Morrison, W. R., & Smith, L. M. (1964) *J. Lipid Res. 5*, 600–608.
Paltauf, F. (1972) *FEBS Lett. 20*, 79–82.
Paltauf, F. (1976) *Chem. Phys. Lipids 17*, 148–154.
Paltauf, F. (1978) *Eur. J. Biochem. 85*, 263–270.
Paltauf, F., & Holasek, A. (1973) *J. Biol. Chem. 248*, 1609–1615.
Paltauf, F., Prough, R. A., Masters, B. S. S., & Johnston, J. M. (1974) *J. Biol. Chem. 249*, 2661–2662.
Privett, O. S., & Nutter, L. J. (1967) *Lipids 2*, 149–154.
Roberts, M. F., Deems, R. A., Mincey, T. C., & Dennis, E. A. (1977) *J. Biol. Chem. 252*, 2405–2411.
Rouser, G., Siakotos, A. N., & Fleischer, S. (1966) *Lipids 1*, 85–86.
Schmid, H. H. O., & Mangold, H. K. (1966) *Biochim. Biophys. Acta 125*, 182–184.
Snyder, F. (1970a) *Prog. Thin-Layer Chromatogr. Relat. Methods 1*, 52–73.
Snyder, F. (1970b) in *The Current Status of Liquid Scintillation Counting* (Bransome, E. D., Jr., Ed.) pp 248–256, Grune & Stratton, New York.
Snyder, F., Blank, M. L., & Wykle, R. L. (1971) *J. Biol. Chem. 246*, 3639–3645.
Tjiong, H. B., Gunawan, J., & Debuch, H. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 707–712.
Van Golde, L. M. G., & Van den Bergh, S. G. (1977) in *Lipid Metabolism in Mammals* (Snyder, F., Ed.) Vol. 1, pp 1–33, Plenum Press, New York.
Waku, K., & Lands, W. E. M. (1968) *J. Biol. Chem. 243*, 2654–2659.
Waku, K., & Nakazawa, Y. (1972) *J. Biochem. (Tokyo) 72*, 495–497.
Waku, K., & Nakazawa, Y. (1977) *J. Biochem. (Tokyo) 83*, 1779–1784.
Wood, R., & Snyder, F. (1969) *Arch. Biochem. Biophys. 131*, 478–494.
Wykle, R. L., & Schremmer, J. M. (1974) *J. Biol. Chem. 249*, 1742–1746.
Wykle, R. L., & Lockmiller, J. M. S. (1975) *Biochim. Biophys. Acta 380*, 291–298.
Wykle, R. L., Blank, M. L., & Snyder, F. (1970) *FEBS Lett. 12*, 57–60.
Wykle, R. L., Blank, M. L., Malone, B., & Snyder, F. (1972) *J. Biol. Chem. 247*, 5442–5447.
Wykle, R. L., Blank, M. L., & Snyder, F. (1973) *Biochim. Biophys. Acta 326*, 26–33.

Structural and Kinetic Studies on the Solubilization of Lecithin by Sodium Deoxycholate†

Dov Lichtenberg,* Yael Zilberman, Peniel Greenzaid,‡ and Shmuel Zamir

ABSTRACT: Mixed dispersions of egg phosphatidylcholine (PC) and the bile salt sodium deoxycholate (DOC) were prepared by various methods, and their turbidities and proton magnetic resonance spectra were studied as a function of time. The spectra of dispersions prepared by dissolving both components in a common organic solvent and replacing the organic solvent by water did not change with time, indicating that the mixed aggregates formed represent "a state of equilibrium". In the ^{1}H NMR spectra of these mixed aggregates, only signals from small mixed micellar structures were narrow enough to be observed. The dependence of the NMR line widths on the molar ratio of DOC to PC (R) is interpreted in terms of a model for the PC–DOC mixed micelles, according to which PC is arranged as a curved bilayer, the curvature of which increases with increasing R. Upon mixing PC with aqueous solutions of DOC, we found that the mixed aggregates formed are slowly reorganized and ultimately reach the same state of equilibrium. This reorganization was found to be a pseudo-first-order process, the rate constant of which depends linearly upon the detergent concentration. This process involves saturation of the outer bilayers of the multilamellar PC by detergent, followed by transformation of these bilayers into mixed micelles. It is concluded that the solubilization occurs through consecutive "peeling off" of lecithin bilayers.

Solubilization of phospholipids by detergents is a widely used process by which bilayered (or multilamellar) structures of phospholipids are broken down to smaller mixed aggregates of phospholipids and detergents (Helenius & Simons, 1975). The mechanism of this process is not clear and only sparce information is available regarding the exact physical properties of the mixed aggregates and their dependence on such factors as concentration, method of mixing, and time.

Mixed dispersions of phospholipids and detergents can be prepared by dissolving both components in organic solvents, evaporating the mixed solutions to dryness, and dispersing the residue in water. However, this procedure [method B, following Yedgar et al. (1974b)] is not the most common for solubilizing phospholipids by detergents. Two other methods are widely employed, namely, mixing of aqueous dispersions of the phospholipids with solutions of the detergent [method A, Yedgar et al. (1974a,b); Helenius & Simons (1975)] or dispersing solid phospholipids in detergent solutions (Dennis & Owens, 1974), denoted herein as method C. A demonstration of the importance of the method of mixing is given in a recent study by Yedgar et al. (1974b). In this study,

† From the Departments of Pharmacology (D.L. and Y. Z.), Biochemistry (P.G.), and Statistics (S.Z.), The Herbrew University, Jerusalem, Israel. *Received March 17, 1978; revised manuscript received May 2, 1979.*

‡ Present address: Makor Chemicals, Jerusalem, Israel.

0006-2960/79/0418-3517$01.00/0

mixtures of sphingomyelin and Triton X-100 prepared by methods A and B were found to form different types of aggregates.

In the present work, mixed dispersions of egg phosphatidylcholine (PC)[1] and the bile salt sodium deoxycholate (DOC) were prepared by the above three methods, and the resultant dispersions were studied by analytical ultracentrifugation, proton magnetic resonance (^{1}H NMR), and turbidity measurements. The spectral properties of the various dispersions as a function of time clearly indicate that PC–DOC mixed dispersions made by method B were "at equilibrium". On the other hand, methods A and C resulted in the formtion of different dispersions, which reorganized with time to form aggregates similar to the ones obtained by method B. Thus, the spectral properties of samples prepared by method B provide information on the composition and structure of the PC–DOC mixed dispersions at equilibrium, whereas the changes with time in the spectra of dispersions prepared by methods A and C shed some light on the mechanism of the solubilization.

Materials and Methods

Egg PC (Makor Chemicals, Jerusalem) and DOC and TDC (Sigma) were chromatographically pure and were used without further purification. Suspensions of the two components were prepared in water or D_2O (99.8%; Merck) at pH 7.4 (pD 7.8; 0.1 M phosphate buffer) by the following three methods. (A) Solutions of PC in chloroform were evaporated to dryness, the residue was dispersed in water or D_2O (Vortex mixer; 2 min), and aqueous solutions of DOC at various concentrations were added to the suspensions. For studies of single-bilayered vesicles, the PC suspensions were sonicated for 5 min at 160 V (Heat Systems sonicator, Model 350) prior to the addition of the detergent solution. (B) Solutions of PC in chloroform were mixed with solutions of DOC in methanol, evaporated, and dispersed in water or D_2O. (C) Aqueous solutions of DOC were added to the solid residue of PC after evaporation of the chloroform, and the suspensions were vortexed in the presence of two small glass beads. Physical measurements were carried out following a 10-min incubation at 37 °C and repeated after the dispersions had been stored at 37 °C for up to 120 h.

Turbidity measurements were carried out in 1-mm and 1-cm cells and determined in a Perkin-Elmer 124 spectrophotometer. Sedimentation coefficients (s values) were determined as described (Yedgar et al., 1974a) in a Spinco Model E analytical ultracentrifuge, equipped with Schlieren optics. The areas of the bands were measured by weighing paper cutouts of blown-up photographs.

^{1}H NMR spectra were obtained on a JEOL MH-100 instrument, the probe temperature being 27 ± 1 °C. Line widths ($\nu_{1/2}$) were measured by using a spectral width of 2 Hz/cm and a sweep rate of 1 Hz/s. Intensities were measured by weighing paper cutouts of the signals.

[1] Abbreviations used: PC, phosphatidylcholine (egg lecithin); DOC, sodium deoxycholate; TDC, sodium taurodeoxycholate; R, the molar ratio of DOC to PC in their mixed dispersions; $\nu_{1/2}$, the width (in Hz) at half-height of the signal; τ_v, the correlation time for the overall Brownian tumbling; n_c, the number of carbon atoms in each paraffinic chain of PC, which is embedded in the hydrophobic core of the mixed micelles; l' ($l' \equiv 2V_c/s$), the height of the paraffinic region of half a flat bilayer which contains the volume of the paraffinic chains (this value indeed represents the average chain length of the PC); l, the dimension of a bile salt molecule which parallels the chain axis of the phospholipid molecules in a mixed micelle of bile salt and phospholipid; a, the approximate radius of curvature of the surface of curved mixed micelles of bile salt and phospholipid (see text and supplementary material for details); δ, $\delta \equiv l' - l$.

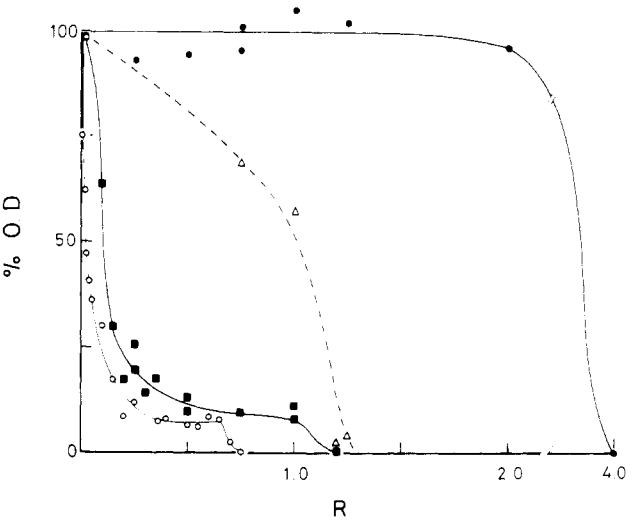

FIGURE 1: Dependence of percent turbidity [= (OD of the sample at 600 nm × 100)/(OD of the sample prepared without DOC)] on the DOC to PC molar ratio (R). (●) Method A, 10 min after preparation; (△) after 20 h at 37 °C; (■) after 60 h at 37 °C; (○) method B, 10 min after preparation; PC concentration was 20 mM throughout.

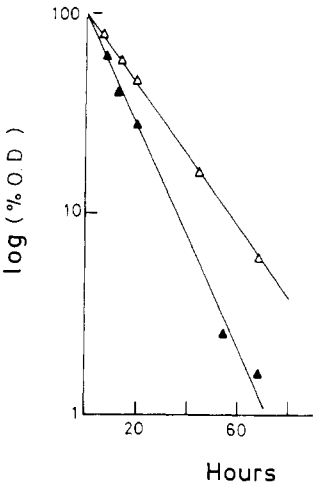

FIGURE 2: The time dependence of log (OD_t/OD_0), for dispersion prepared by method A using 20 mM PC and 15 (△) or 20 mM (▲) DOC with incubation at 37 °C. OD_t, the optical density at time t, is normalized by dividing by OD_0, the optical density at the time of preparation.

Results

In Figure 1, the turbidities of mixed dispersions of PC (20 mM) and DOC (0.2–80 mM), measured in 1-mm cells, are described as a function of R. The turbidities of samples prepared by method B remained unchanged after 120 h of incubation at 37 °C. On the other hand, the turbidities of samples prepared by method A decreased with time and approached those of suspensions of the same composition prepared by method B. The logarithm of the turbidity of dispersions prepared by method A decreased linearly with time, as exemplified in Figure 2. In dispersions with $R > 0.75$, the turbidity approached zero after 120 h at 37 °C. The rate of decrease of the turbidity of such dispersions (with 20 mM egg PC and 15, 20, 25, 30, and 40 mM DOC), given by the slopes of graphs such as those shown in Figure 2, increased linearly with DOC concentration, the slope being equal to about 10^{-9} s^{-1} M^{-1}. Samples prepared by method C showed similar behavior to those prepared by method A.

The NMR spectra of dispersions prepared by the various methods also differed from each other. One major difference

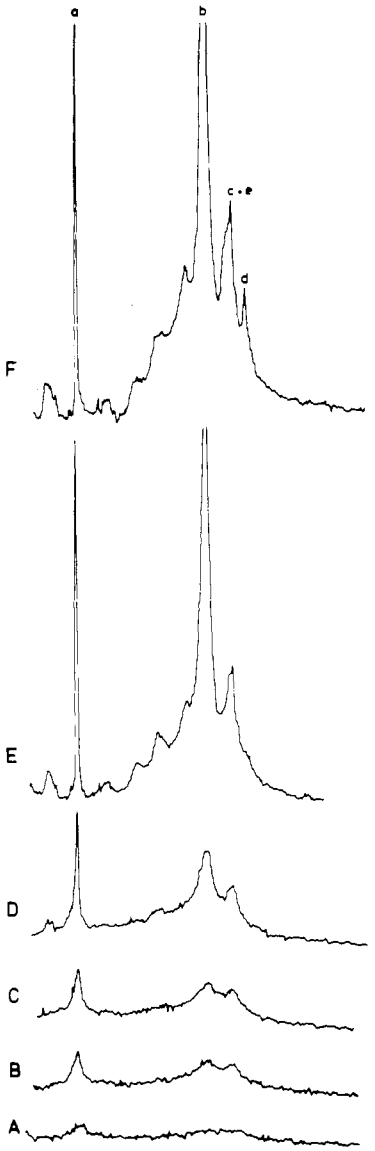

FIGURE 3: ^{1}H NMR spectra of dispersions prepared by method B from 20 mM PC and various DOC concentrations (A, 4; B, 8; C, 10; D, 12; E, 16; F, 32 mM) and measured within 10 min after preparation. Signals are assigned as follows: (a) choline head group; (b) methylene proton of PC, superimposed on some methylene signals of DOC; (c) terminal methyls of PC; (d) 18-methyl signal of DOC; (e) 19-methyl signal of DOC. The chemical shifts of the various signals (in parts per million downfield from external Me_4Si) are as follows: (a) 3.25; (b) 1.32; (c) 0.98; (d) 0.74; (e) 0.93.

was that the spectra of samples prepared by method B (Figure 3) remained unchanged after 120 h of incubation at 37 °C, whereas those prepared by method A (Figure 4) changed with time. Thus, all the signals were broadened and the intensity of the choline head group increased in most dispersions of method A until an equilibrium had been reached. At this point, the NMR intensities (Figure 5), as well as the line widths (Figure 6), were similar to those obtained for dispersions of the same composition prepared by method B. An example of such changes is given in Figure 7. The spectrum of a mixed dispersion of 16 mM DOC and 20 mM PC 10 min after preparation by method A is shown in Figure 7B. With time, the resonances became more intense, although broader. Thus, the 18-CH_3 signal (which appears at the highest field in the spectrum of pure DOC, in Figure 7A) was clearly resolved in the spectrum presented in Figure 7B and 7C but did not appear at all in the spectrum of the same suspension 20 or 45 h later (Figure 7C and 7E).

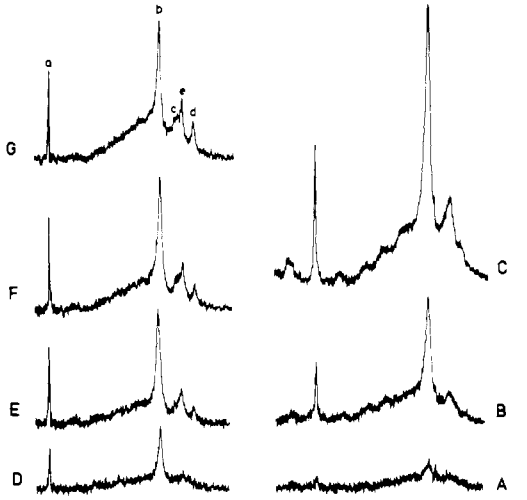

FIGURE 4: ^{1}H NMR spectra of suspensions prepared by the addition of DOC solutions to suspensions of PC (method A) (spectra A–C were recorded with an instrumental amplification fourfold higher than that of spectra D–G). The final PC concentration was 20 mM, in all dispersions. Final DOC concentrations were as follows: (A) 4; (B) 8; (C) 12; (D) 16; (E) 32; (F) 48; (G) 64 mM. Samples were measured within 10 min after preparation. The assignment of the peaks is as described in Figure 3.

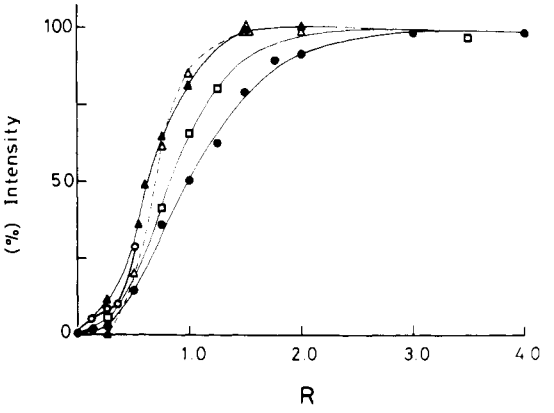

FIGURE 5: Dependence of ^{1}H NMR intensities of the choline head group of PC on the molar ratio of DOC to PC (R). Intensities are expressed as percent of the maximal intensity. (●) Method A, after 20 h at room temperature; (□) after 45 h at 38 °C; (○) after 68 h at 38 °C; (▲) method B; (△) method B with TDC; PC concentration was 20 mM throughout.

When solutions of DOC were added to dispersions of unilamellar (sonicated) PC, the dispersions formed showed the same spectra 10 min and 120 h after mixing. The complete NMR intensity of PC was detected in these dispersions (Figure 8) (Sheetz & Chan, 1972), while the widths of the various signals depended on the molar ratio of DOC to PC. Thus, addition of 16 mM DOC or more to 20 mM of sonicated PC resulted in a narrowing of both the signal of the choline head group and that due to the paraffinic protons of PC (Figures 6 and 8D), whereas addition of lower DOC concentrations had no effect on the line widths (Figures 6, 8B, and 8C). $MnCl_2$ caused broadening (beyond detection) of more than half of the choline head group signal in the spectra of PC vesicles (Figure 8A). The same was observed when $MnCl_2$ was added after the addition of less than 7 mM DOC to 20 mM PC vesicles (Figure $8B_1$). Addition of $MnCl_2$ to a mixed dispersion of 20 mM PC vesicles and more than 8 mM DOC resulted in appreciable broadening of the complete choline head group signal (Figure $8C_1$ and $8D_1$).

In the ^{1}H NMR spectra of PC–DOC dispersions prepared by method B, the intensity of all signals increased upon ad-

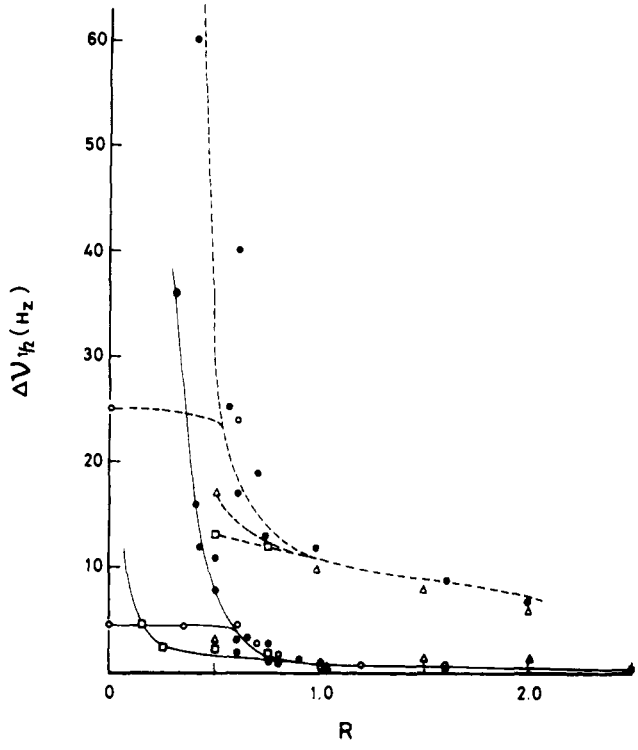

FIGURE 6: The line widths of the signals of the choline head group (full lines) and the paraffinic protons of PC (20 mM) (dotted lines) as a function of R in PC–DOC dispersions. The squares denote spectra of dispersions prepared by method A, full circles denote method B, and empty circles stand for mixtures of DOC with sonicated PC. The symbol Δ represents dispersions prepared by method B from PC and taurodeoxycholate (TDC). In all cases, line widths were measured 10 min after preparation of the samples.

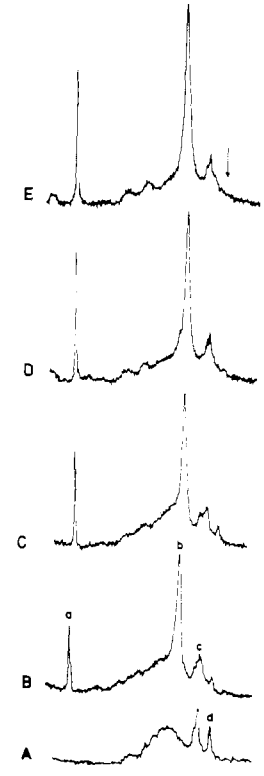

FIGURE 7: ^{1}H NMR spectra of (A) 12 mM DOC and (B–E) a dispersion of 16 mM DOC and 20 mM PC prepared by method A, incubated at 37 °C, and measured at various times after preparation: (B) 10 min; (C) 90 min; (D) 20 h; (E) 45 h. For peak assignment see Figure 3.

dition of DOC (Figure 5). There is no contribution of DOC protons to the intensity of the choline head group signal. The complete intensity of this signal was observed only when $R > 0.75$, while for lower DOC concentrations, the observed intensities (Figure 5) were in accordance with the fraction of small aggregates ($s < 6$) determined by analytial ultracentrifugation. It may therefore be concluded that only these small aggregates contributed to the NMR spectra; the signals from those PC molecules contained in the larger aggregates were probably too broad to be detected under the sweep width employed in our measurements. When PC was solubilized by TDC, practically the same dependence of the choline head group intensity was observed at $R > 0.75$ (Figure 5). However, at $R < 0.5$, no ^{1}H NMR signals were sharp enough to be detected when TDC was used, while much lower DOC relative concentrations were needed to observe the high-resolution signals of PC. The dependence of the line widths of signals obtained both from the choline head group and from the methylene protons on DOC and TDC concentrations is described in Figure 6. Again, at $R > 0.75$, the signals had the same width in dispersions obtained by both TDC and DOC. Below this ratio, solubilization by TDC is accompanied by sharper signals than that by DOC at the same R.

The homogeneity of dispersions prepared by method B was studied by analytical ultracentrifugation. For $R < 0.75$, at least two different populations of aggregates were present. At $R < 0.25$, the suspensions contained aggregates with s values of 20–30, along with some larger particles with s values up to 60. In the range $0.25 < R < 0.75$, an increase in DOC concentration was accompanied by a decrease in the abundance of large aggregates (s = 20–30) and an increase of the smaller aggregates ($s < 6$). Only at $R > 0.75$ did the dispersions

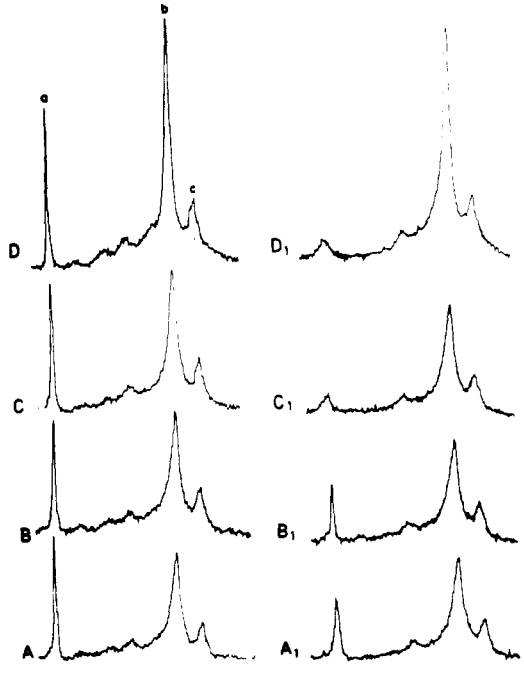

FIGURE 8: ^{1}H NMR spectra of sonicated dispersions of 20 mM PC with various concentrations of DOC (A, 0; B, 6; C, 12; D, 16 mM). Spectra A_1–D_1 were recorded following the addition of $MnCl_2$ to a concentration of 0.1 mM. For peak assignment see Figure 3.

become homogeneous, with the s values decreasing sharply.

The turbidities of dispersions of PC and DOC prepared by method B with $R > 0.1$ were measured in 1-cm cells at various wavelengths and are presented in Figure 9A as a function of R. The dependence of the turbidities on the logarithm of the wavelength was also calculated for the various dispersions.

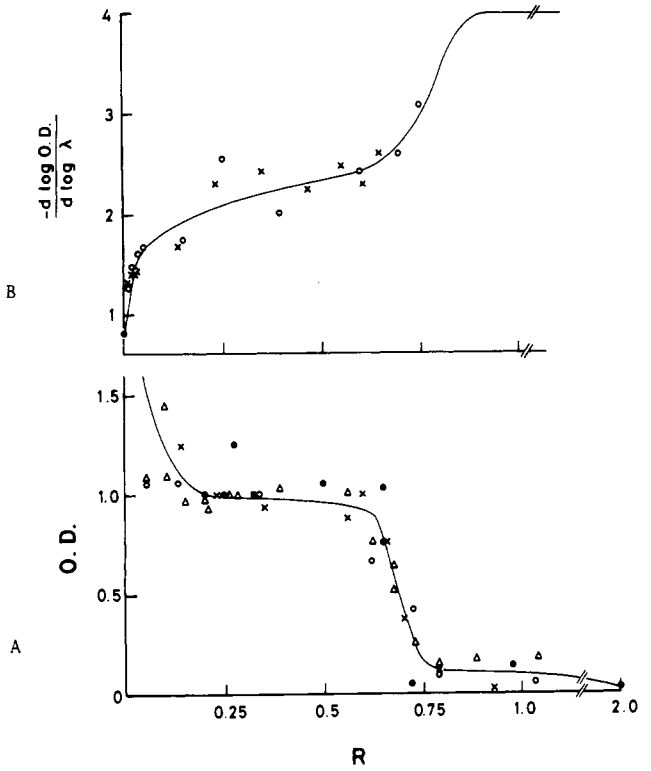

FIGURE 9: The dependence of turbidity parameters (A, OD; B, −d log OD/d log λ) on the molar ratio of DOC to PC (R) for various PC concentrations. (●) 20; (×) 10; (○) 5; (△) 1 mM.

This dependence is known to be rather sensitive to particle sizes, mainly those of large aggregates (Van Holde, 1971). In our experiments, it was found to be linear for all the dispersions, the correlation coefficient being always higher than 0.97. The slopes of these linear dependencies are described in Figure 9B as a function of R. It is of interest to point out that over the range of PC concentrations of 5–20 mM the optical observations did not depend on the absolute concentrations of the components but rather on their relative molar concentrations.

Suspensions with $R < 0.25$ were very turbid, the turbidity depending markedly on R. Suspensions in which $R > 0.75$ were much less turbid and the scattering had the characteristics of real Rayleigh scattering; i.e., the slope of the linear dependence of the optical density on the logarithm of the wavelength approached the value of 4 (Van Holde, 1971). Within the range of $0.25 < R < 0.65$, no significant changes were observed in the turbidity of the suspensions (Figure 9A), as well as in its dependence on the logarithm of the wavelength (Figure 9B).

Discussion

Structural Details of PC–DOC Mixed Aggregates "at Equilibrium". The heterogeneity of the suspensions with $R < 0.75$ is expected from phase diagrams of the bile salt–lecithin–water ternary system (Small et al., 1966; Small, 1971). For any given concentration of phospholipid, a minimum amount of detergent is certainly needed to keep all the phospholipid in "solution". If this bile salt concentration is not reached, only part of the phospholipid will be solubilized and two or more phases will coexist.

Not much information can be gained regarding the size and structure of the DOC–PC mixed aggregates on the basis of the described optical data (Figure 9). The light-scattering properties can, however, be rationalized on the basis of the analytical ultracentrifugation results. When $R > 0.75$, only small mixed aggregates are present in the suspension, while large, probably multilamellar aggregates of PC loaded with DOC are present in suspensions of $R < 0.25$. The sharp decrease in turbidity, which accompanies the addition of up to 0.25 mol of DOC to 1 mol of PC, might reflect division and declustering processes of the PC multibilayers. In the range $0.25 < R < 0.65$, the invariability of turbidity might be an outcome of the addition of DOC per se. More specifically, addition of DOC results in the inclusion of more PC in small micelles and less in the multilamellar structure. Such a change should have been expected to bring about a decrease in turbidity. However, this effect might be balanced by the condensing effect of DOC on the aggregates of both these types. This perhaps is possible due to the large difference in partial specific volume ($\bar{v}$) between DOC ($\bar{v} = 0.75$; Small, 1971) and PC ($\bar{v} = 0.98$; Huang & Mason, 1978). As a consequence of the above balance, the turbidity is not altered in the range $0.25 < R < 0.65$. Only above the latter molar ratio is the DOC concentration large enough to reduce drastically the population of PC multibilayers, and when $R > 0.75$, all the phospholipids are included in mixed micelles.

Figure 5 clearly demonstrates that only when $R > 0.75$ is the full intensity of the choline head group signal observed. Dispersions with lower relative concentration of DOC are heterogeneous, making it very difficult to characterize the species being observed in the NMR spectra, in terms of their size and composition. However, the parallelism between the data obtained by analytical ultracentrifugation and that obtained by NMR intensities suggests that only the small aggregates contribute to the ^{1}H NMR spectra. These aggregates are probably mixed micelles, since addition of trace amounts of $MnCl_2$ to PC–DOC dispersions resulted in a broadening of all the choline head group signals (Lichtenberg & Zilberman, 1979). This indicates the accessibility of all the choline head groups to $MnCl_2$, in contrast to phospholipid vesicles, where only those choline head groups located on the outer surface of the bilayer are affected by the addition of $MnCl_2$ (Kostelnik & Castellano, 1973).

The above data in fact do not rule out the possibility that ^{1}H NMR signals are obtained from PC and DOC molecules contained in perturbed (leaky) bilayers. However, a straightforward interpretation of the NMR data is possible on the basis of a simple model for the PC–DOC mixed micelles. The model proposed by Small and co-workers (Small et al., 1969; Small, 1971) is diagrammatically described, in cross section and longitudinal section, in Figure 10A and 10B. According to this model, the phospholipid molecules are packed as a flat bilayer, covered by the bile salt on the hydrophobic edges of the micelle.

The size and thus the molecular weight of the mixed micelles depend on the molar ratio of the two components. If each PC molecule occupies 70 Å^2 on the surface, then for any given R the cross section of a mixed micelle of radius r includes $\pi r^2/70$ molecules of phospholipid and $2\pi r/8$ molecules of the bile salt [see Figure 10 and Small (1971) for details]. Assuming that the ratio of DOC to PC in the cross section is equal to the ratio (R) of the total concentrations in the dispersion, we can calculate the radius as well as the molecular weight of the mixed micelles as a function of R ($r = 17.5/R$). For $R < 0.75$, an estimate for the size, based on the latter relation, might be somewhat exaggerated. The larger aggregates, that coexist in the dispersions with the small mixed micelles, probably include some DOC, but it is highly improbable that the DOC to PC ratio in them is as high as in the mixed micelles. Thus, this ratio should be higher in the

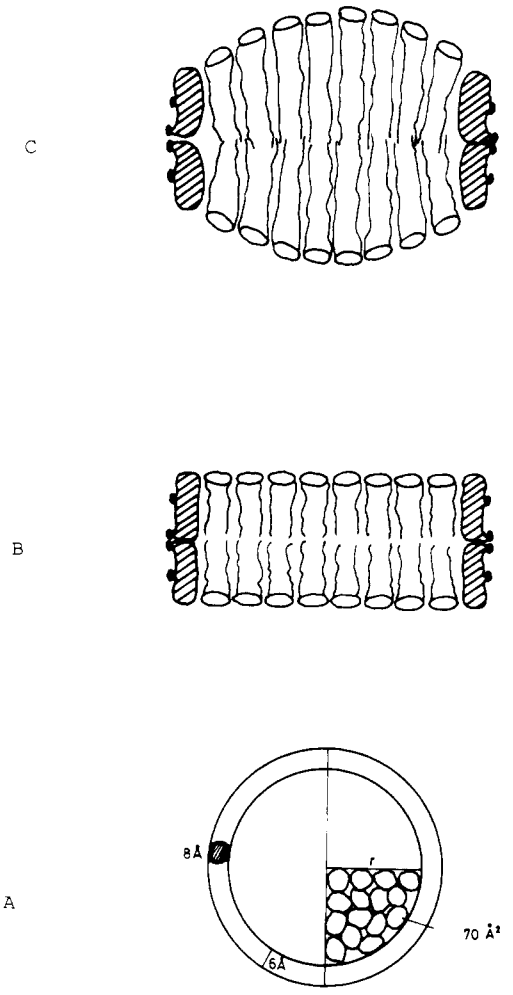

FIGURE 10: Diagrammatic representation of Small's model of mixed micelles of phospholipids and bile salts. (A) Cross section, showing $\pi r^2/70$ phospholipid molecules in half of the bilayer, encircled by $2\pi r/8$ bile salt molecules. (B) Longitudinal section. (C) Longitudinal section of a modification of Small's model, in which the phospholipid bilayer is curved (see text for details).

phase of mixed micelles than in the whole suspension. Consequently, when $R < 0.75$, the total ratio of the components in the suspension is the lower limit for this ratio in mixed micelles, and the calculated size is an upper limit for the radius of the mixed micelles.

Dispersions prepared by method B gave rise to NMR signals only when $R > 0.2$; namely, the radius of the mixed micelles that contributed to the NMR spectra was smaller than 90 Å (Small, 1971). The rate of overall tumbling of such small aggregates should certainly be fast enough to equalize magnetically the various PC molecules contained in the mixed micelles (Seiter & Chan, 1973; Lichtenberg et al., 1975). Actually, if particle tumbling would have been a predominant factor in determining the line width of PC in its aggregates, one would expect the ^{1}H NMR signals of PC in these aggregates to be narrower than in the smallest available PC vesicles [with $r = 105$ Å; Schmidt et al. (1977)]. Since the opposite is observed experimentally, it may be concluded that other factors are involved in determining the line widths in the spectra of these mixed micelles.

One possibility is that DOC might have a tightening effect on the packing of PC in its mixed micelles with DOC. This, however, should also result in increased tightening due to an increase in R, which is accompanied by enlargement of the portion of PC molecules that has DOC as its nearest neighbors. This would mean that the narrowing of the NMR signals, observed upon increasing R, must be due to changes in the rate of overall tumbling due to the decrease in size.

Nevertheless, changes in the rate of tumbling cannot account for the observed narrowing. As an example, when R changed from 0.4 to 0.6, the radius of the mixed micelles should be reduced by a factor of 1.5 at most and the molecular weight of the bilayer should be reduced by less than a factor of 2. Such a reduction in the size should cause a similar increase in the rate of overall Brownian tumbling of the micelles. Therefore, the correlation time associated with this motion (τ_v) should be reduced to approximately half of its value, and accordingly the line widths should be reduced by no more than a factor of about 2 (Lichtenberg et al., 1975). Experimentally, the signal of the choline head group was narrowed from 16 to 4 Hz. This signal is due to a group which is not expected to be tightly packed, and its line width should not be particularly dependent on the rate of micellar tumbling. The experimental fourfold narrowing, which accompanied a twofold decrease in the mass of the mixed micelles, strongly supports the conclusion that the narrowing of the recorded signals is primarily due to changes in the packing rather than changes in the rate of overall tumbling.

The effect of DOC on the packing of PC in the mixed micelles is not known. If it causes "fluidization" of the packing, it could explain the narrowing effect of DOC. However, it would be difficult to explain why the ^{1}H NMR signals are so much broader in the mixed micelles where $R = 0.5$ ($r \leq 35$ Å) than in PC vesicles ($r \sim 105$ Å). This could be understood in light of the disruptive effect of curvature on the packing of PC within bilayers (Seiter & Chan, 1973; Lichtenberg et al., 1975; Petersen & Chan., 1977). Since the bilayer is much more curved in the PC vesicles than in the PC–DOC mixed micelles, the NMR signals of the former are narrower. The line-narrowing effect of inclusion of elevated amounts of DOC might still be due to a fluidizing effect of DOC on the packing of PC in the mixed micelles. However, the line widths can be readily explained on the basis of the curvature expected according to a simple modification of the "planar disc" model proposed by Small (1971) for the structure of the PC–DOC mixed micelles.

In the planar disc model, the volume of half a cylinder is $l\pi r^2$ (Å^3), where l is the dimension of the bile salt, which parallels the chain axis of the phospholipid molecules. In this model, $l' = l$, where l' is the chain length of the phospholipids. This latter parameter is significant as the volume of the described cylinder must accommodate the chains of the phospholipids and therefore should vary with the number of embedded carbon atoms (n_c) in each chain. In all cases where $l' > l$, the hydrophobic chains of PC are too long for a ring of the bile salt "bilayer" to cover the edges of the phospholipid bilayer in a way that will prevent exposure of hydrophobic parts of the bilayer to the aqueous phase. Although it is assumed that some methylene groups near the amphiphile head group may not be contained in the hydrophobic core (Stigter & Mysels, 1955; Clifford, 1965), one would expect prevention of this exposure to have a stabilizing effect.

Several modifications of Small's model for phospholipids with long hydrocarbon chains have been considered and analyzed (see paragraph concerning supplementary material at the end of this paper). On the basis of simple geometrical considerations, it is concluded that in mixed micelles of bile salts and long-chain phospholipids, the latter are probably arranged in curved bilayers (Figure 10C). An approximation for the radius of curvature a of the bilayers is given by $a =$

$2rl'/(r - \sqrt{r^2 - 16l'\delta})$ where r is the radius of the micelles and δ is the difference between the length of the paraffinic chains of PC, l', and the hydrophobic length of the bile salt molecule, l. l is approximately 14–15 Å, since the steroid length of the bile salt is about 11–12 Å and the short hydrocarbon chain adds another 3 Å to this dimension. On the other hand, the thickness of the hydrocarbon part of a monolayer of egg PC can vary from 15 Å in the inner layer of a sonicated PC vesicle to 21 Å in the outer layer (Small, 1967; Huang & Mason, 1978). Consequently, δ can vary over the range of 0–7 Å. When δ is very small, a very small curvature is expected. Nevertheless, the surface curvature is very dependent on the radius of the PC–DOC mixed micelles and thus on the molar ratio of these two components in their mixed micelles. Even if $\delta = 0.5$ Å, the radius of curvature would be 135 Å at $R = 1$ but would be 256 Å at $R = 0.75$ and almost 600 Å at $R = 0.5$. This implies that even when the hydrophobic parts of PC and DOC are very similar in size, the small difference between them has significant implication for the dependence of the micellar surface curvature on R.

In spherical PC vesicles, the radius of curvature is the radius of the vesicles. A line width of about 15 Hz was found for the methylene signal of PC vesicles of 210 Å in diameter (Schmidt et al., 1977). This is also the line width of the same signal in mixed micelles in which $R \simeq 0.6$, i.e., where $r \simeq 30$ Å. This would mean that if the surface curvature is a prime factor in determining the line width, δ (the difference between the PC average chain length and the length of the DOC molecules) should have the value of about 1.5 Å. For $R = 0.4$, where $r \simeq 45$ Å, the radius of curvature, a, is then expected to be about 350 Å. The signal of the paraffinic protons in the spectra of mixed micelles for which $R = 0.4$ is in fact somewhat narrower in comparison to the line width of PC vesicles of a radius of about 500 Å (Sheetz & Chan, 1972; Lichtenberg et al., 1975). This strongly supports the validity of our theoretical treatment as well as the idea that a prime factor in determining line widths in the ^{1}H NMR spectra of phospholipid bilayers (and the "packing" and motion within the bilayers) is the surface curvature [see also Chrzeszczyk et al. (1977) and Huang & Mason (1978)]. It is of interest to point out that for $R > 0.75$, the spectral changes are small, even though one should expect the greatest changes of the radius of curvature in this range. This discrepancy might be due to the inhomogeneous broadening of the NMR signals, due to chemical shift dispersions, spin–spin couplings, and field inhomogeneity. In light of these broadenings, it is highly improbable that at $R > 0.75$, where the signals are quite narrow, changes in $\nu_{1/2}$, due to changes in curvature, could be detected. Thus, even though the curvature might vary over this range, it cannot affect the apparent line widths.

In conclusion, we believe that the line widths are best interpreted in terms of the surface curvature of the PC–DOC mixed micelles. This observation is of special interest since the packing of phospholipids within bilayers is affected by the surface curvature of the bilayers (Lichtenberg et al., 1975). The dependence of the rate of enzymatic reactions on bile salt concentrations might therefore depend on the molar ratio of bile salt to phospholipids through the effect of R on the curvature of the bilayers; as R increases, the packing of the substrate molecules is disrupted and the accessibility of the head groups to phospholipases might be greater.

The curvature of the PC bilayers in PC–DOC mixed micelles might play an important role in mixed micelles with other components, such as proteins. Such mixed micelles are most probably formed when biological membranes are solubilized by bile salts. In these cases, even when the proteins are surrounded by phospholipid molecules, their activity might depend on the molar ratio of bile salt to phospholipid (B. Litman, private communication). This might be due to changes in the surface curvature of the phospholipid bilayers, induced by alteration of R. Such changes may certainly determine the fluidity within the bilayer and influence the activity of the proteins.

As a last remark, it is interesting to note that the comparison between the solubilization of PC by DOC and that by TDC also indicates that the line widths depend on the molar ratio of bile salt to PC. In the presence of excess water, it has been shown that conjugated bile salts solubilize PC less than sodium cholate (Small et al., 1966). Similarly, TDC might solubilize PC less than DOC, which might lead to the fact that at $R = 0.5$, a lower intensity of the choline head group of PC is observed in the spectrum of PC–TDC dispersions compared to that of PC–DOC. However, the observed lines are sharper as the ratio of bile salt to lecithin in the mixed micelles is higher.

Kinetics Aspects of the Solubilization. Reviewing the "solubilization of membranes", Helenius & Simons (1975) suggested that "as increasing amounts of detergent are added to a suspension of phospholipids, the following sequence of events occurs. Stage I: detergent is incorporated into the phospholipid bilayer. Stage II: the bilayers become saturated with detergent and mixed micelles begin to form, resulting eventually in complete lamellar–micellar phase transition. Stage III: the mixed micelles decrease in size." A somewhat more detailed picture of the above stage II is the hypothesis proposed by Dennis & Owens (1973). These authors suggested that only after the concentration of free detergent rises above the critical micellar concentration (cmc) are detergent micelles formed, which are able to dissolve the phospholipid bilayers, saturated by detergent.

This description of the solubilization is consistent with the empirical rule, proposed by Fendler & Fendler (1970), which claims that "little or no solubility increase is observed until the cmc of a surfactant is reached." However, even though this rule is known to hold for the solubilization of hydrophobic molecules in water, it should not necessarily exist for the amphiphilic phospholipids, which form mixed micelles with detergents. Here, the mechanism may involve a spontaneous breakdown of a phospholipid bilayer, saturated with detergent to form a "solution" of mixed micelles. This is consistent with the fact that bilayers constitute the most stable aggregates of phospholipids in water, while micelles are the most stable aggregates in detergent solutions and perhaps in mixtures of phospholipids with detergents above a certain molar fraction of the detergent (Small, 1971).

Upon addition of up to 14 mM DOC to 20 mM unilamellar PC, the NMR spectrum of the sonicated dispersion changed slightly (compare Figure 8A with 8B and 8C; see also Figure 6). Nonetheless, the spectra of the mixed dispersions were not a superposition of spectra of vesicular PC and micellar DOC. For example, the signal of the 18-CH_3 group of DOC (peak d in Figure 7A) is broadened beyond detection in a mixed dispersion of 12 mM DOC and 20 mM sonicated PC (Figure 8C). This indeed suggests that PC and DOC interact with each other. Addition of higher concentrations of DOC resulted in a narrowing of all the signals (Figure 6), and the spectra were similar to those of dispersions prepared by method B with the same composition. This probably means that when $R > 0.75$, the bilayers burst spontaneously and mixed micelles are formed. Addition of $MnCl_2$ to these dispersions was

accompanied by broadening of the choline head group signal (Figure 8D), showing that all the polar groups are accessible to Mn^{2+}. On the other hand, upon addition of $MnCl_2$ to PC vesicles containing low concentrations of DOC ($R < 0.4$), only two-thirds of the choline head group signal were broadened (e.g., Figure 8A,B).

It is likely that when $R < 0.4$, DOC is incorporated into the PC bilayers without altering the permeability of the bilayers to Mn^{2+}, whereas, within the range of $0.4 < R < 0.75$, perturbed bilayers exist through which Mn^{2+} can penetrate. Only when $R > 0.75$ do the bilayers burst and are mixed micelles formed, the ^1H NMR signals of which are narrower than those of molecules contained in PC vesicles. This is especially true for the choline head group signal, which is broadened by about 0.04 ppm in the spectra of vesicles due to the difference in chemical shift between the head groups on the inner and those on the outer surfaces of the bilayer (Kostelnik & Castellano, 1973).

The fast equilibration of dispersions of single bilayered vesicles, following the addition of aqueous DOC solution, indicates that the slow solubilization (by methods A and C) of multilamellar liposomes might reflect a mechanism which involves peeling off of lecithin bilayers, one after another. Such a mechanism is also indicated by several other observations. The signals of both the choline head group and the methylene protons of PC, in the spectrum of a dispersion of 16 mM DOC and 20 mM PC (Figure 7B), became more intense upon "equilibration" (Figure 7C–E). This certainly is a reflection of an additional solubilization of PC by DOC, in the form of mixed micelles. Since the total molar ratio of DOC to PC did not change, the additional solubilization must lead to a decrease of the DOC to PC ratio in mixed micelles. Such a decrease has been previously shown to result in an increase of the micellar size and in broadening of the ^1H NMR signals of both DOC and PC [Small et al. (1969) and Figures 3 and 6]. This in fact is what has been observed, as is evident from comparison of the spectra in Figure 7B with those of Figure 7C–E. Thus, peak d appears in Figure 7B but is broadened beyond detection in Figure 7D,E.

The suggested mechanism also provides a straightforward explanation for the spectral differences between dispersions prepared by the various methods. At equilibrium (method B), with $R = 0.20$, no ^1H NMR signals could be detected (Figure 3A). In spite of this, several high-resolution signals appeared in the NMR spectrum of a suspension prepared by mixing aqueous dispersions of the two components to the same concentrations (Figure 4A). These signals are probably due to protons in molecules contained in mixed micelles which were formed from interaction of the detergent with the outer bilayer of the lecithin. In this interaction, the DOC concentration is high with respect to PC in the outer bilayer, and this bilayer bursts to form mixed micelles. In more general terms, even a relatively low concentration of DOC in the dispersion might constitute a very high excess with respect to those PC molecules which build the outer bilayer. This certainly explains the fact that for some DOC concentrations, dispersions obtained by method A gave rise to sharper (and at some concentrations more intense) NMR signals than do the corresponding dispersion prepared by method B (e.g., compare Figure 3D with 4C).

The suggested mechanism is also consistent with the linear dependence of the logarithm of the turbidity on time (Figure 2). At equilibrium, the optical density, in 1-mm cells, of mixed dispersions of PC and DOC with $R = 0.75$ is zero (Figure 1); i.e., the formed mixed micelles were too small to scatter the light. On the other hand, the mixed dispersions prepared by methods A and C were very turbid immediately after mixing the two components. The decrease in optical density, which reflects reduction of size of the aggregates, can therefore serve as a criterion for the progress of the solubilization process. In the course of this reaction, multilamellar PC, L, interacts with DOC, D, to form small mixed micelles, M. The rate of the reaction

$$L + D \xrightarrow{k_2} M$$

is given by

$$-\frac{d[L]}{dt} = k_2[D][L]$$

If the "effective" concentration of DOC, which is available for solubilization, remains unchanged throughout the course of the reaction

$$k_2[D] = k_1$$

$$\ln [L] = k_1 t + c$$

and ln [L] will depend linearly on time.

We believe that the linear dependence of log OD on time (Figure 2) is indicative of such pseudo-first-order kinetics of the solubilization; thus, its slope reflects the pseudo-first-order rate constant of the reaction, k_1. This conclusion is further supported by the observation that when this parameter, measured for 20 mM PC and 15, 20, 25, and 30 mM DOC, was plotted against DOC concentration, parallel lines were obtained with a slope of about 10^{-9} s^{-1} M^{-1}. This probably is the value of k_2, the second-order rate constant of the solubilization.

The invariability of the effective DOC concentration can be explained in two ways. First, it is possible that transformation of DOC from its mixed micelles with PC to PC bilayers occurs through the solution. In this case, the effective DOC concentration must be the cmc, which can be maintained at its original very low value through equilibria with the various aggregates. This hypothesis can explain the dependence of the pseudo-first-order rate constants of the equilibration on the concentration of DOC, since the cmc is known to vary with the molar ratio of DOC to PC (Small, 1971). Another possibility is that the effective DOC concentration is its total concentration. In this case, micellar DOC may be available for solubilization through very fast equilibria between the various aggregates or through transformation of complete micelles into PC bilayers. At the present stage, no data are available to differentiate between the above possibilities.

In conclusion, mixing phospholipids with aqueous solutions of bile salts initiates a process which is not instantaneous. In the course of this equilibration process, the bilayers of the multilamellar liposomes of PC are probably bursting one after another in a pseudo-first-order reaction, the rate constant of which depends linearly on the detergent concentration. This process is very slow for low bile salt concentrations, a fact which must be considered in order to prevent apparent irreproducibilities, due to major changes in the state of the mixed aggregates, which can be caused by minor changes in the temperature or the length of time between sample preparation and sample usage.

Acknowledgments

We thank Drs. S. Gatt, S. Yedgar, I. Tamir, Y. Barenholz, and M. Heller of our school and Dr. B. Litman of the Department of Biochemistry at the University of Viriginia for

helpful discussions. We also thank S. Amselem and R. Gatt for technical assistance and R. Knafo for the drawings.

Supplementary Material Available

Supplementary material including various modifications to Small's model, the conclusion that for long-chain phospholipids the PC–bile salt mixed micelles are probably curved, and calculation of the N radius of curvature as a function of the chain length and R (6 pages). Ordering information is given on any current masthead page.

References

Chrzeszczyk, A., Wishnis, A., & Springer, C. S. (1977) *Biochim. Biophys. Acta 470*, 161–163.
Clifford, J. (1965) *Trans. Faraday Soc. 61*, 1276–1282.
Dennis, E. A., & Owens, J. M. (1973) *J. Supramol. Struct. 1*, 165–176.
Fendler, E. J., & Fendler, J. H. (1970) *Adv. Phys. Org. Chem. 8*, 271–406.
Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.
Huang, C., & Mason, J. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 308–310.
Kostelnik, R. J., & Castellano, S. M. (1973) *J. Magn. Rèson. 9*, 291–295.
Lichtenberg, D., & Zilberman, Y. (1979) *J. Magn. Reson.* (in press).
Lichtenberg, D., Petersen, N. O., Girardet, J. L., Kainosho, M., Kroon, P. A., Seiter, C. H. A., Feigenson, G. W., & Chan, S. I. (1975) *Biochim. Biophys. Acta 382*, 10–21.
Petersen, N. O., & Chan, S. I. (1977) *Biochemistry 16*, 2657–2667.
Schmidt, C. F., Barenholz, Y., & Thompson, T. E. (1977) *Biochemistry 16*, 2643–2656.
Seiter, C. H. A., & Chan, S. I. (1973) *J. Am. Chem. Soc. 95*, 7541–7553.
Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 4573–4581.
Small, D. M. (1967) *J. Lipid Res. 8*, 551–557.
Small, D. M. (1971) *The Bile Acids* (Nair, P. P., & Kritchevsky, D., Eds.) Vol. 1, Chapter 8, Plenum Press, New York and London.
Small, D. M., & Bourges, M. (1966) *Mol. Cryst. 1*, 541–561.
Small, D. M., Bourges, M., & Dervichian, D. G. (1966) *Biochim. Biophys. Acta 125*, 563–580.
Small, D. M., Penkett, S. A., & Chapman, D. (1969) *Biochim. Biophys. Acta 176*, 178–189.
Stigter, D., & Mysels, K. J. (1955) *J. Phys. Chem. 59*, 45–51.
Van Holde, K. E. (1971) *Physical Biochemistry*, Chapter 9, Prentice-Hall, Englewood Cliffs, New Jersey.
Yedgar, S., Barenholz, Y., & Cooper, V. G. (1974a) *Biochim. Biophys. Acta 363*, 98–111.
Yedgar, S., Hertz, R., & Gatt, S. (1974b) *Chem. Phys. Lipids 13*, 404–414.

Comparison of Fluorescence Energy Transfer and Quenching Methods to Establish the Position and Orientation of Components within the Transverse Plane of the Lipid Bilayer. Application to the Gramicidin A–Bilayer Interaction†

Elizabeth A. Haigh, Keith R. Thulborn, and William H. Sawyer*

ABSTRACT: Fluorescence quenching and resonance energy transfer methods have been used to investigate the position of fluorophores in the lateral and transverse planes of the lipid bilayer. A series of n-(9-anthroyloxy) fatty acids (n = 2, 6, 9, and 12) have been used as energy-transfer acceptors so that apparent transfer distances from a membrane-bound donor (N-stearoyltryptophan) have a transverse as well as a lateral component. Both theory and experiment show that the energy-transfer method is not precise enough to discriminate between the positions of the fluorophores in the transverse plane of the bilayer. The n-(9-anthroyloxy) fatty acids are also susceptible to quenching by the indole moiety of tryptophan. The relative quenching efficiency can provide a semiquantitative measure of the position of quenching molecules in the lipid bilayer. The quenching techniques are applied to the determination of the orientation of gramicidin A in lipid bilayers. The tryptophan residues of gramicidin appear to be located near the membrane surface in agreement with the head-to-head dimeric structure proposed by D. W. Urry et al. [(1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676].

The molecular details of structure–function relationships in biomembranes must involve a description of the topographical distribution of membrane components. This is especially true for such complex processes as membrane transport and energy transduction where the disposition of the participating molecules in the membrane can have an important influence on the kinetics of the processes. The position and orientation of a membrane component in the transverse plane of the membrane, together with the proximity of one molecule to another in the lateral plane, are features of recognized importance; yet, relatively few techniques are available for their examination (for reviews, see De Pierre & Ernster, 1977; Peters & Richards, 1977).

An alternative approach, which is particularly suited to the study of natural as well as model membrane systems, employs spectroscopic probes which locate at different depths in the lipid bilayer and whose spectral characteristics change in response to the proximity of a given membrane component. In this paper we explore the use of fluorescence quenching and

† From the Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia. *Received February 7, 1979.* The work was supported by the Australian Research Grants Committee.

0006-2960/79/0418-3525$01.00/0