios (0.1–0.3) one can observe a strong concentration dependence of the micellar size and a spontaneous micelle to vesicle transition at concentrations below the mixed micellar phase limit (Schurtenberger et al., 1984a,b). An examination of the concentrations and temperatures used in these calorimetry studies suggests that part of the samples were actually in the vesicle region of the phase diagram. The authors probably observed the micelle to vesicle transition (or the reversed process), caused by the temperature changes in the calorimetry experiment, superposed on the lipid phase transition.

Conclusions

The NMR self-diffusion studies of solutions of bile salt and lecithin strongly support the model of coexistence of simple and mixed micelles in the physiologically important concentration range as proposed by Mazer et al. (1980). The results not only are of physiological relevance but also should be seen in the context of a recent theoretical model for multicomponent micelles (Stecker & Benedek, 1984). In particular, the fact that the mixed micelles seem to reach a minimum size in the coexistence region independent of concentration and L/BS ratio is of theoretical interest. This study has also demonstrated the usefulness of the NMR FT-PGSE technique as a tool for investigating mixed amphiphilic systems.

Acknowledgments

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Registry No. TC, 81-24-3.

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