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# Temperature Dependence of the Vesicle–Micelle Transition of Egg Phosphatidylcholine and Octyl Glucoside†

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ABSTRACT: The temperature dependence of octyl glucoside micellization was determined and compared to the phase behavior of the octyl glucoside–egg phosphatidylcholine (PC) mixed system in excess water to help elucidate the process of vesicle formation from mixed surfactant–phospholipid micelles. The critical micelle concentration of octyl glucoside (OG) was determined from the sharp increase of ANS fluorescence at micellization in an NaCl buffer at temperatures ranging from 5 to 40 °C. The cmc decreased with increasing temperature from 31 mM at 5 °C to 16 mM at 40 °C. A similar but less steep temperature dependence is observed for the solubilization of egg PC vesicles by OG as monitored by the surfactant-dependent changes in (1) solution turbidity and (2) the resonance energy transfer between NBD-PE and Rho-PE incorporated in the vesicles. These assays identify two breakpoints, most likely the boundaries of the cylindrical micelle and spheroidal micelle coexistence region. The $[\mathrm{OG}]_{\mathrm{aq}}$ values at these two breakpoints have similar temperature dependencies. However, the cylindrical mixed micelles at the boundary have nearly identical OG:PC ratios over the temperature range studied, whereas the spheroidal mixed micelles have relatively more OG at the higher temperatures (OG:PC ratio increases from 2.92 to 3.72 between 5 and 35 °C). Estimation of the acyl volume to surface area ratio for the compositions observed suggests that this parameter remains constant over temperature. The spheroidal mixed micelles, but not the cylindrical PC–OG micelles, exhibit ideal mixing between the two components at all temperatures (5–35 °C). This temperature sensitivity may be utilized to improve the efficacy of membrane protein reconstitution. A steep drop in temperature at appropriate temperature–concentration regimes induces lamellar formation, making it feasible to control the kinetics or to cycle several times through this transition in the course of a reconstitution protocol.

The need to find effective and predictable means to reconstitute membrane proteins, and to scale these reconstitution protocols for biological research or pharmacological applications, is one reason for interest in the nuances of lipid–surfactant interactions. In addition, studies of the interactions between phospholipids and other amphiphiles, such as surfactants, may be beneficial in both developing techniques and furthering insight into phospholipid phase behavior including the consequences of adding other non-phospholipid amphiphiles to lamellar phase lipids, e.g., diacylglycerols, free fatty acids, anaesthetics, and other drugs. This study examines the temperature dependence of the lamellar–micellar transition of a phospholipid–surfactant mixture, egg phosphatidylcholine (PC)[1] and octyl glucoside (OG), that is frequently chosen for membrane protein reconstitution. The results will both define the specific conditions for and offer some thermodynamic insight into these structural transitions.

Our previous studies on the behavior of the phospholipid, egg phosphatidylcholine, and the nonionic detergent, octyl glucoside, in aqueous solution have defined the stages of vesicle solubilization and formation (Ollivon et al., 1988; Vinson et al., 1989) and described the mixed micellar composition in the region from initial lipid dissolution through complete dispersal of the lipids into individual surfactant micelles (Eidelman et al., 1988). In agreement with others [e.g., see Jackson et al. (1982) and Paternostre et al. (1988)], we have shown that the process of solubilization of egg PC vesicles by octyl glucoside entails partitioning of the surfactant into the lamellar lipid phase until a critical point is reached and large mixed micelles are formed. Between these two states, micellar and lamellar, there are two mixed regions for which the structures have been identified recently (Vinson et al., 1989). First there is a region in which the phospholipid and OG are in both lamellar (vesicular, open vesicular) and cylindrical micellar structures. Then, at somewhat higher relative [OG], the cylindrical micelles coexist with spheroidal micelles (Vinson et al., 1989). The "initial" or phospholipid-saturated spheroidal micelles (~75 mol % OG) are transformed with additional OG to

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[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; cmc, critical micellar concentration; DMPC, dimyristoylphosphatidylcholine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; OD, optical density; OG, *n*-octyl β-D-glucopyranoside; PC, phosphatidylcholine; RET, resonance energy transfer; Rho-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; SUV, small unilamellar vesicle(s).

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smaller structures that contain fewer total amphiphiles and an increasing percentage of OG until each phospholipid molecule is distributed in a single micelle that resembles the pure OG micelle (Eidelman et al., 1988). These structural transitions are reversible [e.g., see Ollivon et al. (1988)] by surfactant removal or dilution. Growth of mixed micelles by lowering the surfactant concentration is relevant to protein reconstitution as, ideally, the protein and lipid become increasingly associated during this process. The critical step is generally considered to be the transition from the micellar to the lamellar state which is now known to include the cylindrical micelle structure; presumably, it is unlikely that proper transbilayer protein insertion will occur after the lamellar state is formed. [However, membrane protein reconstitution can occur from the lamellar state under certain circumstances; see, e.g., Scotto and Zakim (1985) and Rigaud et al. (1988).]

In this study, we examine the temperature dependence of the vesicle micelle transition in egg PC–OG mixed systems and compare this to the temperature dependence of the pure surfactant critical micelle concentration (cmc), using the optical techniques previously described (Ollivon et al., 1988). These results have several practical consequences for reconstitution protocols and establish the feasibility of forming vesicles from mixed micelles by temperature jump, an alternative controlled method of arriving at the lamellar state.

## MATERIALS AND METHODS

*Materials.* Egg phosphatidylcholine (egg PC) and the fluorescently labeled lipids *N*-(7-nitro-2,1,3-benzoxadiazol-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (Rho-PE) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Fluorescent lipids were periodically checked for purity by thin-layer chromatography. The detergent octyl β-D-glucopyranoside (OG) was purchased from CalBiochem (Irvine, CA); the probe 8-anilinonaphthalene-1-sulfonate (ANS) was from Sigma (St. Louis, MO). Phospholipids, detergent, and fluorescent probes were used without further purification. The buffer solution used was 150 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, and 0.02% sodium azide, pH 7.4.

*Vesicle Preparation.* Small unilamellar vesicles (SUV) of egg PC (10 mg/mL in chloroform) or egg PC with both NBD-PE and Rho-PE (1 mol %) were prepared by sonication (Heat Systems-Ultrasonics Model W-375). The chloroform was evaporated under a stream of argon (or nitrogen) to form a thin lipid film which was held for at least 1 h under vacuum. Vesicles were formed by hydrating the lipids with buffer solution and sonication under argon at 4 °C, until the suspension was clear (about 6 min). Final lipid concentration was measured by total organic phosphate (Ames & Durbin, 1960).

*Critical Micelle Concentration.* The cmc of octyl glucoside was estimated from the dramatic increase in ANS fluorescence associated with the appearance of a hydrophobic phase in the suspension (Mast & Haynes, 1975). The concentration of OG in a solution containing 15 μM ANS was increased by continuous addition of concentrated OG (400 mM) to the cuvette which was equipped with a paddle stirrer and held in a water-jacked cuvette holder. Temperature was monitored directly from the solution with a thermocouple (Bailey Instruments) and fluorescence (excitation 380 nm, emission 490 nm) detected with a spectrofluorometer (Perkin Elmer MPF-44B or SLM 8000).

*OG-PC Structural Transitions.* The positions of the two discrete breakpoints representing structural boundaries were determined by using the fluorescent technique described by Ollivon et al. (1988) and/or by changes in the absorbance or the 90° light scattering associated with changes in the size and scattering properties of the OG–PC structures. The fluorescence method is based on resonance energy transfer (RET) between headgroup-labeled lipid probes, NBD-PE and Rho-PE, that, when added at a concentration of close to 1 mol % to phospholipid vesicles, may be used to monitor the distance between the lipid molecules (Struck et al., 1981). RET in the detergent–phospholipid suspensions was measured during continuous OG addition to a suspension of SUV at appropriate temperatures and lipid concentrations as indicated in the figure legends. The excitation and emission wavelengths were 473 and 535 nm, respectively, and a 515-nm high-pass filter was added to the emission path to reduce the effects of scattered light. The rate of OG addition was sufficiently slow to permit equilibration of the sample's fluorescence. A typical trace of the change in fluorescence as OG was added to a suspension of SUV containing NBD-PE and Rho-PE is shown in Figure 1A. The trace is characterized by a small increase in NBD-PE fluorescence as detergent intercalates among phospholipids in the vesicle membranes, followed by a sudden sharp increase over a narrow [OG], followed by a further gradual increase. The beginning and end of the sharp increase (designated as breakpoints B and C) have been associated with the lamellar–micellar phase boundaries (Ollivon et al., 1988) but are now known to mark the region of cylindrical and spheroidal micelle coexistence as indicated in the phase diagram (Figure 1C) (Vinson et al., 1989).

The same boundaries are associated with changes in absorbance or light scattering. Absorbance or turbidity of the samples was measured at 350 nm with a Beckman DU-7 spectrophotometer under conditions identical with those used for the fluorescence experiments. In addition, some turbidity determinations were made simultaneously with the fluorescence measurements at 90° to the sample with a 460-nm notch filter using an SLM 8000 spectrofluorometer in a T-format. The resulting traces show a gradual change in scattering, followed by a sharp peak and precipitous decrease as detergent is added (Figure 1B). The positions of the peak maximum and the initial appearance of a clear solution (very low scattering) correspond to the sharp changes in NBD-PE fluorescence (Figure 1A).

## RESULTS

*Temperature Dependence of the Octyl Glucoside Critical Micelle Concentration.* The cmc of octyl glucoside in a 145 mM NaCl buffer was determined from the increase in ANS fluorescence (Figure 2A) as a function of temperature from 5 to 40 °C. The cmc decreased with increasing temperature (Figure 2B). A set of determinations was made in a low ionic strength buffer (300 mM mannitol, 10 mM Na-HEPES, and 1 mM Na-EDTA, pH 7.2). Both the temperature dependence and the absolute values of the cmc in the mannitol buffer were not significantly different from those observed in the 145 mM NaCl buffer.

The thermodynamic parameters for transfer of a monomeric detergent molecule from aqueous solution to a micelle can be estimated from the temperature dependence of the cmc, if we assume that the micellar structure is a separate phase; this assumption is reasonable in light of our previous results (Eidelman et al., 1988). In addition, OG was assumed to behave ideally in aqueous solution.

The free energy of transfer from water to a micelle is approximated by

$$\Delta G_{\mathrm{w}\rightarrow\mathrm{m}} = -RT \ln \mathrm{cmc} \qquad (1)$$

where $R$ and $T$ have their usual meanings and the cmc is

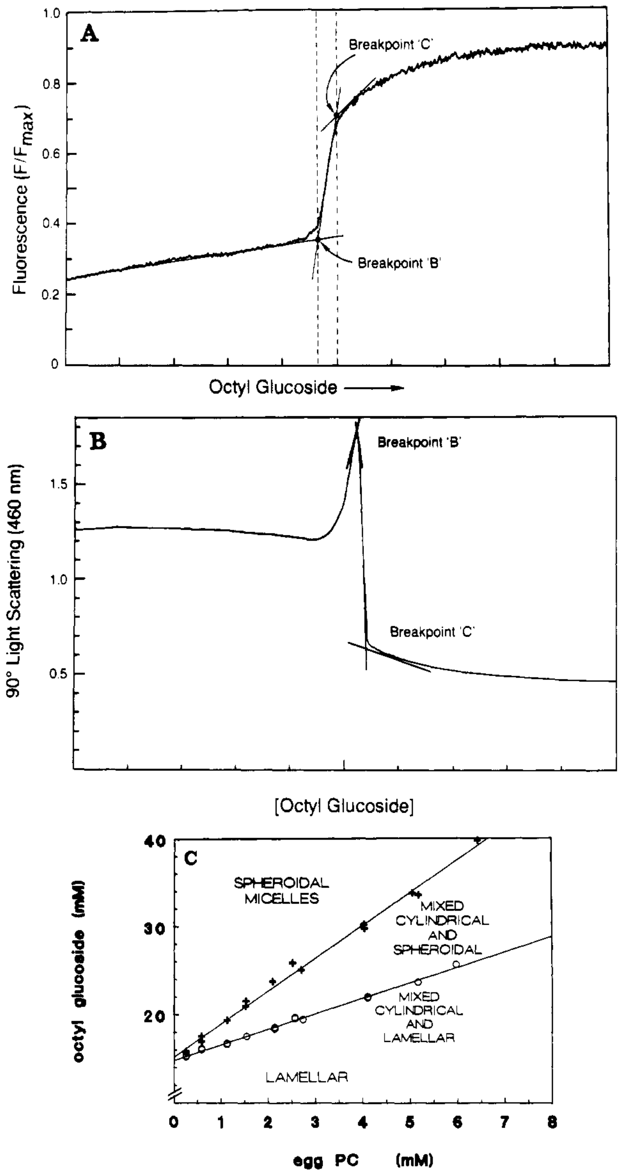

FIGURE 1: (A) Change in RET as a function of time while detergent is added continuously to a suspension of egg PC vesicles labeled with NBD-PE and Rho-PE. Octyl glucoside (400 mM) was added at a rate of 10 μL/min. The fluorescence of NBD-PE was monitored and corrected for nonspecific effects on fluorescence yield and for dilution by dividing the observed signal by that from a sample labeled only with NBD-PE but otherwise treated identically ($\lambda_{ex}$ = 473 nm; $\lambda_{em}$ = 535 nm). (B) 90° light scattering (460 nm) monitored during octyl glucoside addition as described for (A). (C) Phase diagram for octyl glucoside and egg PC taken from breakpoints B (O) and C (+) from RET and 90° light-scattering measurements as shown in (A) and (C) for a range of egg PC concentrations ($T$ = 35 °C). The assignment of the phase boundaries is substantiated in Ollivon et al. (1988) and Vinson et al. (1989).

expressed in mole fraction units [e.g., see Tanford (1980) and Hall (1987)]. The calculated values of $\Delta G$ were more negative with increasing temperature, ranging from −4.1 to −5.1 kcal/mol at 5 and 40 °C, respectively (Table I, column 3).

The enthalpy change associated with moving an OG molecule from water to a micelle ($\Delta H_{w\rightarrow m}$) can be calculated from the temperature dependence of the cmc by using the relationship:

$$\Delta H_{w\rightarrow m} = \left[\frac{d(\ln \text{cmc})}{dT}\right] RT^2 \qquad (2)$$

and, thus, may be estimated from the slope at any point on the curve shown in Figure 2B. Over the range of our data

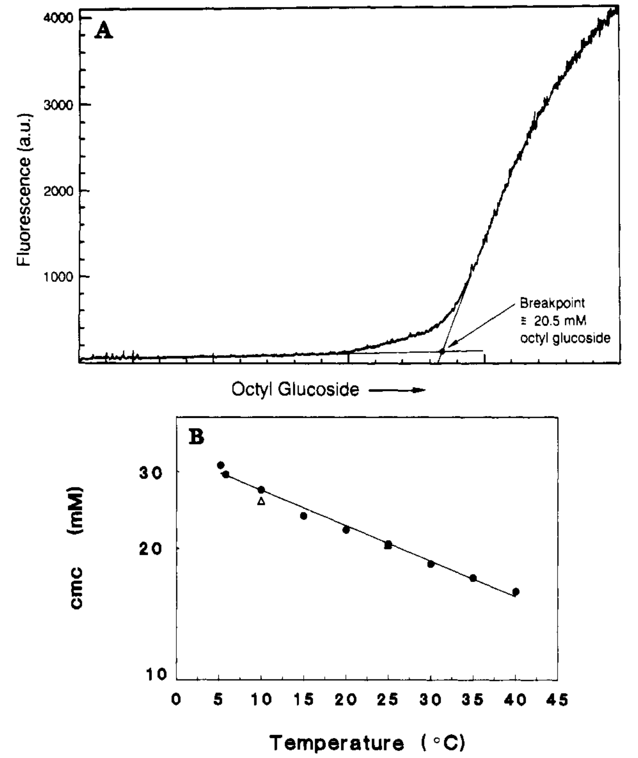

FIGURE 2: (A) Increase in ANS fluorescence was observed with the continuous addition of octyl glucoside, and the breakpoint indicated was assigned as the cmc. Octyl glucoside was added as described in Figure 1. Excitation was at 410 nm, and emission was monitored at 490 nm. (B) Critical micelle concentration of octyl glucoside plotted as a function of temperature. The points represent the observed cmc, and the line is the least-squares fit to the points ($r$ = 0.98, $n$ = 17, the standard error of the slope is 14%; see footnote 2). The triangles are values determined in mannitol buffer and are not included in the regression analysis.

(5–40 °C), this plot was approximately linear, and, thus, a single value for the slope was used to calculate $\Delta H_{w\rightarrow m}$ at each temperature (Table I, column 4).[2]

The change in entropy was calculated from $\Delta G = \Delta H - T\Delta S$. $\Delta S_{w\rightarrow m}$ was positive and increased with increasing temperature (Table I, column 6) as expected for the removal of an acyl chain from water. It was the large negative value of the term $-T\Delta S$ that favored micellization.

*Solubilization of Egg PC by OG Changes with Temperature.* The solubilization of egg PC vesicles by OG was monitored by using fluorescence and light-scattering techniques that permitted identification of the boundaries of the mixed cylindrical and spheroidal micellar states as described under Materials and Methods (Figure 1). The total concentrations of OG at the two breakpoints is plotted as a function of PC concentration and temperature (Figure 3). As observed previously at 25 °C (Ollivon et al., 1988), the [OG] at both boundaries increased linearly with [PC], indicating that for each temperature there was (a) constant composition of the lipid–detergent structure (reflected by the slope of the line) and (b) constant concentration of monomeric detergent (defined as the intercept at zero [PC]) over the range of lipid concentrations examined. The aqueous OG concentrations ($[\text{OG}]_{aq}$) and the compositions of the structures at either

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[2] It is possible that there is a change in the slope in Figure 2B at about 15 °C, although fitting these data with two lines does not give a better correlation than the single least-squares regression fit shown. If two slopes are used, however, to calculate the thermodynamic parameters, the estimates for enthalpy and entropy changes become nearly identical for all temperatures.

Table I

(A) Thermodynamic Parameters for Formation of Octyl Glucoside Micelles in Aqueous Solution[a]

| $T$ (K) | cmc (mM) | $\Delta G_{w\to m}$ (kcal/mol) | $\Delta H_{w\to m}$ (kcal/mol) | $-T\Delta S_{w\to m}$ | $\Delta S_{w\to m}$ [cal/(°C·mol)] |
|---|---|---|---|---|---|
| 278 | 30.9 | -4.14 | 2.92 | -7.06 | 25.4 |
| 278 | 31.8 | -4.12 | 2.92 | -7.04 | 25.3 |
| 278.8 | 29.4 | -4.18 | 2.93 | -7.11 | 25.5 |
| 278.8 | 29.1 | -4.18 | 2.93 | -7.12 | 25.5 |
| 283 | 27.1 | -4.29 | 3.02 | -7.31 | 25.8 |
| 283 | 28.5 | -4.26 | 3.02 | -7.28 | 25.7 |
| 288 | 23.8 | -4.44 | 3.13 | -7.57 | 26.3 |
| 288 | 23.8 | -4.44 | 3.13 | -7.57 | 26.3 |
| 293 | 22.2 | -4.55 | 3.24 | -7.79 | 26.6 |
| 293 | 22.2 | -4.55 | 3.24 | -7.79 | 26.6 |
| 298 | 20.5 | -4.68 | 3.35 | -8.03 | 26.9 |
| 298 | 20.9 | -4.67 | 3.35 | -8.02 | 26.9 |
| 303 | 18.4 | -4.82 | 3.46 | -8.29 | 27.4 |
| 303 | 19.1 | -4.80 | 3.46 | -8.26 | 27.3 |
| 308 | 18.0 | -4.92 | 3.58 | -8.50 | 27.6 |
| 308 | 16.1 | -4.98 | 3.58 | -8.56 | 27.8 |
| 313 | 16.0 | -5.07 | 3.70 | -8.77 | 28.0 |
| | | av -4.53 | 3.23 | -7.77 | 26.5 |

(B) Thermodynamic Parameters for OG Partitioning into Egg PC-OG Mixed Micellar Structures from Aqueous Solutions

| | $\Delta G_{w\to m}$ (kcal/mol) | $\Delta H_{w\to m}$ (kcal/mol) | $-T\Delta S_{w\to m}$ | $\Delta S_{w\to m}$ [cal/(°C·mol)] |
|---|---|---|---|---|
| av values at B | -4.65 | 2.10 | -6.75 | 23.2 |
| av values at C | -4.63 | 2.19 | -6.82 | 23.4 |

[a] Calculations as described in the text.

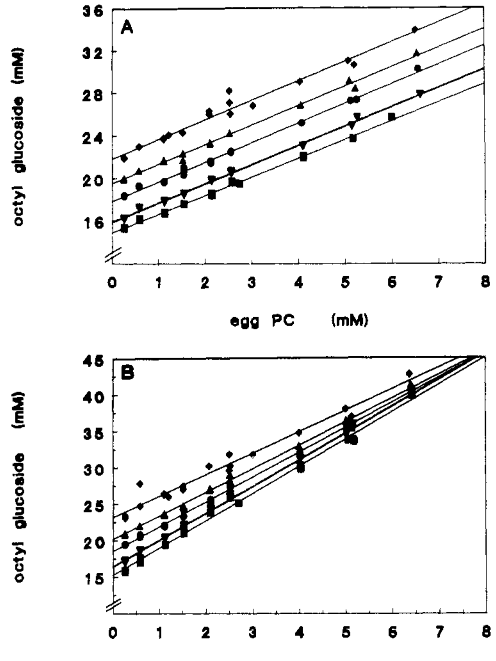

FIGURE 3: Boundaries, marking the upper and lower limits of the cylindrical-spheroidal mixed micelle coexistence region, for octyl glucoside solubilization of egg PC vesicles as a function of temperature. (A) [OG] at the phase boundary B as a function of total egg PC concentration at 5 (◆), 10 (▲), 15 (●), 25 (▼), and 35 (■) °C. The lines are the least-squares regression fit to the data. (B) [OG] at the phase boundary C as a function of total egg PC concentration. The temperatures are as indicated for (A). Data were collected by using both RET and turbidity measurements to determine the concentrations at the respective breakpoints (see Materials and Methods and Figure 1).

Table II: Concentrations of Monomeric Octyl Glucoside and Detergent to Lipid Ratios of the Micellar Structures

| temp (°C) | aqueous octyl glucoside concn (mM ± SEM) cylindrical[a] | spheroidal[a] | OG:PC ratio (mol/mol ± SEM) cylindrical | spheroidal |
|---|---|---|---|---|
| 5 | 21.8 ± 0.6 (20) | 23.2 ± 1.0 (19) | 1.84 ± 0.08 (20) | 2.92 ± 0.13 (19) |
| 10 | 19.6 ± 0.3 (15) | 20.3 ± 0.4 (16) | 1.82 ± 0.04 (15) | 3.22 ± 0.06 (16) |
| 15 | 17.9 ± 0.2 (18) | 18.6 ± 0.3 (16) | 1.84 ± 0.03 (18) | 3.40 ± 0.04 (16) |
| 25 | 15.9 ± 0.2 (18) | 16.5 ± 0.5 (18) | 1.79 ± 0.02 (18) | 3.7 ± 0.06 (18) |
| 35 | 14.9 ± 0.2 (18) | 15.3 ± 0.6 (15) | 1.76 ± 0.03 (18) | 3.72 ± 0.08 (15) |

[a] Cylindrical refers to cylindrical micelles observed at breakpoint B, and spheroidal refers to spheroidal micelles observed at breakpoint C (Vinson et al., 1989). [b] Numbers in parentheses are the number of observations.

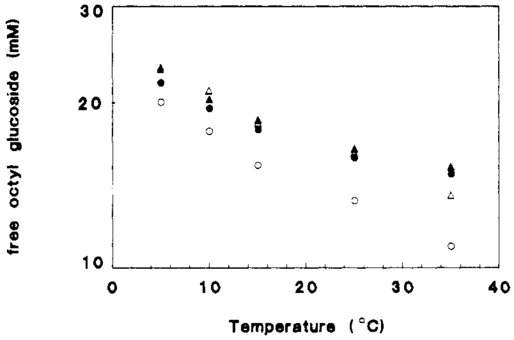

FIGURE 4: Temperature dependence of the aqueous octyl glucoside concentrations at the boundaries shown in Figure 3 observed during OG solubilization of egg PC vesicles. The values of $[OG]_{aq}$ were taken as the intercepts in Figure 3A,B; values at B are shown as (●), and values from C are indicated by (▲). The open symbols indicate the theoretical values for the aqueous OG concentration expected from the composition of the structures at B and C if the components mix ideally as predicted by Raoult's law (eq 3).

boundary of the coexistence region are listed in Table II.

The compositions of the two limiting phospholipid-surfactant structures were calculated from the slopes of the lines in Figure 3 (slope = [OG]/[PC]) and expressed in terms of the mole fraction of OG in each structure. There was no significant change in the average composition of the structures at breakpoint B (Figure 3A, Table II). However, the composition of the lipid-saturated spheroidal micelles did change significantly with temperature, with the higher mole fractions of OG observed at higher temperatures (0.74 at 5 °C to 0.79 at 35 °C) (Figure 3B; Table II). This is equivalent to stating that the ratio of OG per PC molecule in the structure changed from 2.9 to 3.7 over this temperature range. Thus, while about the same fraction of OG was needed for cylindrical micelles, more OG per PC was required to form spheroidal micelles at the higher temperatures.

Like the OG cmc, the aqueous concentration of OG in equilibrium with the two mixed phase structures, i.e., cylindrical and spheroidal micelles, decreased with increasing temperature. However, the magnitude of the change with temperature was less for the mixed system than for OG alone (Figure 4 compared to Figure 2). Moreover, the ln $[OG]_{aq}$ values at the phase boundaries in the mixed OG-PC system have a curvilinear dependence on the temperature, although this cannot be clearly distinguished from a linear dependence statistically. The $[OG]_{aq}$ at both breakpoints is lower than the OG cmc at all temperatures examined but may approach the cmc at higher temperatures; linear extrapolation indicates that these values would coincide at about 60 °C.

*Relationship between $[OG]_{aq}$ and Mixed Micellar Composition.* As expected, the aqueous concentration of OG at which mixed micelles are initially observed was smaller than that of the corresponding cmc for each temperature examined; the addition of an "impurity" (egg PC) to a micelle alters the activity of the major component [e.g., see Tanford (1980)]. For micelles of sufficiently large size that the micellar state can be treated as a separate phase, ideal mixing between the components results in a direct linear relationship between the micellar composition, $X_{\text{mic}}$, the cmc in the absence of added amphiphile, cmc$^0$, and the observed aqueous concentration, $[\text{OG}]_{\text{aq}}$, i.e. [e.g., see Helenius and Simons (1975), Tanford (1980), and Holland and Rubingh (1983)]

$$[\text{OG}]_{\text{aq}} = \text{cmc}^0 X_{\text{mic}} \qquad (3)$$

Comparing the observed values of $[\text{OG}]_{\text{aq}}$ to those predicted from the cmc$^0$ and the micellar composition in mole fraction units indicated that the spheroidal mixed micelles observed at breakpoint "C" fit this relationship well (Figure 4). However, the composition at B was not predicted by eq 3, indicating that OG and PC did not mix ideally in these structures. Another explanation could be that the composition at B represents more than one type of structure, a possibility currently being explored. Another way to think of these data on the "average" composition of the structures is to consider that if mixing were ideal, the nearly constant composition of the structures at B across the temperature range would require that the $[\text{OG}]_{\text{aq}}$ have the same dependence on temperature as the cmc$^0$; clearly, this was not observed.

*Vesicle Formation by a Temperature Change.* The temperature dependence of the structural transitions for OG and egg PC suggested that one practical means for inducing vesicle formation might be to change the temperature of the solution. Since the critical stages of protein reconstitution appear to occur at the lamellar–micellar transition, it is useful to be able to control the rate of transition to be either very fast or very slow depending on the nature of the particular protein–lipid–surfactant mixed micelles [see, e.g., Jackson and Litman (1985)]. Temperature changes offer an alternative means of precisely controlling this rate. As indicated in Figure 3, the formation of mixed micelles from vesicular structures at any given concentration and temperature was favored by raising the temperature. Similarly, formation of the cylindrical mixed micelles and subsequently lamellar states from spheroidal mixed micelles could be induced by dropping the temperature to the point at which the detergent no longer dissolves the lipid. Both RET and 90° light scattering may be used to monitor the transition with temperature (Figure 5A): as expected, cooling a micellar solution results in an increase in scattered light or turbidity and a decrease in NBD-PE fluorescence due to an increase in RET when the two fluorophores come closer together.

Changes in the 90° light scattering associated with the spheroidal to cylindrical micellar transition induced by temperature are shown for four different [OG] in solutions of 0.2 mM egg PC (Figure 5B). The temperature-induced transitions corresponded well with those induced by changing the detergent concentration at fixed temperature. There was a significant time dependence, however; appreciable hysteresis in the transition temperatures was observed at d$T$/d$t$ greater than 0.5 °C/min (not shown).

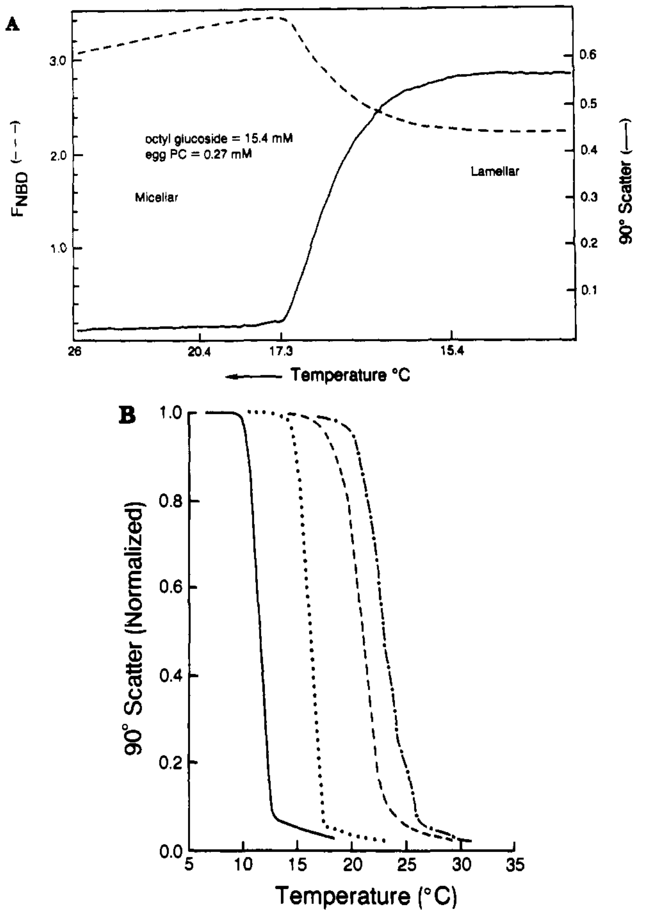

FIGURE 5: Lamellar phase formation from egg PC–OG mixed micelles induced by a change in solution temperature. (A) Fluorescence and 90° light-scattering trace of a fixed mixture of egg PC (0.27 mM) and octyl glucoside (15.4 mM) during cooling from 26 to 10 °C. The temperature in the cuvette was monitored directly as it was decreased from a temperature above to one below the phase transition. (The transition could be cycled repeatedly by alternately cooling and warming the solution.) (B) Structural transition monitored by the change in 90° light scattering, where an increase in scattered light indicates cylindrical micelle formation, for samples containing 0.2 mM egg PC and OG of 16.6 (solid line), 15.4 (dotted line), 14.1 (dashed line), and 13.9 (dot–dash line) mM.

## DISCUSSION

These studies demonstrate that the critical micelle concentration of the nonionic detergent octyl glucoside is very sensitive to temperature in the range used for most biological solubilization and reconstitution protocols. The temperature sensitivity of the cmc is reflected in a similar temperature dependence of the structural transitions observed for aqueous mixtures of OG and egg PC.

*Formation of Octyl Glucoside Micelles.* Absolute solubility is determined by the relative balance of forces favoring aggregation and those favoring solubilization. In general, solubilization is favored at high temperatures; however, it is well-known that for acyl solutes in water, the temperature dependence of solubilization is biphasic with solubility reaching a minimum due to what is referred to as the "hydrophobic effect" [e.g., see Tanford (1980)]. Specifically, it is the unfavorable entropic change associated with water structuring around an acyl chain that dominates the total free energy for solubilization at lower temperatures. Thus, as long as the entropic term is of greater magnitude than the enthalpic term, the solubility will decrease with increasing temperature. Both the absolute solubility and the position of the minimum for an amphiphilic solute will depend on the acyl chain length, the degree of branching, and the nature of the polar headgroup.

In general, ionic detergents often display relatively little change or increasing solubility with temperature from 5 to 40 °C; i.e., thermal effects overcome the hydrophobic forces tending toward micellization [e.g., see Small (1986)]. Nonionic detergents, on the other hand, are reported to have progressively lower cmc's at higher temperatures up to about 60 °C. Octyl glucoside cmc's, as observed here, have a clearly negative temperature dependence up to 40 °C.

The validity of calculating the thermodynamic parameters from the temperature dependence of the cmc is extremely dependent on the assumption that the micelle is a separate phase of discrete size; this is known to be formally incorrect. However, in practice, the error associated with ignoring specific terms for variation of micelle size appears to be small (<3%) relative to the total values (Mueller, 1976). For example, at $T$ = 25 °C for the OG micelle with an aggregation number of about 40 (Eidelman et al., 1988), the correction in $\Delta G$ for dissolution is about 0.5%, certainly less than the error in the measurements. Furthermore, in two examples where the enthalpic term was determined both calorimetrically at the cmc and by the temperature dependence of the cmc, the values were identical within the range of experimental error (Pilcher et al., 1969; Espada et al., 1970). Large variation in micelle size with temperature would be reflected in nonlinear plots of ln cmc vs temperature as observed for the alkyl poly(oxyethylenes) [e.g., see Corkill et al. (1964) and Muller and Platko (1971)]. The near-linearity of $\Delta G$ vs $T$ in this study is a further indication that over this temperature range any changes in the heat capacity term or micelle number were not significant (or clearly discernible given the error in the data[2]). In summary, this method is probably a valid experimental approach despite the theoretical difficulties. In addition, since the focus of this paper is to find differences between OG partitioning from water into an OG micelle, compared to OG partitioning between water and a mixed OG–PC micelle, this particular method avoids the added complications associated with calorimetry with PC as a third component.

The enthalpy change associated with transferring a monomer of octyl glucoside from aqueous solution to a preformed micelle is similar to those observed for other nonionic detergents (Corkill et al., 1964, 1966; Muller & Platko, 1971) and considerably more positive than values for ionic detergents over a similar temperature range [e.g., see Pilcher et al. (1969) and Small (1986)]. In contrast to the present observations, $\Delta H_{\mathrm{w}\rightarrow\mathrm{m}}$ in the studies cited above became more negative at higher temperatures, whereas for OG it increased with temperature. The $\Delta H_{\mathrm{w}\rightarrow\mathrm{m}}$ expected for an eight-carbon alkyl chain is negative (~−4000 cal/mol) if one assumes this process is analogous to removing an eight-carbon alkane from water to a micellar interior (Tanford, 1980). Thus, it is clearly the headgroup solubility properties contributing to a positive enthalpic term that oppose micelle formation.

The entropic term $-T\Delta S$ in the free energy expression is expected to increase in magnitude by about −700 to −800 cal/mol per $-\mathrm{CH_2}-$ ($T$ = 25 °C) [e.g., see Tanford (1980)]. The entropic term for a linear eight-carbon acyl chain can be estimated to be −6.6 kcal/(°C·mol) from an empirical relationship which assigns 2100 cal/(°C·mol) to the terminal methyl group and assumes that the first carbon in the chain is not protected from water: $-T\Delta S = -[2100 + (n - 2)750]$ (Tanford, 1980). This calculated value is about 20% smaller than the average value observed [−7.8 kcal/(°C·mol); Table I]. The discrepancy may indicate that the first methylene group in the OG acyl chain is fully protected from water in the micellar state [then the predicted $-T\Delta S$ would be −7.4 kcal/(°C·mol)] and/or that there is an added positive entropic change associated with concentrating the glucoside moiety and possibly partially removing it from water.

There is no theoretical basis for estimating the contribution of a nonionic polar headgroup to the free energy changes, but any thermodynamic treatment must consider the changes in hydration, interaction among the glucoside moieties, loss of motional freedom, and steric repulsion. It is of interest that neither the cmc nor its temperature dependence is affected by replacing 150 mM NaCl with 300 mM mannitol, suggesting minimal ionic effects on the interactions between the glucoside moieties at this relatively low ionic strength. An important caveat is that the NaCl and the mannitol buffers have identical water activity (osmolality). The effect of *low solute* concentration may be very different.

*Octyl Glucoside Solubilization of Egg Phosphatidylcholine.* Although the lamellar–micellar transition of the mixed detergent–phospholipid system is a different process from the formation of single-component detergent micelles, the processes are closely related, and aspects of the two-amphiphile system may be elucidated by comparison to the pure OG system. The $[\mathrm{OG}]_{\mathrm{aq}}$ values at both boundaries of the mixed micellar region are lower than observed for the pure OG micelle as predicted theoretically [e.g., see Tanford (1980), Holland and Rubingh (1983), and Hall (1987)]. Like the cmc, the OG monomer concentration in equilibrium with the two types of structures decreases with increasing temperatures, but the dependence is shallower than that of the pure cmc and clearly nonlinear (Figures 2 and 4).

That the solubility properties of the surfactant play a significant role in the temperature dependence of the lamellar–micellar transformation is also indicated for the cholates. For example, glycocholate solubilization of dimyristoylphosphatidylcholine (DMPC) vesicles requires higher detergent monomer concentration at 40 °C compared to 25 °C, reflecting the positive temperature dependence of glycocholate, an anionic surfactant (Schurtenberger et al., 1986).

The overall difference in the free energy of removing an OG molecule from water mixed OG–PC cylindrical and spheroidal micelles compared to a pure OG micelle was actually smaller than the changes indicated in the estimates of enthalpic or entropic terms (Table I). The enthalpic and entropic components for either mixed structure were of lower magnitude than equivalent values in the absence of phospholipid (see "average values", Table I). These changes in how the free energy was partitioned had opposite effects of nearly the same magnitude on the net free energy change. An interpretation of these differences is that the mixed-component environments are both more favorable for the OG molecule to form positive interactions (e.g., van der Waals) and more constraining (i.e., limit molecular movement) than the pure micellar structure. Such an interpretation is reasonable given the longer and more ordered acyl chains of the phospholipids contained in the two types of mixed OG–PC micelles compared to the pure OG micelles.

The entropic and enthalpic values calculated here describe only the free energy change for transfer of an OG from water to a mixed micellar structure and not for the formation of the structure per se since they are based on the aqueous OG concentrations. Parameters for the formation of the entire structure are obtained from calorimetric data. The enthalpy of solubilization of DMPC by Triton X-100 monitored at 28 °C (i.e., above the sol–gel phase transition) has been reported to be nearly zero (Kresheck & Nimsgern, 1983). Unfortunately, this value cannot be compared to the present enthalpic calculations for the transfer of OG to a mixed micelle since the two methods measure different processes.

The aqueous or monomer surfactant concentration in equilibrium with the mixed micelles is at equal chemical potential with the micellar surfactant. If mixing is ideal, then the monomer concentration is predicted by Rauolt's law [eq 3 (Tanford, 1980; Holland & Rubingh, 1983; Hall, 1987)]. As noted under Results, the cylindrical micellar structure at breakpoint B does not fit the predictions for ideal mixing, whereas the PC-saturated mixed spheroidal micelles at breakpoint C appear to behave as ideally mixed by these criteria (Figure 4).

Ideal mixing between two molecules as different as phosphatidylcholine and octyl glucoside would not be expected a priori. However, Koynova et al. (1988) determined the effect of the headgroup on miscibility in the lamellar phase using DMPC and 1,2-di-*O*-tetradecyl-3-*O*-$\beta$-D-glucosyl-*sn*-glycerol and observed complete miscibility of these amphiphiles with identical acyl chains. Their system might be reasonably compared to OG–PC spheroidal micelles for which the data are consistent with a model where the acyl region thickness is determined by the detergent (2 × 8 carbons) with the PC interdigitated to permit optimal interactions among the acyl chains despite the disparity in length of the two components (Eidelman et al., 1988; Vinson et al., 1989). This might not be the case for a cylindrical mixed micelle where it is likely that there is segregation of the phospholipid and detergent; the OG might be expected to concentrate on the ends of the cylinders. In addition, the population of cylindrical micelles may show significant size heterogeneity, and/or there may be additional unidentified structures in the mixture.

The compositions of the phospholipid–surfactant structures reflect the ability of the two constituents, OG and PC, to pack into an arrangement that protects the acyl chain from water while meeting the steric constraints of both parts of these amphiphilic molecules. The cylindrical mixed micelles are long flexible rods of egg PC and OG. Breakpoint B represents the point where the first spheroidal micelle appears, and thus the composition at B is thought to represent the cylindrical micellar composition just before these reorganize to form spheroidal micelles. The relative composition at this stage is about 1.8 OG per PC from 5 to 35 °C (Table II). It is of interest that the surface area of lamellar egg PC is about 1.8 times that of an OG molecule which suggests that up to half of the surface area may be associated with single eight-carbon acyl chains before this structure collapses. Moreover, since the relative composition is constant with temperature, the molecular dimensions must not vary significantly.

In contrast, the ratio of OG to PC in the lipid-saturated spheroidal micelles *does* vary from 2.9 at 5 °C to 3.7 at 37 °C (Table II). Since, as seen above, it is not possible to add more detergent to the cylinders at warmer temperatures, the change in composition must reflect the requirements for a stable mixed micelle. If we assume that the relative acyl volume to micelle–water surface area ($V/A$) is an important determinant for the relative stability of a given mixed micelle, then either (a) these OG–PC mixed micelles change size or shape with temperature or (b) an optimal size and shape are maintained while other factors dictate the composition change. If the basic unit of these mixed micelles is considered to consist of one PC molecule and the requisite OG molecules for a given temperature (i.e., 2.9 OG at 5 °C and 3.7 OG at 35 °C), then the weighted-average $V/A$ per "micelle unit" may be calculated from measured and estimated dimensions at each temperature. First, if no temperature corrections are made, $V/A$ may be calculated assuming (1) an area per PC of 71 Å$^2$ (Small, 1986), (2) an area per OG of 38 Å$^2$ (Ollivon et al., 1988), (3) an acyl volume for egg PC of about 1063 Å$^3$ [e.g., see Small (1986)], and (4) an acyl volume of 242 Å$^3$ for octyl glucoside calculated from the empirical relationship that the acyl volume for a linear alkyl chain $n$ carbons long is $27.4 + n(26.9)$Å$^3$ (Tanford, 1980). The value of $V/A$ decreases about 5% from 5 to 35 °C, so clearly no major changes in micelle dimensions or aspect ratio need to be invoked. If the volume estimates are corrected for temperature by using the temperature coefficients for PC and an alkane [i.e., 0.042 Å$^3$ °C$^{-1}$ $-CH_2-^{-1}$ and 0.009 Å$^3$ °C$^{-1}$ $-CH_2-^{-1}$, respectively; taken from Small (1986)], then *no* differences are observed in the $V/A$ parameter for these micelles. Although it is clearly wrong to assume that the area per molecule does not change with temperature, the change for PC is very small (or undetectable) by surface pressure measurements [e.g., see Chapman (1975)]. The conclusion from these calculations is that the shape and size of these OG–PC mixed micelles are invariant in this temperature range despite the change in relative concentrations of the two components.

*Conclusion.* The temperature dependence of OG solubility is steep and negative ($T$ = 5–40 °C), and a similar temperature dependence is found for solubilization of egg PC by OG in excess water. The latter result provides several practical consequences for protein reconstitution from octyl glucoside solutions. First, the temperature of a particular protocol will affect the amount of detergent needed to achieve a particular lipid–detergent phase; any adjustments in temperature must include appropriate adjustments in the detergent concentrations used. Similarly, if a particular protocol involves operations at several temperatures, e.g., solubilization on ice and protein purification at room temperature, it is essential to consider the consequences of changing temperature on the solubilization state of the lipids and, likely, the proteins. Such a protocol may be improved by using a detergent that is relatively temperature insensitive (e.g., MEGA-9; Suchy et al., 1989). Finally, instead of being a problem, the temperature dependence may be exploited to cross the critical lamellar–micellar phase boundary by temperature jump in the absence of chemical composition change. Since this rate may be closely controlled and no dilution is involved, this may be an important tool for membrane protein reconstitution.

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# Role of the Stereochemistry of the Hydroxyl Group of Cholesterol and the Formation of Nonbilayer Structures in Phosphatidylethanolamines[†]

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ABSTRACT: The phase behavior of mixtures of cholesterol or epicholesterol with phosphatidylethanolamine was studied by differential scanning calorimetry and by X-ray diffraction. Discrete domains of cholesterol are detected by X-ray diffraction in the $L_\alpha$ phase of phosphatidylethanolamine from egg yolk and synthetic dielaidoylphosphatidylethanolamine beginning at mole fractions of 0.35–0.4 cholesterol. Separate domains of crystalline epicholesterol can also be detected in the $L_\alpha$ phase of dielaidoylphosphatidylethanolamine by X-ray diffraction at as little as 0.16 mole fraction of epicholesterol. This is a result of poor miscibility of the epicholesterol with dielaidoylphosphatidylethanolamine. Epicholesterol does not alter the $L_\beta \rightarrow L_\alpha$ transition or bilayer spacing. Epicholesterol also has little effect on the diameter of the cylinders in the hexagonal phase. Formation of the inverted hexagonal phase is facilitated by addition of small amounts of cholesterol (mole fraction less than 0.2) in both egg phosphatidylethanolamine and dielaidoylphosphatidylethanolamine. However, at higher mole fractions of cholesterol, the stability of the liquid-crystalline phase is found to increase markedly for dielaidoylphosphatidylethanolamine but not for egg phosphatidylethanolamine, indicating the importance of the structure of the acyl chains in controlling the relative stability of the lamellar and nonlamellar phases in these systems. In contrast to cholesterol, epicholesterol markedly lowers the $L_\alpha \rightarrow H_{II}$ phase transition temperature at low mole fraction of sterol. This result demonstrates the importance of the orientation and motional properties of an additive in determining the $L_\alpha \rightarrow H_{II}$ transition temperature.

Although many phospholipids in aqueous dispersion spontaneously form bilayers, it has long been recognized that some of the major lipid components of biological membranes, e.g., phosphatidylethanolamine (PE),[1] spontaneously form nonbilayer phases in purified form and that nonbilayer structures may be important intermediates in membrane function (Cullis & De Kruijff, 1979; Cullis et al., 1980; Hui et al., 1981; Verkleij, 1984; De Kruijff, 1987). The polymorphism of natural and synthetic PE has been reported, and the phase diagrams are well characterized (Shipley, 1973; Seddon et al., 1983; Harlo & Eibl, 1980, 1981; Boggs et al., 1981). Extensive calorimetric studies of the interaction of sterols with synthetic

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[1] Abbreviations: EYPE, egg yolk phosphatidylethanolamine; DEPE, dielaidoylphosphatidylethanolamine; PE, phosphatidylethanolamine; $L_\beta$, lamellar–gel phase; $L_\alpha$, lamellar–liquid-crystalline phase; $H_{II}$, hexagonal phase, type II; Tris, tris(hydroxymethyl)aminomethane; $X$(chol), mole fraction of cholesterol; $X$(epi), mole fraction of epicholesterol; PS, phosphatidylserine; PC, phosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine.

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