Kinetic and Structural Aspects of Reconstitution of Phosphatidylcholine Vesicles by Dilution of Phosphatidylcholine–Sodium Cholate Mixed Micelles†

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ABSTRACT: Dilution of mixed micellar dispersions of egg phosphatidylcholine (PC) and sodium cholate beyond a critical value results in formation of cholate-containing PC vesicles. The structure of the resultant vesicles and some mechanistic aspects of this process have been investigated by the use of light scattering and nuclear magnetic resonance techniques. The main findings and conclusions are the following: (1) Both the state of aggregation (micellar or vesicular) and the apparent equilibrium size distribution of micelles or vesicles obtained by dilution of the PC–cholate mixed micellar dispersions are a function of the cholate to PC molar ratio in the mixed aggregates (micelles or vesicles). When this effective ratio (R_e) is higher than 0.4, the dispersion is micellar, and the size of the mixed micelles increases with decreasing R_e; when $R_e < 0.3$, the dispersion is essentially vesicular, and the mean hydrodynamic radius of the vesicles is an increasing function of R_e; in dispersions with $0.3 < R_e < 0.4$, mixed micelles and vesicles coexist. (2) Addition of cholate to vesicular dispersions, to R_e values below 0.3, results in vesicle size growth through a concentration-independent lipid-exchange mechanism. Addition of cholate to higher R_e values results in micellization (solubilization) of the vesicles. On the other hand, dilution of vesicular dispersions does not affect the size of the vesicles. (3) Apparent equilibration of a mixed micellar dispersion following dilution to R_e values below 0.3 is slow (many hours). The overall process involves a series of three subsequent categories of steps: (i) a rapid (~1–2 min) prevesiculation equilibration of micellar sizes; (ii) a relatively rapid (20–30 min) membrane closure due to cholate depletion from large ("mixed-disc") mixed micelles (this results in the formation of small unilamellar vesicles); (iii) a slow postvesiculation size growth of the initially formed small cholate-containing vesicles. This series of vesicle size growth processes probably involves a net lipid exchange from small vesicles to larger (more stable) ones, through a residual fraction of PC–cholate mixed micelles. We suggest that for any given R_e apparent equilibrium is approached when the "off rate" of PC molecules from the vesicles becomes extremely slow. Thus, the apparent equilibrium size distribution may be a metastable state of aggregation.

Much of the recent progress in the understanding of biological membranes is due to the possibility of solubilizing them by detergents, which enables the purification of their various water-insoluble components. Subsequently, if the detergent used for solubilization is removed in the presence of phospholipids (with or without other components), lamellar structures (artificial membranes, model membranes, liposomes, vesicles) are formed.

A detergent commonly used for membrane solubilization and reconstitution is sodium cholate, although other bile salts have also been used. The size of the vesicles formed upon removal of these detergents appears to depend on the method of detergent removal. Small unilamellar vesicles can be formed by passing mixed dispersions of phospholipids and bile salts through a Sephadex G-50 column (Brunner et al., 1976). Larger vesicles are formed by dialysis of various detergents from mixed lipid–detergent dispersions. Fast (hollow-fiber) dialysis of sodium cholate has been used for the preparation of egg PC[1]–cholesterol vesicles with mean diameters varying form 340 to 1280 Å (Rhoden & Goldin, 1979). Another procedure, based on a fast and controlled dialysis of sodium cholate from PC–cholate mixed micelles (ca. a 2:3 molar ratio), yields homogeneous spherical unilamellar vesicles of a diameter of about 600 Å (Milsman et al., 1978). Dilution procedures have also been applied [e.g., Racker et al. (1975) and Wickner (1976)]. In some proteoliposome preparations, procedures based on dilution are more successful than cholate dialysis (Winget et al., 1977), perhaps because the detergents are rapidly removed from lipid–protein–detergent mixed aggregates in which the protein might be denatured by the detergent.

Formation of PC vesicles by dilution of PC–bile salt mixed micelles has recently been studied systematically (Startk et al., 1986; Schurtenberger et al., 1984, 1985). These studies showed that the radius of vesicles, formed upon dilution of mixed micellar dispersions, is a monotonic function of the bile salt to PC molar ratio in the bilayer of the resultant vesicles

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[1] Abbreviations: PC, phosphatidylcholine; cmc, critical micellar concentration; R_e, effective ratio, molar ratio of nonmonomeric cholate to PC in mixed aggregates; K_d, distribution coefficient of cholate between PC vesicles and the aqueous medium; QLS, quasi-elastic light scattering; NMR, nuclear magnetic resonance; OD, optical density; ϵ, molar optical density (OD/mol); $\bar{R}_h$, mean hydrodynamic radius of micelles and vesicles.

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(here denoted R_e). Moreover, if bile salt was added to the diluted (vesicular) dispersions at a concentration that was insufficient to cause solubilization, the added detergent caused an increase of the vesicle size. The resultant vesicles were similar to those obtained in dilution experiments to the same R_e. While these studies clearly demonstrate that the most dominant factor in determining the size of the vesicles is R_e, no conclusive explanation for this dependency has yet been offered. Furthermore, thermodynamic considerations failed to explain the fact that after formation of large vesicles by dilution of mixed micelles removal of residual bile salt had no significant effect on the vesicle size. Since in all these studies the formed vesicles were analyzed only after prolonged equilibration of the diluted dispersions (24–48 h after dilution), the mechanistic aspects of vesicle formation could not have been explicitly addressed.

We believe that a better understanding of the processes involved and more knowledge of the factors that govern the characteristics of model membranes will constitute a major contribution to the foundation of a more rational basis for reconstitution experiments. In this work, kinetic and structural studies of PC vesicle formation due to dilution of PC–cholate mixed micelles are presented and shed some light on the mechanistic aspects of vesicle reconstitution.

Materials and Methods

Materials. Phosphatidylcholine (PC) was prepared from egg yolk according to the description by Singelton et al. (1965). Its purity was confirmed by thin-layer chromatography. Sodium cholate was purchased from Sigma (St. Louis, MO) and used without further purification.

Preparation of Micelles and Vesicles. Mixed micellar dispersions of PC and cholate were prepared by codissolving the appropriate amounts of each lipid in a chloroform–methanol mixture (1:1) and then dried for 2 h in a rotavapor under reduced pressure at room temperature and subsequently lyophilized for additional 18 h. The lipid films were hydrated in 135 mM NaCl solution. PC–cholate mixed vesicular dispersions of different compositions were prepared by dilution of mixed micellar dispersions either with 135 mM NaCl solution or with 135 mM NaCl solution containing different amounts of sodium cholate.

Turbidity Measurements. Turbidity was measured usually at 450 and 330 nm on a Varian 635D double-beam spectrophotometer equipped with a Varian strip-chart recorder. The molar absorbance (turbidity per mole of lipid, here denoted ϵ) of a dispersion of vesicles of a given composition increases linearly with the hydrodynamic radius ($\bar{R}_h$) of the vesicles. For vesicles made of PC and cholate, $\bar{R}_h$ in angstroms can be estimated from the empirical relationship

$$\bar{R}_h = 8.33\epsilon_{450} + 64 \quad (1a)$$

or

$$\bar{R}_h = 2.63\epsilon_{330} + 45 \quad (1b)$$

for molar absorbance measured at 450 and 330 nm, respectively. This result is in accord with calculations of turbidity that employ the expression of Kerker (1969) for hollow spheres. The value of ϵ is therefore a measure of vesicle size. The validity of this conclusion was tested by studying the vesicle sizes in the equilibrated dispersions by electron microscopy and quasi-elastic (dynamic) light scattering (QLS). These techniques also provided data on the polydispersity of the dispersions.

Quasi-Elastic Light Scattering (QLS) Measurements. Mean hydrodynamic radius ($\bar{R}_h$) and polydispersity (V) of mixed PC–cholate vesicles were measured by a QLS apparatus as describes by Somjen & Gilat (1983). Prior to being measured, the samples were usually centrifuged at 100000g for 5 min to remove dust particles.

Electron Microscopy. Negatively stained vesicle preparations were examined on carbon-coated Formvar films on copper grids in a Jeol 100 B electron microscope operating at 80 kV, usually at 33000× magnification. The samples were mixed isotonically with ammonium molybdate solution (2%). After 1 min of incubation, it was applied to the grid, the excess solution was drained, and the preparation was examined immediately.

Nuclear Magnetic Resonance (NMR) Measurements. The NMR measurements were performed at room temperature at 8.4 T (145.7 MHz for ^{31}P and 360 MHz for ^{1}H) by using a Bruker AM 360-WB Fourier-transformation spectrometer. All spectra contained 4K and 8K data points for ^{31}P and for ^{1}H measurements, respectively. ^{1}H NMR spectra were obtained by using an 8 ppm sweep width, 1-s delay between acquisitions, and 1-μs pulse width. Proton-decoupled ^{31}P NMR spectra were obtained by using a 40–100 ppm sweep width, 1–2-s delay between acquisitions, and a 30-μs pulse width. NMR data at 90 MHz were obtained at room temperature with a Bruker WH-90 Fourier-transformation spectrometer.

Determination of Cholate to PC Ratio in Lipid Bilayers, R_e. For a mixture containing L mM phospholipid and D_t mM bile salt (D_w mM in the aqueous medium and D_b mM in the bilayer), R_e is given by $R_e = D_b/L$. Assuming an equilibrium distribution of the detergent between the bilayer and the aqueous medium, a distribution coefficient K has been defined (Schurtenberger et al., 1985) as

$$K = D_b/(LD_w) = R_e/D_w \quad (2)$$

Hence

$$D_t = D_b + D_w = R_eL + R_e(1/K) \quad (3)$$

and

$$R_e = D_t/(L + 1/K) \quad (4)$$

To obtain the effective ratio R_e, the distribution coefficient of cholate between PC bilayers and saline (K) has been determined as follows: Vesicles were made by cholate removal either by dialysis or dilution of PC–cholate mixed micellar dispersions. Following 48 h of incubation at room temperature, the mixtures were diluted with cholate-containing media and then reequilibrated for an additional 24 h. Subsequently, the dispersions were centrifuged to remove the vesicles. The supernatants of all the mixed dispersions studied contained no PC, as tested according to Stewart (1980). Their cholate concentrations (D_w), as well as the total cholate concentrations (D_t), were assayed in triplicate by the enzymatic method of Turnberg and Anthony-Mote (1969) with 3α-hydroxysteroid dehydrogenase and β-NAD^+ (Sigma). The plot of the cholate level associated with the vesicles ($D_b = D_t - D_w$) against the product of concentrations of lipid and aqueous detergent yields a straight line ($r^2 = 0.998$), which passes through the origin of the axes (not shown). The slope of this line $[D_b/(LD_w)]$ is the distribution coefficient defined above. It has a value of $K = 0.05$ mM^{-1} (or $1/K = 20$ mM) compared to $1/K =$ 13.5–16 mM obtained by Schurtenberger et al. (1984, 1985) for the distribution of glycocholate between egg PC vesicles and a saline solution. R_e is calculated from this value of $1/K$ according to eq 4: $R_e = D_t/(20 + L)$.

Results

Size of Vesicles in Diluted and Equilibrated PC–Cholate Mixtures: Dependence on R_e. Three different modes of varying R_e were used:

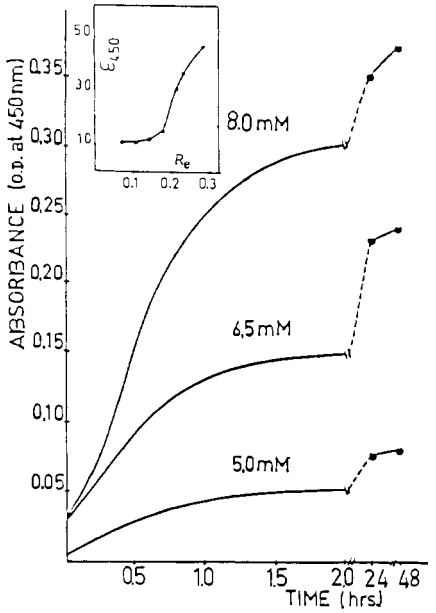

FIGURE 1: Time course of vesicle formation upon cholate removal from PC–cholate mixed micelles by dilution. A mixed micellar dispersion containing 100 mM PC and 100 mM cholate was diluted 1:12.5, 1:15.4, and 1:20 with 135 mM NaCl to yield mixed vesicular dispersions of different concentrations. The inset presents the molar absorbance (ϵ_{450}) of the resultant mixed vesicular dispersions at equilibrium, as a function of the cholate to PC effective ratio (R_e).

Table I: Mean Hydrodynamic Radii ($\bar{R}_h$) and Polydispersities (V) of Equilibrated PC–Cholate Mixed Vesicles, As Measured by QLS[a]

[PC] = [cholate] (mM)	R_e	$\bar{R}_h$ (Å)	V (%)
8.0	0.285	540	8.2
6.5	0.236	340	2.6
5.0	0.200	215	1.3
3.0	0.130	201	34.6
2.0	0.091	170	30.5

[a] Vesicles were prepared by different dilutions of a mixed micellar dispersion containing 100 mM PC and 100 mM cholate with 135 mM NaCl solution. The cholate to PC molar ratio in bilayers (R_e) was calculated according to eq 4. Polydispersity is as defined by Mazer et al. (1980).

(1) Cholate–PC mixed micellar dispersions were diluted serially such that the total cholate/PC ratio remained constant while the absolute concentrations varied, thus causing variation of R_e. For equal total concentrations of PC and cholate, any mixture with 8 mM or less of each component was clearly vesicular. Thus, a dilution of a micellar mixture containing 100 mM of each of these components by a factor of 12.5 (or more) resulted in vesiculation (Figure 1). Following dilution and equilibration, electron microscopy of the dispersion made by a 12.5-fold dilution (R_e = 0.285) revealed vesicles of an average radius of ca. 500 Å, whereas the radius of vesicles formed by a 50-fold dilution (R_e = 0.091) was ca. 150 Å (not shown). These values are consistent with the mean hydrodynamic radii as measured by QLS (Table I). QLS and turbidity measurements were also performed for vesicles made by other dilutions. Over the whole range of $0.091 < R_e < 0.285$, the polydispersities of the vesicle preparations were relatively low and $\bar{R}_h$ was an increasing function of R_e (Table I). The same conclusion can be reached on the basis of turbidity measurements (inset to Figure 1).

(2) PC–cholate mixtures were diluted into media containing varying concentrations of cholate to yield vesicles of a constant PC concentration and varying levels of cholate. In these experiments, for any given PC concentration, the turbidity reached a maximum at a certain cholate concentration (Figure 2). Noteworthy is the finding that the maximal value of the molar turbidity (ϵ_{450} = 52 ± 1 OD units/mol of lipid) is independent of PC concentration (inset to Figure 2) and occurs at a constant R_e value of 0.30.

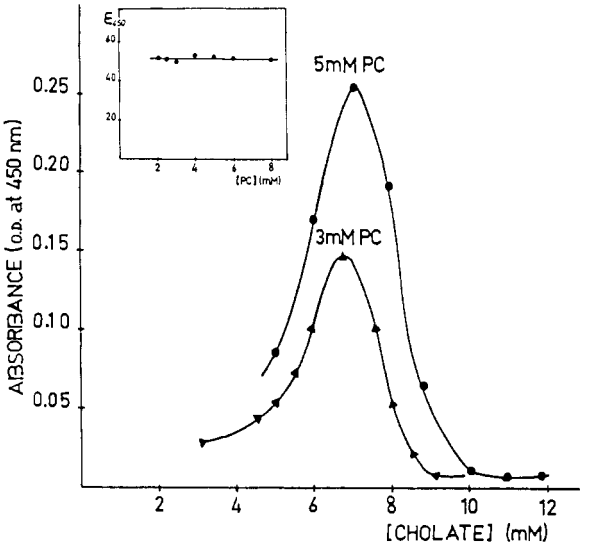

FIGURE 2: The dependence of turbidity on the cholate concentration in equilibrated PC–cholate mixed dispersions containing constant PC levels and varying levels of cholate. The samples were made by a series of dilutions of a mixed micellar dispersion of 100 mM PC and 100 mM cholate in cholate-containing 135 mM NaCl solutions. For each PC concentration (2.0–8.0 mM), the final cholate concentrations were 2.0–12.0 mM. The triangles represent the turbidity of equilibrated samples with 3 mM PC, and the circles are of dispersions of 5 mM PC. The inset presents the maximal molar absorbance as a function of the PC concentration for all these experiments.

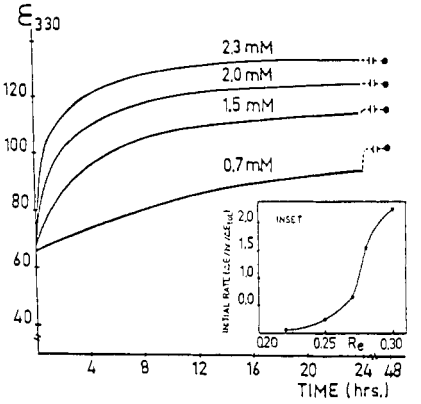

FIGURE 3: Time course of size growth of vesicles upon cholate addition. Cholate (0.7, 1.5, 2.0, 2.3, and 2.8 mM) was added to equilibrated mixed vesicular dispersions containing 5 mM PC and 5 mM cholate. Cholate incorporation into the bilayers resulted in R_e values from 0.20 to 0.31. Size growth was followed by turbidity measurements at 330 nm and is expressed in terms of the time-dependent increase of the molar absorbance (ϵ_{330}). The initial rate of each of these size growth processes is expressed in terms of the fraction of the total increase in turbidity that occurred in the first hour after dilution [$\Delta\epsilon\ h^{-1}$ (total $\Delta\epsilon)^{-1} = (\epsilon_{1h} - \epsilon_0)/(\epsilon_{48h} - \epsilon_0)$]. The dependence of initial rate on R_e is presented in the inset.

(3) Addition of cholate to diluted and equilibrated dispersions of vesicles resulted in a time-dependent increase of the molar turbidity, provided that R_e increased to a level still lower than 0.31. A new apparent equilibrium was established in less than 48 h (Figure 3). At this point, the value of ϵ was again an increasing function of R_e. Dilution of mixed micellar dispersions beyond the micellar → lamellar phase boundary (Figures 1 and 2), as well as cholate-induced size growth of equilibrated vesicles (Figure 3), resulted in the formation of vesicles whose molar absorbance (thus the hydrodynamic ra-

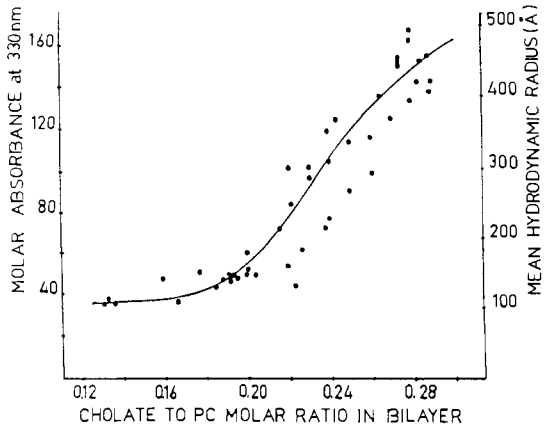

FIGURE 4: The dependence of molar absorbance of vesicular dispersions (ϵ_{330}) and mean hydrodynamic radius ($\bar{R}_h$) on the molar ratio of cholate to PC in the bilayers of vesicles (R_e). ϵ_{330} and R_e values were taken from the experiments shown in Figures 1–3 as well as several similar experiments. The mean hydrodynamic radius was calculated according to eq 1b.

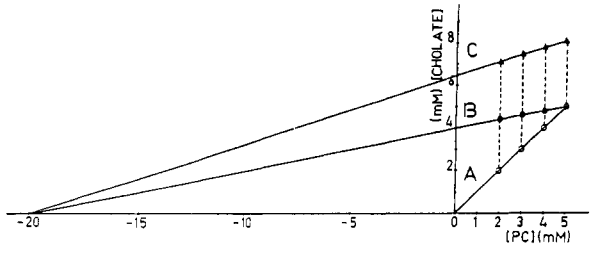

FIGURE 5: Protocol of an experiment designed to evaluate the dependence of vesicle size on R_e and postvesiculation dilution. A mixed vesicular dispersion containing 5 mM PC and 5 mM cholate (R_e = 0.20) was prepared by a 20-fold dilution (with 135 mM NaCl) of a mixed micellar dispersion and equilibrated for 48 h. Serial dilutions of this dispersion (with 135 mM NaCl) yielded dispersions with compositions indicated by line A. ϵ_{330} of all these dispersions was 66 ± 1 OD units. To reestablish an R_e of 0.20 for all the diluted dispersions, different amounts of cholate were added, 24 h after dilution. The compositions of the resultant dispersions are indicated by line B. This cholate addition had no effect on ϵ_{330}. Twenty-four hours later, a second portion of cholate was added to each of the dispersions to increase R_e to a value of 0.31. The new compositions are presented by line C. All the latter cholate additions resulted in time-dependent increases of ϵ_{330}. After 24 h of equilibration, all the dispersions had ϵ_{330} values of 149 ± 5 OD units.

dius) was a monotonic function of R_e. This is evident from Figure 4 in which data of Figures 1–3, as well as of several other similar experiments, were plotted in terms of the dependence of ϵ (and $\bar{R}_h$) on R_e.

Dilution of PC–Cholate Mixed Vesicles. In the experiment described in Figure 5, vesicles were prepared by a 20-fold dilution of a mixed micellar dispersion of 100 mM PC + 100 mM cholate. Following equilibration, this dispersion was diluted to yield a series of PC concentrations (4, 3, and 2 mM; line A). This did not result in any change of the molar absorbance (ϵ_{330} = 66 ± 1 OD units; not shown), indicating that decreasing R_e by dilution of dispersions containing large vesicles did not result in a decrease of vesicle size upon further equilibration. Similar results were obtained when an equilibrated dispersion of 8 mM PC and 8 mM cholate (R_e = 0.285) was serially diluted to yield dispersions with varying R_e values ($0.086 < R_e < 0.285$) and with a constant molar absorbance (ϵ_{450} = 45 ± 2 OD units; not shown).

Electron microscopy of vesicles made by a two-step dilution of the mixture of 100 mM PC and 100 mM cholate (12.5-fold dilution, 48-h equilibration, and subsequent 4-fold dilution) revealed vesicles of a mean radius of about 500 Å, similar to

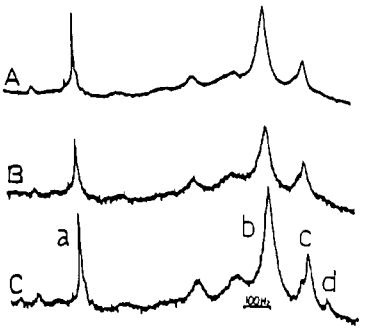

FIGURE 6: 360-MHz ^{1}H NMR spectra of PC–cholate mixed vesicles of different sizes and R_e values. Spectrum A was obtained from vesicles of an R_e of 0.285 and a mean hydrodynamic radius of 540 Å (Table I) prepared by 1:12.5-fold dilution of a mixed micellar dispersion containing 100 mM PC and 100 mM cholate. Spectra B and C are of vesicular dispersions containing 2 mM PC and 2 mM cholate (R_e = 0.091) prepared by two different procedures. Spectrum B is of a dispersion of vesicles of $\bar{R}_h$ = 540 Å made by a 4-fold dilution of a mixed vesicular dispersion of 8 mM PC and 8 mM cholate. Spectrum C is of a vesicle dispersion of $\bar{R}_h$ = 170 Å (Table I) made by a 50-fold dilution of a mixed micellar dispersion of 100 mM PC and 100 mM cholate. Peak assignment is as follows: (a) choline head group; (b) methylenes of PC superimposed on cholate signals; (c) terminal methyl groups of PC, superimposed on the 19-CH_3 of cholate; (d) 18-CH_3 of cholate.

the vesicles obtained after the first step of dilution (with R_e = 0.285) and quite different from the vesicles made by a 50-fold dilution (to R_e = 0.091) of the same micellar dispersion (not shown).

However, the difference in R_e did affect the ^{1}H NMR spectrum of the vesicles. Dispersions of different R_e values prepared by dilutions of mixed vesicles exhibited significantly different NMR spectra, even though the molar turbidities were identical and the sizes similar. Thus, all the signals in the ^{1}H NMR spectrum of a mixture with R_e = 0.285 (8 mM PC + 8 mM cholate; Figure 6A) were narrower than that of a mixture with R_e = 0.091 (2 mM PC + 2 mM cholate; Figure 6B), in spite of the similar hydrodynamic radius of the vesicles. The most informative signal (due to interpretation difficulties of other resonances) is the one of the *N*-methyl (choline) group of PC. For vesicles of a constant $\bar{R}_h$ (540 Å), this signal is broadened from ca. 4 to ca. 10 Hz upon dilution from an R_e = 0.285 to an R_e = 0.091 (not shown). This effect is consistent with previous NMR studies that have indicated that inclusion of bile salts in PC bilayers results in narrowing of all the NMR resonances as a result of reduction in the restriction of local motion within the bilayers (Stark et al., 1986; Brouillette et al., 1982; Lichtenberg et al., 1979). A similar effect might have been expected to result from a reduction in vesicle size (increase in vesicle curvature), but this effect is rather small. The vesicles made by a 50-fold dilution of a mixture of 100 mM PC + 100 mM cholate, despite being much smaller than those made by the two-step dilution procedure, gave rise to NMR signals (Figure 6C) that were only slightly narrower than those in the spectrum of the much larger vesicles of the same composition (Figure 6B). Thus, the choline head group in Figure 6C had a line width of 9 Hz, as compared to 10 Hz in Figure 6B.

The ^{31}P NMR line width was more sensitive to the size of the vesicles. As an example, a vesicle suspension made by a 25-fold dilution of a dispersion of 100 mM PC and 100 mM cholate to an effective ratio (R_e) of 0.167 gave rise to a ^{31}P resonance of a line width of 14 Hz. On the other hand, a two-step dilution procedure (12.5-fold dilution followed by equilibration, then a subsequent 2-fold dilution and an additional 6-h equilibration) resulted in a broader ^{31}P resonance

of 23 Hz (not shown), as could have been expected for vesicles of a larger average size.

Kinetic Studies on Vesicle Formation. To further explore the mechanism of vesicle formation and the possible dependence of size on kinetic factors, we examined the time dependence of the turbidity increase and NMR signal broadening that accompany the vesiculation of the PC–cholate mixture. Immediately after the addition of the micellar dispersions to the diluting aqueous medium (time zero in Figure 1), the turbidity was much higher than that of the original undiluted mixed micellar dispersions. About 10 min later, the dependence of log OD on the logarithm of the wavelength (log λ) was linear ($r^2 > 0.99$) with a slope of −4, as expected for Rayleigh scattering of light.

Since vesiculation of the PC–cholate mixed micelles may be preceded by a series of "micellar equilibration" processes, caused by the removal of cholate from the mixed aggregates (and a consequent growth of micellar size; Mazer et al., 1980), we found it of interest to investigate the time course of such equilibration. A 6.25-fold dilution of a mixture of 100 mM PC and 100 mM cholate did not result in vesiculation of the mixed micelles, yet the turbidity increased. The molar turbidity of the resultant micellar dispersion was about 7.5 at 330 nm, i.e., much lower than that of a dispersion of the smallest obtainable vesicles (about 35 OD units at 330 nm, 10 OD at 450 nm). The interesting finding is that equilibration was reached within 1 min (not shown). This fast reaction could not be followed by NMR. The NMR spectrum of the equilbrated dispersion was quite typical for micelles (not shown). As a conclusion, we suggest that in those experiments in which vesicles are being formed, the first step, namely, micellar equilibration, is too fast to be regarded as a major contributor to the time-dependent turbidity increase that follows dilution.

Another possible time-dependent process is the removal of cholate from PC–cholate mixed vesicles. This process did not affect the molar turbidity of vesicle dispersions (e.g., the experiment described in Figure 5). Therefore, it cannot have a major effect on the increase of turbidity observed after dilution. Moreover, this process is too rapid to make a significant contribution to the overall time-dependent increase of turbidity. This conclusion is based on an NMR experiment in which we have studied the time dependency of the choline head group line broadening (from 5 to 8 Hz) caused by depletion of cholate from preformed PC–cholate vesicles (of R_e = 0.285), brought about by dilution to an R_e value of 0.167. This process is likely to be slower than dilution-induced depletion of cholate from mixed micelles. However, even the cholate depletion from vesicles is apparently completed in less than 12 min (not shown) and can therefore play only a minor role in the overall process, as reflected by the increase of turbidity (e.g., Figure 1).

In the course of the overall process, it is rather difficult to determine the particular time at which the arrangement of the lipids in the diluted mixtures becomes mostly vesicular. However, the following experiments indicate, indirectly, that this occurs in a relatively short period (10–20 min). First, if cholate is added prior to equilibration, the turbidity of the equilibrated dispersion is similar to that of a dispersion of the same composition made by including the additional cholate in the diluting medium or by adding the same cholate concentration after apparent equilibration (Figure 7). It is of interest that the time differences between all these size-transformation processes are very small. And, most importantly, in our view, the sigmoidal character of the increase of

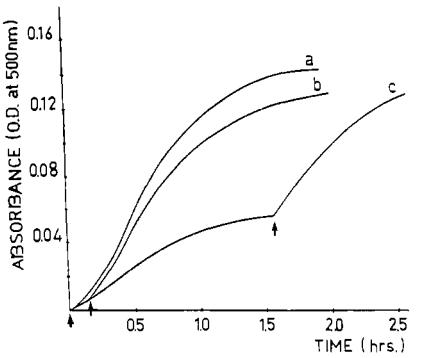

FIGURE 7: Time course of vesicle formation upon cholate addition to PC–cholate dispersions at different times after dilution. A mixed micellar dispersion containing 100 mM PC and 100 mM cholate was divided into three aliquots and diluted 1:20 with 135 mM NaCl. In (a), the dilution was immediately followed by addition of 1 mM cholate. The second and third aliquots were treated as indicated above, but in (b) and (c) cholate was added 0.15 and 1.6 h after dilution, respectively, as shown by the arrows. The turbidity of all samples was measured at 500 nm and is expressed in OD units.

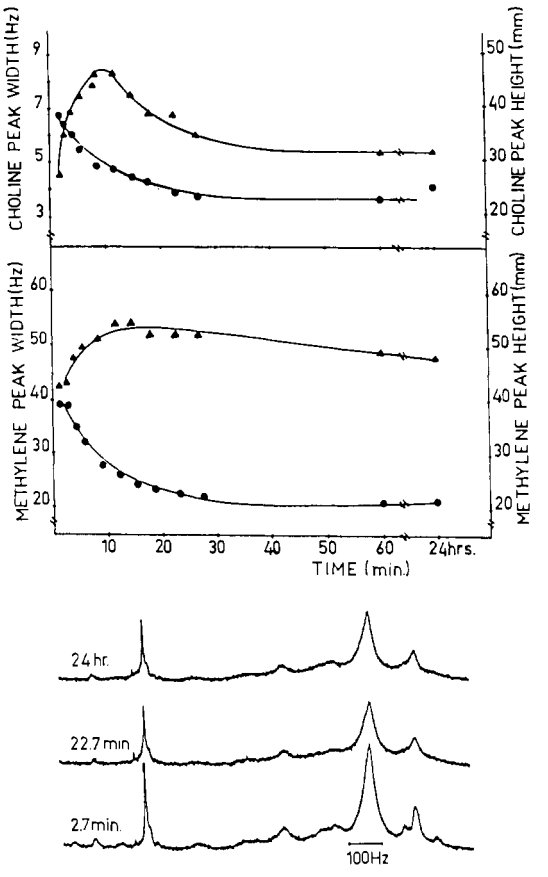

FIGURE 8: Time course of vesicle formation upon dilution of PC–cholate mixed micelles as studied by 360-MHz ^{1}H NMR spectroscopy. A micellar dispersion of 100 mM PC and 100 mM cholate was diluted 12.5-fold. ^{1}H NMR spectra were measured at various times. The figure shows three representative spectra taken 2.7 min, 22.7 min, and 24 h after dilution. Apparent peak heights (circles) and peak widths (triangles) of the resonances due to the choline head group and methylene protons are also presented as a function of time.

turbidity is only apparent if cholate is added less than 15 min after dilution. This finding may be explained if vesiculation takes about 15 min.

This explanation is consistent with the ^{1}H NMR measurements of this transformation (Figure 8). In general terms, given all the uncertainties imposed on the system by its polydispersity during postdilution equilibration, two empirical parameters, the peak heights and peak widths, can be extracted

for each of the ^{1}H NMR resonances. The heights of both the choline head group and the methylene signals decreased monotonically in about 20 min to an apparent equilibrium. Twenty-four hours later, the heights were still similar to those observed 20 min after dilution. On the other hand, the half-widths of these signals increased to a maximal value in about 10 min (ca. 55 Hz for the "methylene envelope" and about 8.5 Hz for the choline resonance). Subsequently, both these resonances became narrower, approaching an apparently constant level (ca. 49 Hz for the methylene and 5.5 Hz for the choline signal) in about 30 min. This complex behavior is due to the different sensitivity of these two parameters measured as a function of time to the processes involved in the equilibration of the diluted sample. The data of Figure 6 show that for cholate-containing vesicles of a given cholate content, the size of the vesicles over the range of 170 Å < $\bar{R}_h$ < 540 Å affects the apparent line widths only slightly. However, for smaller vesicles, the different packing of phospholipid molecules in the inner and outer monolayer of vesicles causes a difference between the chemical shifts of protons of these lipids (10 Hz for the two choline head group signals in vesicles of $\bar{R}_h$ = 80 Å and 5 Hz in vesicles of $\bar{R}_h$ = 130 Å; Brouillette et al., 1982).

The data of Figure 8 can therefore be interpreted in terms of a relatively rapid vesiculation (ca. 10 min) followed by size growth. This vesicle size growth affects only slightly the intrinsic widths of the various signals but causes an apparent narrowing by reducing the chemical shift difference between the signals of phospholipid molecules located in the inner and outer monolayers. This interpretation is strengthened by the ^{1}H NMR measurements at 90 MHz (not shown), in which the line width of the choline head group signal increased monotonically, to about 7 Hz in less than 30 min. No subsequent line narrowing was observed, probably because the expected change of chemical shift difference between inner and outer monolayer signals (from ca. 2 to ca. 1 Hz upon increase in $\bar{R}_h$ from 80 to 130 Å) is not sufficient to considerably affect the overall apparent line width.

On the basis of our kinetic studies, we suggest that the very rapid "micellar equilibration" is followed by a rapid phase transformation (vesiculation), which is most likely completed in about 10 min. Following this stage, the time-dependent increase of turbidity (Figure 1) is therefore due to "postvesiculation" size growth.

Postvesiculation Size Growth Processes. Postvesiculation time-dependent size transformation can occur either through cholate-induced vesicle–vesicle fusion or through cholate-catalyzed lipid-exchange steps, in the course of which a part of the vesicles grow at the expense of the disintegration of other vesicles. Differentiation between the two possible modes of vesicle size growth is difficult because the cholate-containing vesicles are permeable to various solutes that could have been used to monitor fusion-induced mixing of aqueous compartments of the vesicles. In an attempt to shed light on the mechanism of size growth, we have designed experiments to evaluate the effect of two parameters on the rate of this process. First, we have studied the effect of R_e on size growth of vesicles of a fixed concentration and size. We added 0.7–2.8 mM cholate to a series of samples of an equilibrated vesicle dispersion of 5 mM PC and 5 mM cholate (R_e = 0.20; $\bar{R}_h \simeq$ 180 Å) to increase R_e to values in the range of 0.23–0.31. The resultant time-dependent increase of turbidity is accelerated by increasing R_e (Figure 3). Of special interest is the very marked acceleration observed upon increasing R_e from 0.27 to 0.30.

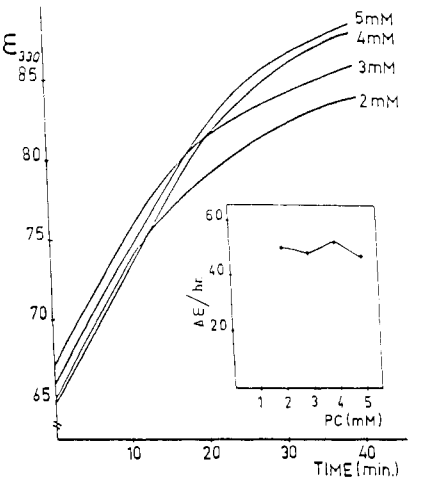

FIGURE 9: Time-dependent cholate-induced size growth of vesicles. In all the experiments, the vesicles had the same initial size ($\bar{R}_h \simeq$ 215 Å) and the same initial and final R_e values (0.20 and 0.31, respectively). The various recordings differ in the total lipid concentration, as indicated in this figure. The details of the experiment are described in Figure 5, lines B and C. The inset to the figure presents the intial slopes (calculated from the increase of ϵ_{330} in the first 15 min and expressed as $\Delta\epsilon_{330}$/h) as a function of (vesicular) PC concentration.

Second, we investigated the dependence of the rate of size growth on vesicle concentration. This was done by preparing vesicles of constant size ($\bar{R}_h \simeq$ 215 Å) and cholate to PC effective ratio (R_e = 0.20) at various concentrations. The protocol of this experiment is described by line B in the inset to Figure 5. Addition of cholate to the same R_e (0.31; line C) resulted in a time-dependent size increase, which ultimately yielded vesicles of the same size ($\bar{R}_h \simeq$ 540 Å). Remarkably, the rate of this process did not depend on the vesicle concentration (Figure 9, inset).

Discussion

The complete dependence of the turbidity of equilibrated bile salt–lecithin mixtures on R_e is described schematically in Figure 10. On the basis of the data of experiments such as the ones described by Figure 2, it can be concluded that the maximal (apparent equilibrium) turbidity is obtained at R_e = 0.3. At higher R_e values, solubilization begins, and at R_e > 0.4, all the lipid is contained in PC–cholate mixed micelles. In any mixture with 0.3 > R_e < 0.4, mixed micelles and vesicles coexist.

Since distribution of added detergent between the bilayers and the medium is rapid, solubilization (of unilamellar vesicles) is also quite fast. On the other hand, a reduction of R_e, brought about by dilution of a PC–cholate mixed micellar dispersion beyong the micellar to lamellar phase boundary, triggers a complex series of processes, ultimately yielding vesicles whose radius increases with the ratio of cholate to lecithin in these lamellar aggregates (Schurtenberger et al., 1984, 1985; Stark et al., 1986; Figure 4).

The initial driving force for these transformations is the removal of bile salt molecules from the lipid–detergent mixed micelles caused by dilution. To avoid the excess energy that stems from the consequent exposure of PC hydrophobic regions to the aqueous medium (Tanford, 1980), a series of micelle–micelle interactions is initiated. This leads to micellar equilibration, which will result in large (mixed) disclike micelles. Apparently, this series of processes is faster than any alternative mechanism. According to Lasic (1982), a subsequent "bending" of large micelles to form "curved micelles"

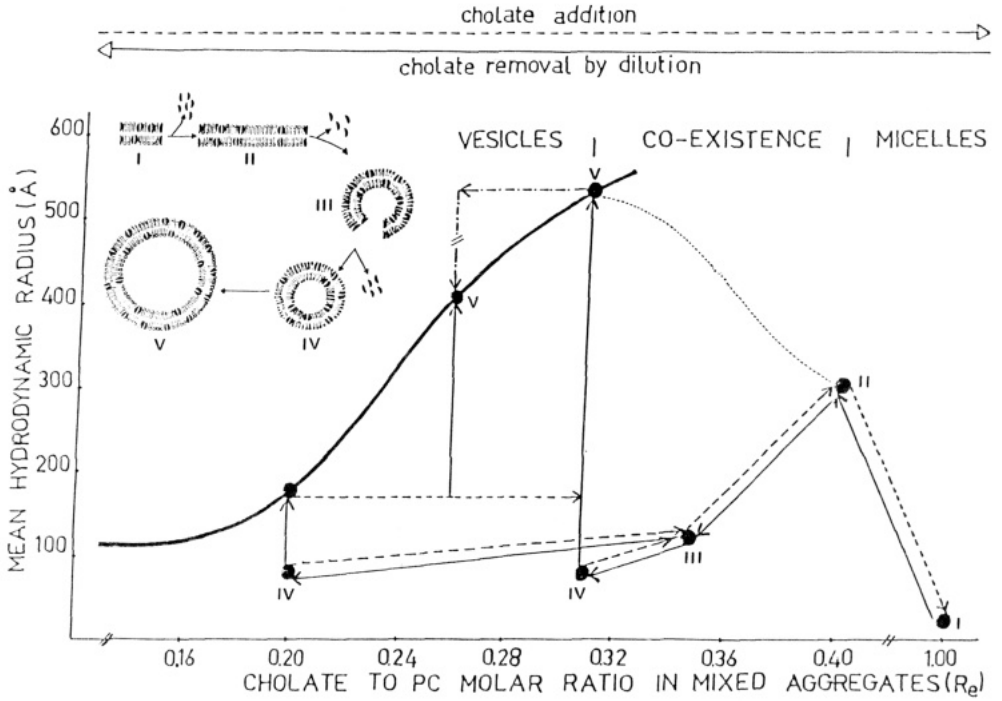

FIGURE 10: Schematic representation of the aggregational states and micellar–lamellar phase transformations in PC–cholate mixed dispersions. The hydrodynamic radius of PC–cholate mixed aggregates ($\bar{R}_h$) is described as a function of the cholate/PC molar ratio in mixed aggregates (R_e). The thick solid line (taken from Figure 4) presents the apparent equilibrium hydrodynamic radii of vesicles in the pure vesicular phase ($R_e < 0.3$). The size of mixed micelles in the pure micellar phase ($R_e \geq 0.4$) decreased with increasing R_e (Mazer et al., 1980). At $R_e \simeq 0.35$, 50% of the lipid is contained in each of these types of aggregates. For $0.3 < R_e < 0.4$, mixed vesicles and micelles coexist. Dilution of micelles with $R_e > 0.4$ to $R_e < 0.3$ results in removal of cholate from the micelles, causing transformation of small micelles (I) to larger ones (II), which bend upon further removal of cholate to form curved mixed micelles (III). Subsequently, small vesicles are formed (IV) in which R_e rapidly approaches its final value. These vesicles then spontaneously undergo size growth through lipid-exchange steps, ultimately yielding larger unilamellar vesicles (V). These processes, induced by cholate removal from mixed micellar dispersions, are described by solid lines. Cholate addition to vesicles (broken lines) to R_e values below 0.3 results in a corresponding vesicle size growth (solid lines at constant R_e values). Addition of cholate to R_e values above 0.4 results in solubilization and subsequent decrease of micellar size. Dilution of vesicles (broken lines and dots) results in a decrease of R_e without affecting $\bar{R}_h$.

can occur. The amplitude of this bending is likely to increase as the micelles become larger. Finally, at a critical micellar size, the amplitude of the bending is sufficient to cause bilayer closure. This mechanism would lead to vesicles of sizes proportional to that "critical micellar size". Nonetheless, the initially formed vesicles still undergo size transformation processes, similar to those obtained upon addition of subsolubilizing detergent concentrations to postdilution-equilibrated vesicular dispersions (Figures 3, 5, and 7). Similar size growth was also observed for PC sonicated vesicles upon addition of subsolubilizing concentrations of deoxycholate (Enoch & Stritmatter, 1979), sodium taurocholate (Lichtenberg et al., 1984), and sodium glycocholate (Schurterberger et al., 1985).

Thus, three categories of steps occur in the overall apparent equilibration of the diluted cholate–PC mixtures, namely, micellar equilibration, vesiculation, and postvesiculation size growth. In addition, if the cholate concentration in the initially formed vesicles is higher than the equilibrium concentration of cholate in the bilayer, the depletion of cholate from these vesicles has also to be considered. NMR data indicate that upon dilution of PC–cholate mixed vesicles, reequilibration of cholate levels in the bilayers is completed in less than 15 min (not shown). This suggests that equilibration of cholate levels in vesicles formed upon dilution of PC–cholate mixed micelles is much faster than the postvesiculation size growth, which may take many hours.

The postvesiculation time-dependent size transformation is independent of vesicle concentration (Figure 9). This concentration-independent process is inconsistent with a fusion mechanism. Studies on the kinetics of vesicle fusion (Wilschut et al., 1980; Nir et al., 1983) demonstrated an increase in the overall rate with vesicle concentration. It has been established that vesicle fusion consists of a second-order aggregation step, followed by the actual first-order fusion step (Nir et al., 1980, 1983; Bentz et al., 1983). An independence of the overall fusion rate on vesicle concentration implies that the aggregation step does not affect the overall rate of fusion, which means that after a certain period all the vesicles are aggregated. It was shown (Bentz & Nir, 1981) that dilution of a suspension of aggregated vesicles must result in a certain reduction in molar turbidity due to a reduction in average aggregate size. However, a dilution of our suspensions did not result in changes in the molar turbidity (Figure 5). Furthermore, aggregated large vesicles (hollow spheres of radii of 400–500 Å) cannot satisfy the observed result that log OD is linearly dependent on log λ with a slope of -4 [see expressions in Kerker (1969)]. We therefore suggest that cholate-induced vesicle–vesicle fusion plays a minor role in this size growth. Accordingly, we suggest that the size growth occurs through cholate-catalyzed lipid-exchange steps, in the course of which a part of the vesicles grow at the expense of the disintegration of other vesicles. This mechanism is more likely to be independent of vesicles concentration, especially if the rate-limiting step in the overall process is the "off-rate" of lipids from lipid–detergent mixed vesicles [similar to various other lipid-exchange processes; e.g., Frank et al. (1983) and references cited therein]. Throught this (rate-limiting) step, the concentration of aqueous PC (monomeric or in PC–cholate mixed micelles) is maintained constant throughtout the process. Hence, an apparent zero-order kinetics is observed. If PC–cholate mixed micelles are involved in the size growth, the concentration independence of this process may be the result

of fusion of vesicles with these PC–cholate mixed micelles, whose concentration is maintained constant through a (rate-limiting) self-rupture process of the small vesicles.

This rate-limiting process may ultimately lead to the most stable distribution of particle sizes in mixtures of any given composition. However, as the size becomes larger, the stability of the vesicles increases; thus, self-rupture becomes less likely. In addition, packing of PC molecules in the larger vesicles is tighter (Sheetz & Chan, 1972; Suurkuusk et al., 1976; Lichtenberg et al., 1981). Consequently, the off-rate of PC molecules can be expected to decrease. Therefore, the size transformation may be expected to be considerably slowed down and, in practical terms, it may stop prior to establishing a true state of equilibrium, yielding a metastable state of aggregation. The presence of cholate in the bilayers is likely to increase the off-rate of PC from vesicles of any size. Consequently, at high R_e values only the formation of significantly larger vesicles will be sufficient to result in a reduction of the off-rate to levels below a critical value, which is apparently zero. Hence, for any given bile salt to lecithin ratio, the vesicles undergo size transformation processes until the size is such that the off-rate of lecithin molecules from the bile salt containing vesicles is too slow to allow for a further size growth. After an apparent equilibrium has been reached, additional "subsolubilizing" concentrations of cholate to a vesicular dispersion increase the off rate, leading to a further growth in size. On the other hand, dilution of a dispersion containing large vesicles (with high R_e values) and the consequent reduction of R_e do not result in a decrease of $\bar{R}_h$.

The dependence of vesicle size on the bile salt to lecithin effective ratio has been previously analyzed by Schurterberger et al. (1985) in terms of the theory of Israelachvilli et al. (1977), which is based on equilibrium thermodynamics. The calculations based on this theory led to reasonable predictions of the dependence of $\bar{R}_h$ on R_e only if the specific volume of the hydrocarbon region of the bile salt was at least 1500 Å^3, as compared to the estimate of 600 Å^3 based on the geometrical dimensions and partial specific volume (Rowland & Woodley, 1980). This discrepancy may be explained by the different structure and shape of the bile salt molecules in comparison to the phospholipid molecules, as proposed by Schurtenberger et al. (1985). However, we suggest that while there is an equilibrium distribution of bile salt between the bilayers and the aqueous medium, the size *does not* represent a state of equilibrium and its dependence on R_e stems from the factors described above. Therefore, the vesicle size cannot be analyzed in terms of the model of Israelachvilli (1977) or any other model based solely on equilibrium thermodynamics. However, the alternative explanation of Schurtenberger et al. (1985) cannot be ruled out at the present time.

In conclusion, we believe that our data can best be interpreted if it is assumed that the most stable arrangement of PC in aqueous media is that of large unilamellar vesicles of $\bar{R}_h > 600$ Å. Experimentally, vesicles of $\bar{R}_h$ close to 600 Å are formed only when the membranes are rich in cholate ($R_e \simeq 0.3$). We postulate that for any mixture with $R_e < 0.3$ the apparent equilibrium of the dispersion (with $\bar{R}_h < 600$ Å) represents a metastable (nonequilibrium) state of aggregation. Larger vesicles would have been formed if there would have been an efficient mechanism through which size transformation could have occurred. Lipid exchange constitutes such a mechanism provided that the rate of removal of PC from the cholate-containing vesicles is sufficiently rapid. This is the case for small vesicles with high cholate content. However, reduction of R_e and/or an increase of $\bar{R}_h$ would reduce this off-rate. Thus, the size of vesicles formed by any cholate-removal procedure is likely to be primarily a function of the rate of detergent removal since this factor will determine at what state the size growth due to lipid exchange will stop.

The generality of this conclusion, with respect to its applicability to other lipids and detergetns and the effect of various proteins on the different processes involved and, consequently, on the ultimate size and heterogeneity of the reconstituted membranes, has yet to be studied.

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Registry No. Sodium cholate, 361-09-1.

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Mixtures of a Series of Homologous Hydrophobic Peptides with Lipid Bilayers: A Simple Model System for Examining the Protein–Lipid Interface†

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ABSTRACT: The interactions of several members of a homologous series of peptides with the phospholipid bilayer have been examined by using fluorescence and deuterium NMR spectroscopy, differential scanning calorimetry, and measurements of water-to-bilayer partition coefficients. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers and tripeptides of the form Ala-X-Ala-*O-tert*-butyl are used as a model system to probe the influence of amino acid side-chain substitution on the insertion of peptides into membranes and the behavior of peptide/bilayer mixtures. Tripeptides with X = Gly, Ala, Phe, and Trp have been examined. All of the tripeptides are water soluble, and all partition into DMPC bilayer vesicles to some extent. The Gly-containing peptide is the least soluble and the Trp-containing peptide the most soluble in the bilayer. The extent of perturbation of the bilayer structure induced by the peptides parallels their bilayer solubility: the Gly and Ala peptides act as simple impurities while peptides containing bulky aromatic rings cause a phase separation. Changes in the fluorescence properties of the Trp analogue upon incorporation into the bilayer indicate that the Trp side chain is probably immersed in the hydrocarbon region of the bilayer. Peptides of this form should serve as easily modifiable model systems with which to examine details of how the bilayer environment affects peptide conformation, as well as how hydrophobic peptides affect the bilayer structure.

Most physical models for the insertion and translocation of proteins and peptides into and across membranes invoke relatively simple partitioning processes that rely upon knowing the free energy of transfer of the hydrophobic portions of proteins from water into the lipid bilayer (Engelman & Steitz, 1981; Jahnig, 1983; Sabitini et al., 1982; von Heijne & Blomberg, 1979; Wickner, 1979). Thus, it is of fundamental importance to understand how the hydrophobic portions of proteins interact with their surroundings. Almost all analyses of the protein insertion/translocation problem make four assumptions: (1) The interior of the bilayer can be treated as a simple bulk hydrophobic phase. (2) Partitioning data for the transfer of hydrophobic amino acid side chains from water to bulk organic phases provide an adequate quantitative basis for the partitioning process. (3) All membrane-spanning proteins are comprised largely of either α or 3_{10} helixes. (4) Protein/water interfacial area analyses which are useful for examining globular proteins in water (Chothia, 1976; Richards, 1977; Guy, 1985) are equally applicable to membrane proteins. These assumptions are not necessarily unreasonable, and some parts of the analyses based upon them are compelling. On the other hand, direct experimental data adequate for the evaluation of these assumptions are lacking. The complexity of intact biological membranes makes it difficult to use them for this purpose. One must therefore turn to simpler systems to examine questions of the solubility of portions of proteins (i.e., peptides) in the bilayer, lipid perturbation induced by the incorporation of peptides, location of the peptides in the bilayer, peptide conformational and motional changes induced upon transfer from H_2O to the bilayer, and the effects of changes in the primary structure of the peptide.

Several model systems aimed at elucidating one or more of these properties are being investigated in various laboratories. Davis and co-workers have employed ^2H NMR and other techniques to examine leucine oligomers that span the bilayer (Huschilt, Hodges, & Davis, 1985; Davis et al., 1983).

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