Micelle–Vesicle Transition in Phospholipid–Bile Salt Mixtures. A Study by Precision Scanning Calorimetry†

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ABSTRACT: A systematic study of the micelle–vesicle transformation in phospholipid–bile salt mixtures using differential scanning calorimetry (DSC) indicates that the lipid undergoes a variety of changes in its thermal properties as mixed micellar solutions are diluted to concentrations at which vesicles form. In the experiments, micellar solutions of 50 mg/mL total lipid, containing sodium taurocholate (TC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), are diluted to concentrations corresponding to differing extents of aggregation of the TC with phospholipid. Turbidity and equilibrium dialysis measurements are used to establish boundaries between where micelles persist and where vesicles are formed and to determine the extent of aggregation of the TC with DPPC. At molar ratios R_e of bound TC to DPPC greater than 0.3, micellar solutions are formed, while at $R_e < 0.15$ vesicles are evident upon dilution. As the transformation from micelles to vesicles occurs, the thermal transitions in the lipid change from broad, low C_p (max) peaks in the micelle region to multiple peaks of high cooperativity in regions of composition where lamellar structures and vesicles form. The DSC curves show that in the composition region corresponding to where bilayer micelles exist a new thermal phase forms, which has a melting transition near 32 °C, if the solutions are allowed to equilibrate for 48 h at 21 °C. Furthermore, at compositions between $R_e = 0$ and 0.25, there is metastability in the lipid when equilibrated at 21 °C, but heating the lipid through the thermal transitions leads to reversible behavior. Interpretation of the enthalpy effects for the two transitions that occur between $R_e = 0$ and 0.2 shows that there could be a specific complex formed between phospholipid and bile salt that immobilizes 3 mol of lipid per mole of bile salt. Also, the van't Hoff enthalpies of several of the peaks show discontinuities at the boundaries between where vesicles are present and where micelles are formed. These results indicate that cooperativity in the lipid is affected by the gross morphology of the lipid–surfactant aggregates.

The behavior of micellar mixtures of surfactants and phospholipids upon dilution is of considerable interest in lipid biophysics. Dilution of the mixed micelles leads to formation of large unilamellar vesicles (Schurtenberger et al., 1985; Stark et al., 1985; Almog et al., 1986; Ollivon et al., 1988), and this process can be used for reconstitution of model membrane systems (Milsmann et al., 1978). Interest in various ways to prepare vesicles has been stimulated by the potential for using lipid vesicles in drug delivery (Szoka & Papahadjopoulos, 1980). In addition, the specific changes in morphology, composition, and properties of mixed micelles and vesicles, particularly in bile salt–phospholipid mixtures, are of vital importance in understanding the mechanisms of lipid transport and fat digestion in biliary physiology (Carey et al., 1983).

The properties of mixed micelles and mixed vesicles in bile salt–lipid systems have been studied by a number of methods under a variety of stages of dilution and composition. The early work of Small (1967) and of Mazer and Carey (1983) indicates that, in bile salt–lecithin mixtures in which the ratio of bile salt to lipid is high, bilayer disk micelles form. Dilution or dialysis of these bilayer micelles to remove the detergent from the solubilized lipid ultimately leads to the formation of large unilamellar vesicles (Schurtenberger et al., 1985). The reverse process of solubilizing vesicles through addition of detergent has also been carefully studied (Schubert et al., 1986; Schubert & Schmidt, 1988; Malloy & Binford, 1990).

The transformation from micelles to vesicles is a complex process. Quasielectric light scattering has been used to define the sizes of the aggregates and the boundaries in the transition region between micelles and vesicles in bile salt–lecithin mixtures (Schurtenberger et al., 1985, 1986). Nuclear magnetic resonance measurements have helped to characterize the transition region and to confirm that vesicles do form upon dilution (Stark et al., 1985). Recent reports of small-angle neutron scattering on bile salt–egg lecithin mixtures indicate that there may be rods and extended sheet structures present, depending on the specific composition and state of dilution of the mixtures (Hjelm et al., 1988, 1990). Important to understanding the behavior of mixed surfactant–lipid mixtures is the aggregation equilibria between the water-soluble detergent component and the insoluble lipid. The binding equilibria between bile salts and lecithin have been studied by dialysis and centrifugation methods, so that the actual composition with respect to the two components in the aggregated state is known (Duane, 1975, 1977; Spink et al., 1982; Schubert & Schmidt, 1988). Similar studies have been reported for octylglucoside–phospholipid mixtures (Ollivon et al., 1988). These results reveal that it is vitally important to have actual compositional data on the aggregated structures to interpret results of physical measurements on the systems.

Because the state of aggregation and the particular forms present in bile salt–phospholipid mixtures are quite variable and concentration dependent, it is desirable to have physical methods that can respond to the subtle changes that occur as the composition and the structures of the aggregated forms change upon dilution of the micellar solutions. Differential scanning calorimetry (DSC)[1] has been used to study a variety

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of systems in which thermally active bilayer phospholipids are involved. It has been shown that bile salt–lecithin bilayer micelles are thermally active if the phospholipid component is a synthetic diacylphosphatidylcholine such as dipalmitoylphosphatidylcholine (DPPC) (Spink et al., 1982). Vesicles of DPPC and related lipids also show clear thermal transitions (Schullery et al., 1980; Suurkuusk et al., 1976; Stankowski et al., 1979). The work outlined below was undertaken to examine the thermal transitions at a variety of phospholipid–bile salt compositions of the aggregated forms that occur as the mixed micelles are diluted from the micellar region through intermediate concentrations and to compositions corresponding to unilamellar vesicles. Because the thermal transitions of DPPC have been well characterized, this phospholipid was chosen for study in the bile salt mixtures. The experiments are designed to use the thermal activity of DPPC as a probe of the structures and state of aggregation in the bile salt–phospholipid system upon dilution of micellar solutions of the two components. The calorimetric and van't Hoff enthalpies and the temperature at which the various thermal transitions occur are sensitive to the lipid environment and are evaluated for the various observed transitions. Equilibrium dialysis measurements on the mixtures are made to provide actual compositional data on the aggregated forms. Since the thermal transitions contain information about the physical state of the aggregated lipid, the data are used to monitor the changes in the lipid–bile salt interactions as transformation from micelles to vesicles occurs.

Experimental Procedures

Materials. Sodium taurocholate was obtained from Sigma Chemical Co., St. Louis, MO, and DPPC and egg lecithin were from Avanti Polar Lipids, Birmingham, AL. The lipids and bile salt were examined by thin-layer chromatography ($CHCl_3/MeOH/H_2O$ 65:30:5) and showed only a single spot when visualized by sulfuric acid charring. Thus, both reagents were used as received. All buffer components were reagent grade, and the organic solvents used in sample preparation were of spectroscopic quality.

Sample Preparation. Stock micellar solutions of taurocholate and DPPC were prepared by mixing appropriate volumes of a chloroform solution of DPPC with a methanol solution of taurocholate, evaporating the organic solvents under argon, and removing the last traces of the solvent in a vacuum oven overnight. The micellar solution, usually at a concentration of 50 mg/mL total lipid and at TC/DPPC ratios of 1.8:1 or 1:1, was constituted by adding a buffer solution consisting of 0.02 M phosphate and 0.15 M NaCl at pH 7.4, heating to 45 °C, and mildly vortexing the solution to aid the dissolution process. These stock solutions were then diluted to the appropriate level by mixing with additional buffer and storing under argon in the dark for a minimum of 48 h at 21 °C.

Equilibrium Dialysis Measurements. In order to determine the actual stoichiometry of the taurocholate–DPPC aggregates, equilibrium dialysis measurements were performed with either 1 or 5 mL of cells according to the basic method of Duane (1977). The membranes were Technitron membranes with a 10000 MW cutoff obtained from Fisher Scientific, Pittsburgh, PA, and were boiled in dilute sodium bicarbonate, followed by copius rinsing with distilled water. The membranes were stored in buffer prior to use. The dialysis cells were loaded with 1 or 5 mL of the sample solution and dialyzed against an equal volume of buffer. The sample solutions were prepared by diluting a stock micellar solution (50 mg/mL total lipid) to the appropriate level, heating briefly to 45 °C, and storing at 21 °C with gentle agitation. After a minimum of 48 h, samples (20 μL) of dialysate from the buffer side were analyzed for taurocholate by liquid chromatography. A C-18 reverse-phase column (25-cm length and 6-mm diameter) was used with a Perkin-Elmer series 4 chromatograph, detection of the bile salt being accomplished with a Spectroflow variable wavelength detector set at 205 nm. The carrier liquid was 35% methanol and 65% water. Peak areas were determined and compared with standard curves of millimolar bile salt vs peak area; the standards were made from solutions of taurocholate in buffer. Constant bile salt concentrations were found in the dialysate side after about 36 h, but the cells were allowed to equilibrate for 48 h at 21 ± 1 °C. Since the concentration of bile salt in the dialysate and the total amount added to the sample are known, the amount of bile salt aggregated with DPPC can be determined along with the actual mole ratio of bile salt to lipid, R_e.

Turbidity Measurements. In order to determine the boundaries between micellar solutions and solutions in which vesicles are forming, turbidity was measured at 480 nm with a Hewlett-Packard Model 8452 spectrophotometer. As above, the solutions were prepared by dilution of 50 mg/mL total lipid samples to lower levels, heating to 45 °C, and then equilibrating for 48 h at 21 °C. The molar turbidities were obtained by dividing the measured absorbance by the molar concentrations of DPPC.

DSC Measurements. Approximately 1 mL solutions of diluted micellar stock solutions (50 mg/mL total lipid), which had been equilibrated at 21 °C for 48 h, were taken for DSC analysis. Measurements were made with a Microcal MC-2 precision scanning calorimeter at a scan rate of 0.4 deg/min. Several scans were made on a given sample in order to observe the effect of repeated heating of the samples through the thermal transitions. As a check on reproducibility, a number of scans were made from different stock micellar solutions. Data were acquired and analyzed with a computer interfaced to the instrument, and overlapping DSC transitions were deconvoluted with software provided by Microcal.

Results

Equilibrium Dialysis and Turbidity Results. It has been shown by several authors that the effective ratio, R_e, of bile salt associated with phospholipid bilayers to the lipid concentration is directly proportional to the aqueous bile salt concentration:

$$R_e = K \times [BS]_{aq} \quad (1)$$

where $[BS]_{aq}$ is the concentration of bile salt not associated with lipid and K is the distribution coefficient of the bile salt between aqueous phase and the bilayer, $K = [BS]_{bil}/([DPPC][BS]_{aq})$ (Schurtenberger et al., 1985; Lichtenberg, 1985; Almog et al., 1986). This equation can be expressed as

$$R_e = R_{st}/1 + (KC_L)^{-1} \quad (2)$$

where in this case R_{st} is the stoichiometric ratio of bile salt

[1] Abbreviations: C_L, millimolar concentration of DPPC; cmc, critical micelle concentration; C_p, excess heat capacity; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; H_c, calorimetric enthalpy; H_v, van't Hoff enthalpy; K, distribution constant; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, diacyl-*sn*-glycero-3-phosphocholines; R_e, ratio of bile salt to phospholipid in the aggregated state; R_{st}, stoichiometric ratio of bile salt to phospholipid; TC, sodium taurocholate; T_m, temperature at the midpoint of the thermal transition.

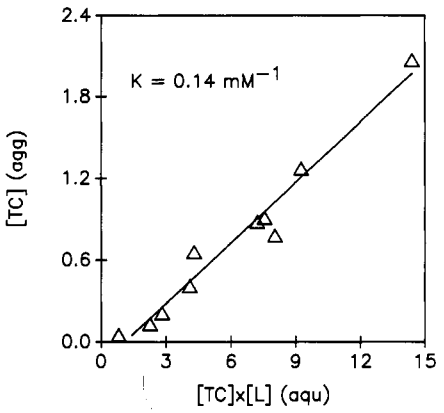

FIGURE 1: Equilibrium dialysis results for TC–DPPC mixtures. [TC](agg) is the millimolar concentration of TC aggregated with DPPC in vesicles or other lamellar structures. The abscissa is the product of TC concentration in the aqueous phase and the total DPPC concentration. The slope of the least squares line in the figure is K (mM^{-1}), the equilibrium constant for the association of TC with phospholipid.

to phospholipid mixed together and C_L is the total phospholipid concentration. Thus, from knowledge of the distribution coefficient, it is possible to find the actual mole ratio of bile salt to lipid in the aggregates that form at given values of R_{st} and C_L. With this information, the DSC thermal transitions reported below for particular mixtures can be discussed in terms of the actual state of aggregation of the lipid, R_e, even though they may be prepared from differing amounts of the components.

Equilibrium dialysis provides a method for obtaining the distribution coefficient. Figure 1 shows a plot of the experimentally determined concentration of bile salt aggregated in bilayers versus the product of the total lipid content and the aqueous bile salt concentration, determined at 21 °C by the equilibrium dialysis method (see Experimental Procedures). The slope of the least-squares line, shown in the figure, is the distribution coefficient K. The value obtained is 0.14 mM^{-1} (±0.02), with a correlation coefficient of 0.96. This distribution coefficient compares with 0.076 mM^{-1} obtained for glycocholate–egg lecithin mixtures (Schurtenberger et al., 1985) and with 0.065 mM^{-1} found for glycocholate–dimyristyrylphosphatidylcholine under similar conditions (Schurtenberger et al., 1986). Malloy and Binford (1990) found values of K of about 0.1 for the saturation binding of cholate to DMPC small unilamellar vesicles, which is close to the values of our binding constants for DPPC. The values of K are measured in the range of compositions in which either vesicles or extended bilayer structures are likely to persist, so that the K represents an average value for the structural forms present. The fact that the data in Figure 1 fit a linear function with good precision suggests that there is little difference in the distribution behavior of the bile salt into the various bilayer forms present in solution.

It has been pointed out by Almog et al. (1986) that the critical solubilization ratio, R_e^C, where solubilization of bilayers to micelles occurs is

$$R_e^C = K \times \text{cmc} \quad (3)$$

where K is the distribution coefficient and cmc is the critical micelle concentration of the detergent. Since the cmc for taurocholate under our buffer conditions is about 2.7 mM (Small, 1971), eq 3 predicts that the value of R_e corresponding to solubilization should be 2.7 × 0.14, or 0.37.

In order to monitor the solubility changes that are occurring upon dilution, we have used the turbidity method of Almog et al. (1986). The turbidity measured at 480 nm shows low values in the micellar region but increases dramatically when aggregation to vesicles or vesicle precursors occurs. Figure 2 shows turbidity values for TC–DPPC mixtures of 1.8:1 (open circles) and 1:1 (closed circles) ratios of bile salt to lecithin at different levels of dilution. These curves help to define the boundary between where the solution is homogeneous as a result of the presence of solubilized mixed micelles and the region where aggregation to large lamellar forms begins to occur. The turbidity curves show an increase for $R_e < 0.3$ for both 1.8:1 and 1:1 ratios, but the molar turbidity for the 1:1 curve increases more dramatically than that for the 1.8:1 dilutions. This latter observation suggests that the size of the aggregates in the 1:1 series is larger than that for the 1.8:1 solutions. Note, however, that there is less DPPC in the mixtures at a given R_e value in the 1.8:1 series. Malloy and Binford (1990) have shown that the turbidity at a given bile salt content is dependent on phospholipid content, and this is consistent with smaller sized particles for less DPPC in the mixtures. The inset in Figure 2 shows a turbidity curve for egg lecithin–taurocholate mixtures under similar states of dilution. The R_e values for the egg lecithin case were calculated from the equilibrium constant data in the literature. [Almog et al. (1986) report $K = 0.050$ for cholate–egg lecithin mixtures, while Schurtenberger et al. (1985) find $K = 0.076$ for the egg lecithin–glycocholate system. We use the latter number since the bile salt component is conjugated as is taurocholate.]

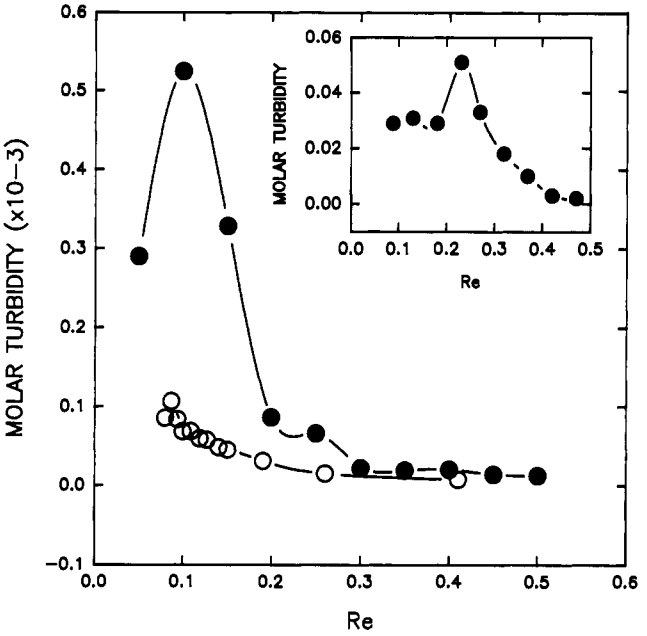

FIGURE 2: Representative molar turbidity data for TC–DPPC mixtures as a function of R_e, the ratio of TC to DPPC in the aggregated forms. The molar turbidity is calculated by dividing the measured absorbance at 480 nm by the molar concentration of lipid. The samples were heated to 45 °C after dilution and then equilibrated for 48 h at 21 °C prior to turbidity measurements. The closed circles show the turbidity values for dilution of a 50 mg/mL total lipid micellar solution with 1:1 stoichiometric ratio of TC to DPPC, while the open circles are for a 1.8:1 ratio. The inset shows similar turbidity data for dilution of a 50 mg/mL total lipid egg lecithin–TC micellar solution with 1:1 ratio of TC to egg lecithin.

The increase in turbidity near $R_e = 0.4$ for the egg lecithin system occurs at a somewhat higher ratio of bile salt to lecithin than for DPPC. Also note that the molar turbidity values for the egg lecithin mixtures are much smaller than for the DPPC dispersions, again suggesting that the DPPC vesicles that form are much larger than egg lecithin vesicles, at least in the 1:1

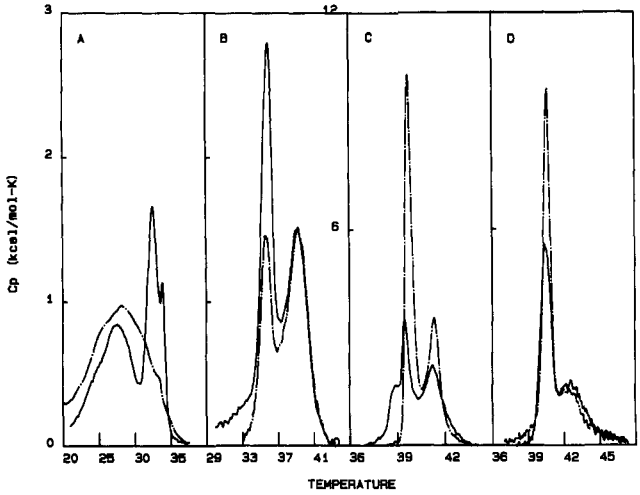

FIGURE 3: DSC transition curves for TC–DPPC mixtures formed by dilution of 50 mg/mL total lipid content with a stoichiometric ratio of TC to DPPC indicated in parentheses. The solid curve is the first scan of the solution, and the dotted curve is the second scan. Conditions: (A) 10 mg/mL total lipid (1.8:1), R_e = 0.81; (B) 4.3 mg/mL total lipid (1.8:1), R_e = 0.50; (C) (note scale change!) 1.3 mg/mL total lipid (1.8:1), R_e = 0.18; (D) (the scale is the same as in C) 0.70 mg/mL total lipid (1:1), R_e = 0.07.

mole ratio series. From this information and the turbidity results above, we define the micellar boundary to be at an R_e value of 0.30 in the DPPC–TC system, so that below 0.30 we expect significant changes in the state of aggregation of the lipid and, further, that at about R_e = 0.15 or below vesicles form. It should be pointed out that, in repeating the turbidity measurements with fresh micellar stock solutions, there was a 10–30% variation in molar turbidities at a given R_e, but the boundaries were repeatable within a few hundredths of an R_e unit. Using the turbidity data and with the boundaries defined, we can discuss the DSC data by using the compositional quantity, R_e, that is representative of the true state of aggregation of the bile salt and lipid, and we also have an idea of the expected structural forms present in the mixtures.

DSC Results and Dilution Experiments. DSC scans were made on solutions obtained by dilution of 50 mg/mL total lipid micellar solutions to compositions corresponding to those reported above. Thus, one stock solution was made up to a 1.8:1 stoichiometric ratio of TC to DPPC, and the dilutions were made to ranges similar to those reported by Hjelm et al. (1988). Another series of solutions with a 1:1 ratio of TC to DPPC was also prepared and scanned, in order to have samples of different total lipid compositions and yet the same values of R_e (Almog et al., 1986).

Figure 3 shows DSC curves for several of the diluted solutions of the TC–DPPC micellar stock solution of 50 mg/mL total lipid. The curves are representative data for the first and second (repeat) scans of the solutions that were originally equilibrated after dilution for a minimum of 48 h at 21 °C. Transition curves were repeatable within about 10% when fresh micellar stock solutions were used and comparisons were made on second or higher scans. The total lipid content is between 10 and 0.21 mg/mL (R_e = 0.81–0.04). There are significant differences between the first and second scans in most cases, but subsequent scans show no further change. The differences between the first two scans imply that equilibrium in the thermally active lipid has not been attained and that heating promotes conversion to reproducible transition curves. The fact that the equilibrium dialysis experiments and turbidity of the solutions reach equilibrium within 48 h suggests that the gross structural features of the vesicles or micelles are at equilibrium but that DPPC requires heating to attain equilibration of the physical phases available to the lipid. The nature of the changes in the thermally active lipid upon rescanning is dependent upon the R_e values at which comparisons are made. For example, at higher R_e values (Figure 3A,B), in the micellar region the differences between the first scan and the second are the result of the presence of a transition that disappears or significantly decreases upon rescanning. Observe that in Figure 3A the first scan shows two transitions, a broad structureless peak at 26 °C and a second transition between 31 and 35 °C, which disappears upon a rescan of the solutions. The 26-°C peak is similar to that observed previously for micellar solutions by Spink et al. (1982) and shifts to very low temperatures (near 5 °C) when the bile-salt content is high. The 33-°C peak appears as a result of the longer equilibration times used in the experiments reported here. It was found that during the course of equilibration of the samples at 21 °C the generally low molar turbidities (near 20) for the higher total lipid samples were found to increase during the sample equilibration (to near 40, e.g., for the 10 mg/mL sample). The disappearance of the 33-°C peak upon rescanning is also accompanied by a decrease in turbidity back to the original value near 20. Thus, the peak is associated with a phase that forms on standing at lower temperatures. If the samples are equilibrated at 35 °C, this peak never appears, turbidity remains low, and the broad transition is persistent for the first and subsequent scans. The nature of the peak arising as the turbidity increases in the micelle region is discussed below.

For samples with R_e between 0.5 and 0.3 (see Figure 3B, for example), there is a low-temperature peak that persists upon rescanning and whose C_p (max) and T (max) increases as R_e decreases. That this transition remains in the low-turbidity solutions suggests that it is associated with a new phase of the lipid in the micelles. The effect of a rescan on these solutions is again a decrease in the magnitude of the lower temperature peak, while the higher temperature transition is hardly affected at all.

Upon higher dilution of the stock solutions, there are new differences in the thermal transitions between the first and second scans. Figure 3, panels C and D, shows data for concentrations of 1.3 and 0.4 mg/mL total lipid (R_e = 0.18 and 0.08) in the samples, respectively, that by the boundary criteria presented above correspond to the vesicle region. In this region of composition, the peaks in the second scan have a generally larger C_p (max) with a highly cooperative transition dominating the DSC pattern. This behavior is observed for all of the thermograms with $R_e < 0.2$ and is just the opposite to the rescan behavior for mixtures with $R_e > 0.2$. Upon additional rescans of these samples, there is virtually no change from the transitions observed in the second scan. Also, samples that have been scanned and allowed to sit at 21 °C for long periods show no further changes. For example, a sample with R_e = 0.25 was scanned twice and then allowed to sit at 21 °C in the dark under argon for 30 days. The sample was then scanned again, and the curve was virtually superimposable on the second scan of the sample. These results suggest that heating causes a change from an initial state in which the lipid is not totally equilibrated to a state that is thermally reversible.

The DSC measurements reveal several other aspects of the equilibration of synthetic PC's at 21 °C. First, in the mixed micellar region with R_e values above 0.40, the thermal transitions were quite reproducible after a few hours, except for the appearance of the 33-°C peak mentioned above. That is, rescans of these mixtures after 4 h are virtually identical with

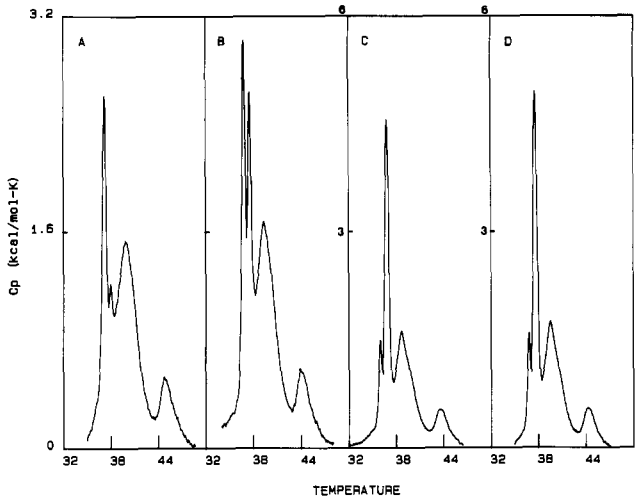

FIGURE 4: DSC transition curves for TC–DPPC mixtures equilibrated at 21 °C for different times. The sample was prepared from 50 mg/mL total lipid (1:1 ratio), diluted to 3.0 mg/mL total lipid, R_e = 0.25. Conditions: (A) after 3.5 h; (B) after 96 h; (C) second scan of the solution after 3.5 h; (D) second scan of the solution after 96 h (note the scale change for curves C and D).

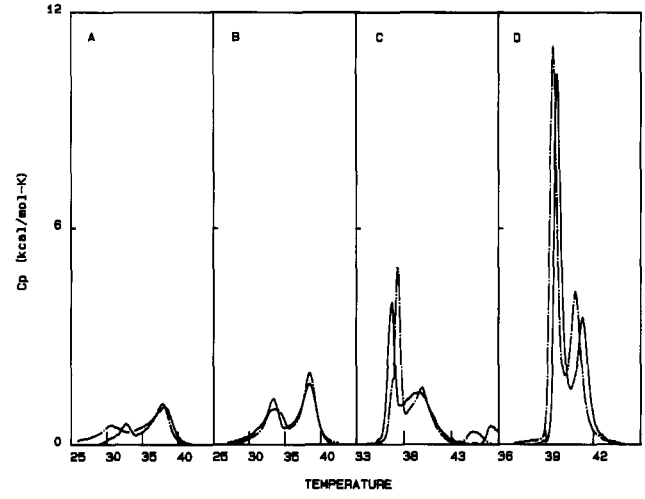

FIGURE 5: DSC transition curves for TC–DPPC mixtures formed by dilution of 50 mg/mL total lipid to concentrations of comparable R_e values. Solid curves are for 1.8:1 and dotted curves for 1:1 stoichiometric ratios of TC to DPPC. (A) R_e = 0.51; (B) R_e = 0.40; (C) R_e = 0.25; (D) R_e = 0.17.

scans obtained after 48 h or longer. The second scans in Figure 3, panel A or B, would be the same for rescans of the solutions any time after 4 h. The large transition at 31–35 °C, of course, gets larger upon sitting at 21 °C for longer times. But the transitions we associate with the mixed bilayer micelles equilibrate relatively rapidly as determined from the DSC behavior [see also Spink et al. (1982)]. In the region of composition where aggregation to larger structures begins to occur, at R_e values less than 0.40, there are some general features of the equilibration process that emerge. In mixtures with R_e values between 0 and 0.25, there are changes that occur both with time and upon rescanning. Figure 4 shows traces for a mixture with R_e = 0.25 at different times after dilution from a 50 mg/mL stock solution and after equilibrating at 21 °C. Enough sample was prepared so that scans could be made on fresh samples after equilibrating for the prescribed times. Some samples were rescanned immediately after the first scan. As the figures show, the broad higher temperature peak and the 45-°C peak do not change much with time, but the sharp lower temperature transition initially shows a large peak at lower temperature, and, as time progresses, a second slightly higher temperature transition emerges. Even after 92 h, neither has become the dominant transition. An important point is that if any of the samples are rescanned, they transform to the transition curves shown in Figure 4, panel C or D, with the higher temperature peak dominant. It appears not to matter at which time the peaks are rescanned. The same curve is obtained upon rescanning at 4 h or at 96 h. These data suggest that the sharp transition becomes equilibrated to a reversible transition upon scanning to 45–50 °C and that there is significant metastability in the lipid when equilibrated at 21 °C, even if allowed to stand for over 90 h.

Similar behavior was observed for more dilute solutions with R_e values below the vesicle transition point. It was found that the first scans (after 48 h of equilibration at 21 °C) were basically quite similar to second scans, except that the large vesicle peak was 50–75% larger on second or subsequent scans. Figure 3D shows this effect. One concludes from this that heating the samples of low R_e promotes equilibration of the lipid in the vesicles and that mixtures left at room temperature at compositions where significant aggregation to the large lamellar forms occurs can have metastable lipid present over quite long time periods. With this information and the results in the micellar region in mind, it is now possible to look at the effect of composition of the mixtures on the DSC behavior by using the second scan as an indication of equilibrated samples.

DSC Results and the Effect of Composition. Figure 5 shows DSC scans of two series of dilution experiments, one with a mole ratio of TC to DPPC of 1.8 (solid curves) and the other with a 1:1 ratio (dotted curves). All of the curves are for second or higher scans. It is apparent that changing the stoichiometric ratio of bile salt to lecithin changes the thermal behavior at a given total lipid content. In Figure 5, the curves for 3 mg/mL total lipid for the 1.8:1 and 1:1 series are in panels B and C, respectively, and are clearly quite different. However, if the DSC behavior is simply related to R_e, the actual ratio of bile salt to lecithin in the aggregated forms, and not to the total lipid content, then the thermal transitions observed for mixtures of differing stoichiometric ratio or phospholipid concentration should be the same if the R_e value for the mixtures are the same. Figure 5 clearly shows the similarities between the two dilution series, as long as comparisons are made at the same R_e value. The detailed similarities are remarkable considering the rather different amounts of lipid and bile salt in the solutions that are compared in Figure 5. These comparisons provide additional support to the hypothesis that on dilution of micellar mixtures of bile salts and phospholipids the particular structures that form are only determined by the ratio of detergent to lipid aggregated in the bilayer system (Schurtenberger et al., 1985; Almog et al., 1986).

The data for the 1:1 dilution series allow measurements at lower amounts of associated bile salt in the diluted solutions, so that DSC scans can be made at higher lipid content in regions where the vesicles should be prominent. Figure 6 shows DSC scans for the more dilute concentrations in the 1:1 ratio series. With these mixtures, at high dilution one can see the vesicle transition clearly, the peaks being of high excess heat capacity (note the scale change) and very narrow, as expected for large unilamellar vesicles (Schullery et al., 1980).

Thus, in summary, in the vesicle region there are two thermal transitions, a sharp highly cooperative transition and a smaller transition that becomes more evident at $R_e > 0.1$. On the other hand, the micelle region shows a very broad transition that changes its T (max) strongly with composition. In addition, as R_e gets near 0.4, another peak is seen that

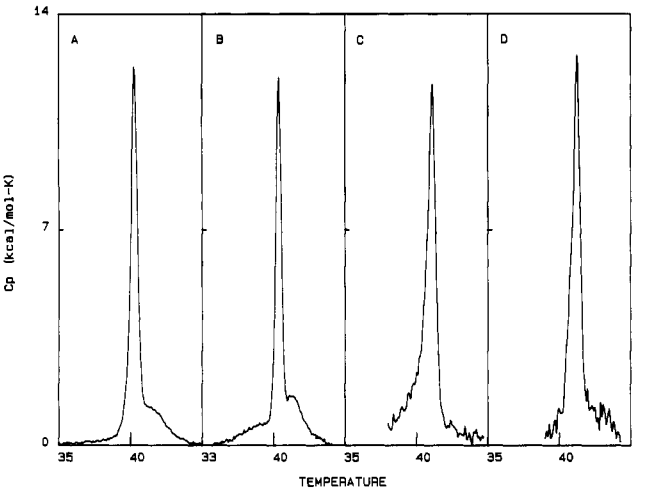

FIGURE 6: DSC transition curves for TC–DPPC mixtures formed by dilution of 50 mg/mL total lipid content with a 1:1 stoichiometric ratio of TC to DPPC. (A) 1.1 mg/mL total lipid, R_e = 0.11; (B) 0.7 mg/mL, R_e = 0.07; (C) 0.2 mg/mL total lipid, R_e = 0.02; (D) 0.1 mg/mL total lipid, R_e = 0.01.

becomes more highly cooperative as dilution proceeds. In the region between micelles and vesicles, there are several transitions that are markedly composition dependent. The nature of these transitions will be discussed below.

Discussion

In order for the DPPC thermal behavior to be useful for the study of bile salt–lecithin mixtures, it is important that the general behavior of DPPC–TC mixtures is similar in behavior to that of other model systems that have been used for this purpose. Egg lecithin and various bile salts have been commonly used for the study of the micelle–vesicle transition. It is thus necessary to consider the relative behavior of egg lecithin and DPPC with respect to the phase boundaries and other compositional properties of mixtures of the phospholipids with bile salts. Our turbidity data for TC–DPPC dilutions show that highly scattering vesicle dispersions form until the R_e value is greater than about 0.15–0.20, at which point the turbidity decreases. This vesicle boundary point is less than the egg lecithin point (R_e = 0.25, as shown in Figure 2). The recent small-angle neutron-scattering data of Hjelm et al. (1990) show that for dilution of glycocholate–egg lecithin mixtures vesicles are evident up to R_e = 0.2, but beyond that point bilayer sheets or rods prevail. Schurtenberger et al. (1986) found that the dilution of micellar solutions of DMPC and glycocholate formed vesicles at R_e about 0.10. Malloy and Binford (1990) have used dimyristoyl-PC in the study of solubilization of vesicles by bile salts, and if one calculates vesicle boundary in terms of R_e from their data, solubilization of DMPC by cholate commences when R_e is near 0.12. It thus appears that, for the saturated synthetic phospholipids, vesicles form at somewhat higher dilutions than for egg lecithin. So the observation that DPPC vesicles are found in the DSC measurements by dilution of micellar solutions below $R_e < 0.15$ seems consistent with other model studies of the micelle–vesicle transition.

From the behavior of the turbidity of DPPC suspensions (Figure 2), we have concluded that for R_e greater than 0.30–0.35, micellar solutions form. This is true also for egg lecithin, the turbidity being quite low at about R_e = 0.4 [see also, Almog et al. (1986) and Schurtenberger et al. (1985, 1986)]. Thus, the micelle boundary falls in the range of R_e = 0.3–0.4 for a variety of phospholipid components. A further point to make about comparisons of the behavior of DPPC and other synthetic saturated PC's with egg PC is that when aggregation occurs to form the large lamellar structures and vesicles, the vesicles that form appear to be larger for DPPC or DMPC than for egg lecithin. Schurtenberger et al. (1986) found that the hydrodynamic radii of vesicles formed from dilution of DMPC–glycocholate micelles were about twice as large as those formed when egg PC was used. As Figure 2 shows, the turbidity for DPPC–TC mixtures is significantly greater than that for egg PC mixtures with TC at comparable degrees of association with the lipid. Thus, in examining the DSC data for DPPC–TC mixtures, we should keep in mind that the transition region between micelles and vesicles is somewhat larger (R_e = 0.35–0.15 compared with 0.40–0.30 for egg PC) and that the structures that form upon dilution into the aggregation region will be larger.

Another issue that must be confronted about the DSC experiments with DPPC is the nature of the temperature dependence of the various compositionally related quantities, since in the DSC experiment the temperature is scanned in order to determine the phase-transition temperatures and enthalpies. For example, it is important to consider the temperature dependence of the association constant, K, which determines the effective ratio of bile salt to lecithin in the aggregated structures. Several studies have indicated that there is only a small temperature effect on K for bile salt–lecithin interactions. For egg PC and glycocholate, K was found to be the same at 40 °C as at 20 °C (Schurtenberger et al., 1985). Malloy and Binford (1990) found K for cholate binding to DMPC at 20 °C, the lipid of which is in its gel state, also to be virtually the same as at 27 °C where the lipid is liquid crystalline. These authors found that the boundary for solubilization of DMPC vesicles was also independent of temperature. DMPC vesicles at temperatures above the phase transition do show a slight dependence of K on temperature (Schurtenberger et al., 1986). In the micellar region, K for association of TC with DPPC was found to have a slight temperature dependence (Spink et al., 1982). The enthalpy changes for the binding of bile salt to vesicles, −6.6 kcal/mol (Malloy & Binford, 1990), or micelles, −4.5 kcal/mol (Spink et al., 1982), lead to contributions of less than 0.2 mcal/K·g in the excess heat capacity across the temperature range of a typical DSC scan, on the basis of estimates of the change in extent of binding with temperature. Since that small effect is below the experimental error of measurement, it is unlikely that the temperature effects on K contribute significantly to the measured DSC transitions.

It is clear from the results presented on the effect of rescanning that in the vesicle and vesicle-fragment region there is considerable metastability in the lipid undergoing the thermal transitions. A question arises as to whether that metastability arises because of changes in the size or distribution of structural forms in the mixtures upon heating. Schurtenberger et al. (1986) have shown that with glycocholate–dimyristoyl-PC the hydrodynamic radii of vesicles formed at 43 °C increase substantially when cooled to 25 °C. The radii of the cooled vesicles are about the same as the radii of those formed by dilution of micellar solutions at 25 °C into the vesicle region. On the other hand, the radii of vesicles formed by dilution at 25 °C change less than 10% when heated to 40 °C. This size hysteresis was shown in egg lecithin vesicles formed by dilution from mixed micelles with glycocholate (Schurtenberger et al., 1985). Presuming that the TC–DPPC vesicles behave the same way with respect to size changes on heating, it would appear that in the vesicle region the me-

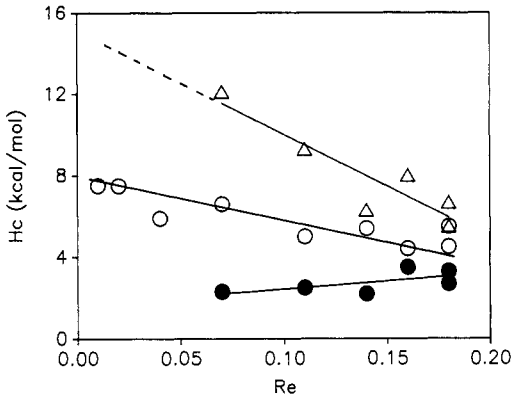

FIGURE 7: Calorimetric enthalpies for resolved transitions in the vesicle region vs R_e of the mixture. Symbols: (○) main vesicle transition; (●) broad vesicle transition; and (△) calculated complex of TC and DPPC (see text for details of the calculation).

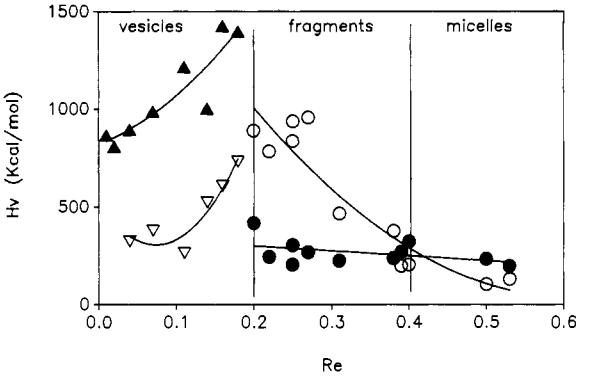

FIGURE 8: Resolved van't Hoff enthalpies for observed transitions in TC–DPPC mixtures vs R_e of the mixture. Symbols: (▲) main vesicle transition; (△) broad vesicle transition; (○) sharp transition in micelle and fragment region; and (●) broad transition in the micelle and fragment region.

tastability observed in the thermal transitions is largely a consequence of the unequilibrated lipid within the vesicles, which is altered upon heating. Since the samples in this study are prepared at 21 °C, we assume that the size of the vesicles on scanning in the calorimeter will not change much. The changes in the thermal transitions upon rescanning would then be a result of the DPPC undergoing some internal reorganization such that repeat cycling of the temperature has no effect. The formation of the vesicles or vesicle fragments at low temperature seems to lead to domains of lipid that are not at equilibrium, even if allowed to stand for long periods of time. Heating through the thermal transitions leads to reproducible conformations of lipid that then reflect a state of the lipid that is characteristic of the structural and environmental features of the system.

An additional feature of the micellar region that deserves discussion is the appearance of the 33-°C transition in those micellar solutions that were equilibrated at 21 °C for 48 h. Hjelm et al. (1990) reported that at higher total lipid content in glycocholate–egg lecithin mixtures the solutions showed the presence of rod-like structures in the neutron-scattering data. These rod structures of about 27-Å radius were formed after equilibration of the micellar solutions (about 5 mg/mL total lipid) at 20 °C for 48 h. As mentioned above, the TC–DPPC solutions showed the 33-°C transition at comparable compositions, but the DSC scans returned to the normal micelle transitions upon heating to about 31–33 °C (see Figure 3). It is thus possible that the rod structures seen in neutron scattering correspond to the entity responsible for the 33-°C transition. Schurtenberger et al. (1985) report that mixed bilayer micelles exist in this region, the disks having radii of about 30 Å. If the observation by Hjelm et al. (1988, 1990) that the rods result from a stacking of the bilayer disks into extended structures is correct and if these structures do melt at about 33 °C, then neutron-scattering measurements at 34 °C should show only bilayer disk micelles and not rods. The thermal transition at ca. 31–32 °C in this region would correspond to the conversion of the rod structures back to the simple bilayer micelles.

Transition Peak Parameters. From the DSC transition data, one can obtain the calorimetric enthalpy H_c, the van't Hoff enthalpy H_v, and the transition temperature T_m for the individual peaks in the cases where overlap occurs (Biltonen & Friere, 1978). Using the deconvolution software provided by Microcal, we have evaluated the specific transition enthalpies and peak temperatures for the data on the bile salt–DPPC mixtures. The calorimetric enthalpy corresponds to the energy of melting of the gel phase, and H_v gives a measure of the temperature dependence of the fraction melted during the transition and thus measures the sharpness of the transition.

Figure 7 shows the variation with R_e of the calorimetric enthalpies of the resolved, second scans of the mixtures in the vesicle region. The *total* calorimetric enthalpy in the vesicle region is constant within the experimental error at about 8.4 kcal/mol and then drops abruptly at compositions at which fragments or micelles exist. In Figure 7 for the vesicle region, the large sharp peak shows a steady decrease in calorimetric enthalpy up to the vesicle boundary, while the broad transition, on the other hand, has low enthalpy but increases somewhat in this region. This latter peak is not clearly evident until the R_e value reaches 0.08–0.10. The decrease in the area of the sharp transition could be a result of conversion of DPPC to a specific complex with bile salt. Mabrey et al. (1978) have shown that multilamellar suspensions of cholesterol and DPPC have two transitions at compositions between 0 and 20 mol % cholesterol. The main gel to liquid crystal transition decreases with increasing cholesterol, disappearing by 20% cholesterol, and a second broad transition persists to around 50 mol %. The results are interpreted that an immiscible packing complex forms between DPPC and cholesterol, and this complex is responsible for the observed broad transition. Since TC has a basic steroidal skeleton similar to cholesterol, by analogy it is possible that a similar packing complex could be responsible for the broad transition in the vesicle region of bile salt–DPPC mixtures. If it is assumed that the sharp transition is almost pure DPPC (T_m varies from 41.3 to 39.7 °C between R_e = 0 and 0.2), then one can calculate the amount of DPPC that is tied up in the bile salt complex (Mabrey et al., 1978). The ratio of the enthalpy for the sharp transition at any composition to that of pure DPPC, which extrapolates to 8.0 kcal/mol in Figure 7, represents the fraction of DPPC in the main transition. The remaining DPPC can be used to calculate a corrected enthalpy for the bile salt complex with DPPC, also shown in Figure 7. Although the scatter is fairly high, the result provides an interesting comparison with the cholesterol–DPPC complex discussed above. The extrapolated enthalpy for the complex is about 15 kcal/mol, which is identical with the 15 kcal/mol for the cholesterol complex (Mabrey et al., 1978). Also, from the slope of the main transition enthalpy in Figure 7, one can determine the number of molecules of lipid complexes with bile salt, which is three molecules of DPPC per TC, compared with 4 mol of DPPC per mole of cholesterol. These similarities between bile salt and cholesterol behavior in the presence of phospholipid support previous work suggesting that the basic cholesterol steroid ring system interacts very specifically with

lipid (Shimshick & McConnell, 1973; Rectenwald & McConnell, 1981; Mabrey et al., 1978; Ipsen et al., 1987).

Figure 8 shows the behavior of the van't Hoff enthalpies, H_v, for the major resolved transitions between $R_e = 0$ and 0.5. The graph is separated into three regions, the vesicle, bilayer micelle, and the intermediate region, labeled as fragments. These regions are defined on the basis of the turbidity data (see Figure 2), and the approximate boundaries are defined on similar systems (Hjelm et al., 1988; Schurtenberger et al., 1985; Stark et al., 1988; Mazer et al., 1980; Almog et al., 1986). There are two major thermal transitions in the micelle or micellar fragment regions that are evident between R_e = 0.5 and 0.2 (see Figure 3A,B or 5A,B). In the vesicle region there are also two transitions, a major sharp, highly cooperative one and a smaller, broader transition (Figure 3D or 6A,B). These are the transitions whose van't Hoff enthalpies are presented in Figure 8. The van't Hoff enthalpy is related to the cooperativity of the melting transition; that is, H_v measures the sharpness of the transition. Formally, the cooperativity parameter, CUP = H_v/H_c, relates the observed enthalpic properties to cooperativity or loosely to the size of the melting unit in the transition. It is useful to see how the van't Hoff enthalpies are varying; and since H_c for the individual transitions, except for the main vesicle peak, are close to constant, H_v gives a reflection of the trends in cooperativity.

The H_v values for the transitions above $R_e = 0.4$ are very low, which is expected since the lipid domains in the small bilayer micelles would be small and ought to be highly perturbed by bile salt around the perimeter and within the bilayer of the micelles and thus would have low cooperativity. As the turbidity begins to increase due to aggregation of the bilayer micelles below $R_e = 0.4$, the van't Hoff enthalpy for the lower temperature transition increases markedly, while the lipid phase associated with the higher temperature (perturbed lipid) transition maintains low H_v. Thus, the large bilayer fragments forming in the intermediate region have increasingly larger areas of lipid that are able to melt with high cooperativity, as reflected in the increase in H_v for the lower temperature peak. One can envision that the two phases of lipid are associated one with the perimeter of the large fragments and of low thermal cooperativity, and the other with the internal large areas of less perturbed lipid with high H_v.

The two vesicle transitions below the $R_e = 0.2$ boundary show high cooperativity near the transformation boundary and then decrease to lower values near $R_e = 0$. The size of the vesicles prepared by dilution is known to decrease with decreased R_e in the vesicle region (Hjelm et al., 1988; Schurtenberger et al., 1986). In addition, Stankowski et al. (1979) and Suurkuusk et al. (1976) found that small vesicles made from synthetic PC's had both smaller calorimetric and smaller van't Hoff enthalpies than larger vesicles. In the region between $R_e = 0.1$ and 0.2, on the basis of the neutron-scattering data of Hjelm et al. (1988), the radii of the vesicles increase by about a factor of two. This would mean that the surface area of the vesicle would increase by a factor of four, providing the possibility for larger domains of lipid and increased cooperativity in the gel-melting process for both thermal transitions in the vesicle region. In the case of TC–DPPC mixtures, the van't Hoff enthalpies of the two vesicle transitions show a clear effect of increased cooperativity as the size increases to the micelle–vesicle boundary. Thus, the thermal data seem to be consistent with the observations from other work that vesicles made by dilution or dialysis do decrease in size with increased dilution. The van't Hoff enthalpy is very sensitive to the state of the lipid in this region of composition where the vesicle size is changing significantly.

Conclusions. This study leads to the following conclusions about the thermal behavior of the phospholipid in TC–DPPC mixtures:

(1) The DSC transitions in TC–DPPC mixtures are very sensitive to the composition and structure of the bilayer structures that form in these systems and are useful probes of the nature of interactions between bile salt and phospholipid. Several thermal transitions are observed in the micelle and vesicle regions that can act as probes of the nature of the physical state of the lipid in these environments. DSC also reveals a unique phase that forms in the micelle region upon standing at room temperature for extended periods and could relate to rod structures that have been detected by other methods.

(2) The calorimetric and van't Hoff enthalpies, as well as the T_m for the transitions, change as the micelle and vesicle size and composition changes. The changes correlate with structural variations observed by other methods used to study the behavior of phospholipid as it undergoes transformation from micelles to vesicles upon dilution or dialysis of the mixtures.

(3) Thermally active phospholipids, such as in the synthetic saturated PC's, in vesicles or in vesicle fragments are not at equilibrium when formed at room temperature and allowed to sit for 48 h or even longer times. Heating through the thermal transitions does convert the lipid to forms that give reproducible thermal scans that are very characteristic of the particular mixture.

On the basis of the findings in this paper, the DSC behavior of lipids in combination with surfactants could provide useful information on such processes as reconstitution by dialysis or dilution methods of model membranes in the presence of protein or other membrane components. The sensitivity of the thermal transitions to composition makes the DSC study of reconstituted vesicles useful for probing phospholipid drug delivery systems. And finally, the nature of biliary lipid mixtures, which are largely phospholipid and bile salt, can be studied to see how the presence of cholesterol or other biliary components affect the thermal behavior and thus the lipid environment in these systems.

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