Vesicle-micelle transition of phosphatidylcholine and octyl glucoside elucidated by cryo-transmission electron microscopy

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ABSTRACT Vesicle-micelle transition structures of egg phosphatidylcholine (PC) and octyl glucoside (OG) mixtures were observed in the vitrified hydrated state by cryo-transmission electron microscopy (cryo-TEM) and correlated with the macroscopic and molecular changes previously associated with micellization monitored by 90° light scattering and resonance energy transfer between fluorescent lipid probes. Several distinct structural changes occurred as OG was added to the PC vesicles. First, the average vesicle size decreased from 160 nm to <66 nm with no apparent change or decrease in optical density (OD). Then, associated with a small rise in OD, samples with open vesicles were observed coexisting with pieces of lamellae and long cylindrical micelles; more micelles were seen at higher [OG]. This mixture of vesicles and cylindrical micelles occurred in the region of the phase diagram previously attributed to vesicle opening, and possibly vesicle size increase. At higher [OG], small spheroidal micelles coexisting with cylindrical micelles correlated with a decrease in OD and changes in the fluorescence signal. At high [OG] when the solution appeared clear, spheroidal micelles were the dominant structure. By using cryo-TEM, a technique which preserves the original microstructure of fluid systems and provides direct images at 1 nm resolution, we have elucidated the vesicle-micelle transition and identified intermediates not known previously in the PC/OG system.

INTRODUCTION

Since the introduction of phospholipid bilayer vesicles as a tool to study the properties of cell membranes (Bangham, 1968), the use of and sophistication in preparing such vesicles have both increased. One important use of phospholipid vesicles is as an appropriate milieu for the study of membrane proteins. Although there are some examples of effective membrane protein reconstitution into pre-existing phospholipid vesicles (e.g., Newton et al., 1983; Scotto et al., 1985), many effective membrane protein reconstitution protocols start from systems containing surfactant-phospholipid-protein mixed micelles, and require removal of the surfactant component in such a way that the micellar-vesicular transition permits functional protein insertion in the newly formed lipid bilayer vesicles. It is for this reason that describing the phase behavior of mixed surfactant-phospholipid systems in excess water should provide a rational basis for developing reconstitution schemes. Also, these surfactant-phospholipid systems are just one example of mixtures of two different amphiphilic molecules that may play a role biologically: the effects of free fatty acids, of bile salts and of some anaesthetics which partition into the bilayer may be analogous to some effects of detergents at low concentration. In addition, similar phenomena are known to occur in synthetic mixed amphiphile systems making examination of PC and OG interactions quite widely applicable.

Solubilization of phospholipid vesicles by surfactants has been considered to occur in three general stages. At low surfactant concentrations, the surfactant partitions into the vesicle structure in a way that may be described by a vesicle/water partition coefficient (e.g., Jackson et al., 1982). According to previous work, when the phospholipid bilayer becomes "saturated" with surfactant, the system undergoes a structural transition from lamellar structure (proposed to be large bilayer sheets) to surfactant micelles saturated with lipid (e.g., Helenius and Simons, 1975; Wrigglesworth et al., 1987). The transition is not infinitely sharp, and there is a region where both of these mixed amphiphilic structures are thought to coexist. As surfactant concentrations are further increased, the mixed micelles contain increasing mole fractions of the surfactant, until at very high relative surfactant concentration, the phospholipid is fully solubilized. The concentrations at which these microstructural changes occur, the effects of low surfactant levels, and the nature of the mixed micelles depend on the type of surfactant. For example, micellization of egg PC occurs at a surfactant-to-phospholipid ratio in the micelles of 1.3–2.5 for Triton X-100 (e.g., Goni et al., 1986; Paternostre et al., 1988), of 0.9–2 for sodium cholate (e.g., Müller, 1981; Paternostre

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et al., 1988), and 3–3.8 for octyl glucoside (e.g., Jackson et al., 1982; Ollivon et al., 1988; Paternostre et al., 1988). At low surfactant levels, sodium cholate in the presence of egg PC facilitates vesicle growth to characteristic sizes for each surfactant:phospholipid ratio (Almog et al., 1986). An apparently unique state is postulated for bile salt-egg PC mixtures; this is a small mixed disc of lipid and bile salt(s) with bile salt stabilized edges (e.g., Mazer et al., 1980; Fromherz and Rüppel, 1985). The structural changes have not been as well characterized for other mixtures of sufactants and lipid.

Our work has focused on octyl glucoside solubilization of egg phosphatidylcholine vesicles. Octyl glucoside is a nonionic surfactant frequently chosen for biological reconstitutions due to its relatively high critical micelle concentration (CMC) of 21.5 mM (25°C). In previous studies three phase boundaries were identified using optical density and resonance energy transfer methods in combination with other measurements (Ollivon et al., 1988). The physical meanings ascribed to these boundaries have been, in accordance with Helenius and Simons (1975), at increasing octyl glucoside concentrations: (*a*) vesicle opening, (*b*) "detergent-saturated lamellar" structure formation, and (*c*) "lipid-saturated micelle" formation. A fluorescent technique was developed to determine the relative composition of the mixed surfactant-phospholipid micelles. This technique showed that the mole fraction of OG in the micelles gradually increases while exhibiting ideal mixing between the octyl glucoside and phosphatidylcholine components, and that the total number of molecules per micellar structure decreases with increasing OG concentration (Eidelman et al., 1988). The phase boundaries are sensitive to temperature, showing a dependence similar to that of the octyl glucoside CMC (Miguel et al., 1989). In addition, the compositions at the boundaries seem to be somewhat determined by molecular packing constraints (Miguel et al., 1989).

Despite this detailed information about the egg PC-OG mixed system, none of the above studies addresses directly the microstructure of the aggregates. Thus, it is not clear that the boundaries observed are due to the transitions ascribed to them. Hence, here, we have correlated the phase boundaries observed by fluorescence and 90° light scattering with long-lived structures observed using cryo-transmission electron microscopy (cryo-TEM). The samples are prepared as a thin film in a controlled (temperature, humidity) environment, rapidly quenched in liquid ethane at its freezing point, and examined in the vitrified hydrated state. This technique, which avoids the artifacts of staining and drying procedures, permits observation of relatively undistorted samples. It has been applied to the study of viruses (Adrian et al., 1984), vesicular and liposomal dispersions (Talmon, 1986) phospholipid phase transitions (Talmon et al., 1989; Siegel et al., 1989), spherical micelles (Burns and Talmon, 1987; Vinson, 1988; J.L. Burns, Y. Cohen, and Y. Talmon, manuscript submitted for publication), cylindrical micelles (Bellare et al., 1986; Vinson, 1988), and microemulsions (Vinson, 1988). Cryo-TEM is the only experimental tool that provides direct microstructural information of complex fluids over the 1–1,000 nm size range. The insight this allows toward the microstructural elucidation of supramolecular assemblies and dynamics in liquids is evident.

The optical and correlative measurements from our previous work and that of others (Jackson et al., 1982; Ollivon et al., 1988; Eidelman et al., 1988; Paternostre et al., 1988; Miguel et al., 1989) have raised several specific structural questions that we wished to address in the present experiments. In particular, the existence of large pores in vesicles or open lamellar sheets is suggested by some data, but is controversial. The specific structure of structures responsible for the turbidity peak just before solubilization are not known, although vesicle fusion, open vesicle sheets and multilamellar vesicle formation have been suggested. It is clear that the mixed micelles change size with changes in detergent levels, but neither the shape nor the progression with surfactant concentration is known. Finally, the phospholipid and surfactant rearrangements at the lamellar-micellar transition have not been described. It will be seen below that the present results address these issues and reveal other unexpected structural changes. These results have been presented in an abstract (Vinson et al., 1989).

MATERIALS AND METHODS

Phosphatidylcholine-octyl glucoside mixtures

Egg phosphatidylcholine (egg PC) vesicles were formed either by sonication (SUV) (model 200; Branson Sonic Power Co., Danbury, CT) or by extensive dialysis (DUV) from octyl glucoside (OG) solutions. The requisite amount of lipid was added to a clean glass vessel from a stock solution in chloroform and the chloroform evaporated under a stream of nitrogen. The dry lipid was held under vacuum for at least 1 h to remove any traces of solvent before being suspended in aqueous buffer. Sonication was done in an ice bath and the vesicle suspension spun at low speed immediately after sonication to remove any titanium particles from the suspension. For the dialysis preparations, OG was added from a 400 mM stock solution to bring the OG to PC ratio in the mixed micelles to ~5, and the mixture was dialyzed against at least five changes of 100-fold excess buffer solution. The aqueous buffer was filtered (0.2 μm nucleopore membranes), degassed and subsequently bubbled with nitrogen. The buffer composition was 150 mM NaCl, 10 mM Na-Hepes, (sometimes 10 mM MES), 1 mM EDTA, and 0.02% sodium azide, pH 7.2.

Some vesicle preparations were composed of only egg phosphatidylcholine, others contained 1% (wt/wt) *N*-(7-nitro-2,1,3, benzooxadiazol-4-yl) phosphatidylethanolamine (NBD-PE) or 1% each NBD-PE and *N*-(lisamine rhodamine B sulfonyl-phosphatidylethanolamine (Rho-PE). The fluorescent groups were attached to the ethanolamine head-

group of the lipid. All lipids were from Avanti Polar Lipids (Birmingham, AL) and OG from CalBiochem Behring Corp., La Jolla, CA; all were used without further purification.

Mixtures of egg PC and octyl glucoside were prepared for cryo-TEM by addition of octyl glucoside from a 400 mM stock solution, generally during simultaneous observation of optical density or fluorescence as described elsewhere (Ollivon et al., 1988). The mixtures were held in sealed containers under nitrogen until they were prepared for EM observations. The lipid concentration was determined from total hydrolyzed phosphate, following the method of Ames and Durbin (1960).

In order to compare the micrographs taken of different preparations of OG and egg PC, it was necessary to determine the mole fraction of OG in the structures using the appropriate value for the aqueous OG concentration based on the compositional diagrams developed by Ollivon et al. (1988) and Eidelman et al. (1988). This is because the OG partitions between the aqueous and lipid regions. At concentrations below the point where the solution begins to clear, OG:PC interactions may be described by a simple partition coefficient, Kp, of 58 (Jackson et al., 1982; Ollivon et al., 1988).

Cryo-transmission electron microscopy

Cryo-TEM specimens of the egg PC-OG mixtures were prepared in the Controlled Environment Vitrification System (CEVS). A brief description of the sample preparation methods utilized is given below. See Bellare et al. (1988) for a detailed description of sample preparation with the CEVS.

Preparation of thin vitrified specimens of the egg PC-OG mixtures was relatively simple. A 3 μl drop of sample was placed on the surface of a holey carbon film-covered TEM grid (also called holey carbon grid or HCG) held by tweezers and mounted on the spring-loaded plunger of the CEVS. Before introducing the sample into the CEVS, the environmental chamber was equilibrated at the desired temperature (25°C) and humidity (95–99% rh). Thin (10–500 nm) liquid specimen films spanning the 2–8 μm holes of the HCG were formed when excess sample was removed by touching a filter paper to the grid. A synchronous double-cable release triggered first a camera shutter in the bottom of the CEVS chamber, and then the CEVS plunger. This action plunged the specimen through the opening shutter and into liquid ethane at its freezing point, where the thin specimen films were vitrified. Vitrification, i.e., solidifying the specimen without a change of phase, prevented microstructural artifacts due to crystallization. The vitreous specimen was transferred under liquid nitrogen into a TEM cold-stage and into the TEM, where the specimen temperature was maintained between 100–108 K. The specimen was imaged using normal and low-dose techniques. Specimens were examined in either an analytical electron microscope (model 120CX; JEOL Electron Optics, Div., Peabody, MA) with a Cryoholder (model 626; Gatan Inc., Warrendale, PA) or an analytical electron microscope (model 100CX) with an EM-Specimen Cooling Holder (JEOL Electronic Optics Div.). Micrographs were recorded with a nominal underfocus of 3.9 μm on SO-163 film and developed for 12 min in full-strength D-19 developer (Eastman Kodak Co., Rochester, NY).

RESULTS

Structure of the pure components

To establish a phase diagram, it was useful to first determine the structures of each component alone in aqueous solution. The surfactant, octyl glucoside, forms micelles at 25°C in aqueous solution. These were shown by cryo-TEM to be spheroidal particles with diameters of ~2–8 nm (Fig. 1 *a*). The resolution in our micrographs is limited to ~2 nm by chromatic aberration, mechanical stability of the cold stage, and the defocus of the objective lens.

The egg phosphatidylcholine vesicles formed by dialysis from a mixture of phospholipid and OG in aqueous solution, appeared by cryo-TEM to be spherical shells enclosing the buffer with an average diameter of 150 nm (Figs. 1 *b*, 5 *a*). The vesicles had clearly delineated 5 nm lipid bilayer walls that did not appear to vary in thickness. There was a small percentage of bi- or trilamellar vesicles in a freshly prepared sample; the number of multilamellar structures and very large irregular vesicles tended to increase with the age of the sample (not shown), especially if the initial preparation was by sonication. Despite these differences in the morphology of the initial preparation, the sequence of structures observed after the addition of octyl glucoside was the same for all six preparations examined.

In some micrographs the vesicles were invaginated (Fig. 2 *a*), even when vesicles from the same cryo-TEM specimen are spherical in other micrographs. The different vesicle images in Fig. 2 *a* can all be realized by considering projections of an invaginated vesicle at different orientations with respect to the electron beam. Vesicle invagination is thought to occur during specimen preparation. If there is a difference between the activity of water in the sample and in the vapor in the CEVS, then there will be an exchange of water between the sample and the vapor. In the present situation, the exchange is most likely to be from the sample to the surrounding vapor, which will in turn increase the osmotic pressure of the sample buffer and induce vesicle shrinkage due to water efflux. Vesicle shrinkage at nearly constant surface area results in the invaginated structures. Dubochet et al. (1988) compared cryo-TEM specimens of lipid vesicles in 100 mM NaCl prepared without evaporation and with intentional evaporation. Specimens prepared with intentional evaporation showed invaginated structures, whereas specimens prepared without evaporation did not show the osmotic effects. Similarly, we induced osmotic shrinkage of vesicles by mixing a drop of concentrated salt solution with a drop of the vesicular suspension on the grid before blotting and vitrification. These specimens showed invaginated structures as expected (data not shown).

In general, specimens prepared in the CEVS are adequately safeguarded against sample evaporation. Any areas containing invaginated vesicles, however, may indicate insufficient evaporation control for that specimen. Sometimes invaginated and unaffected vesicles existed at different locations on the specimen, indicating that evaporation is not uniform across the specimen or that invagination may arise by other mechanisms.

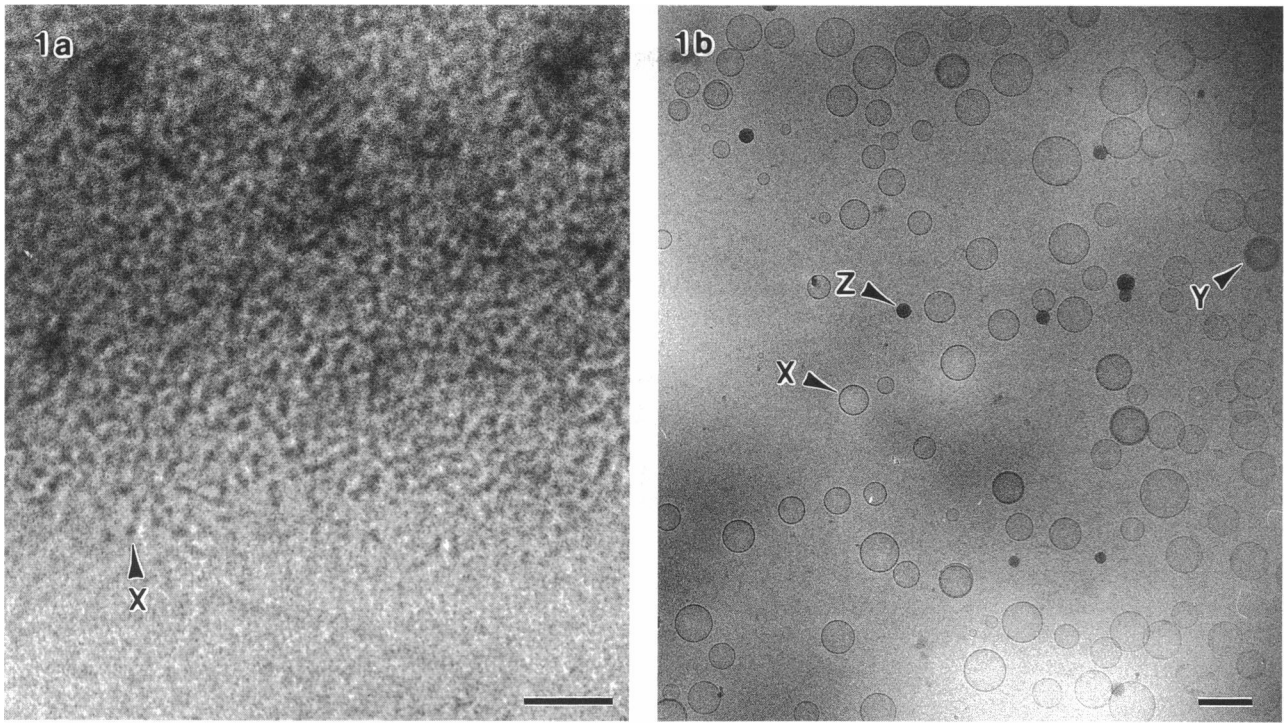

FIGURE 1 (*a*) TEM micrograph of vitrified 200 mM OG micelles. The spheroidal images (X), 2–8 nm in diam, are projections of the micelles. Bar = 50 nm. (*b*) Egg PC vesicles, unilamellar (X) and multilamellar (Y) with ~5-nm thick bilayers. (Z) denotes frost that deposited during specimen transfer into the microscope (recognized from the electron diffraction and diffraction contrast which makes the frost appear dark); vesicle size distribution is shown in Fig. 5 *a*. Bar = 250 nm.

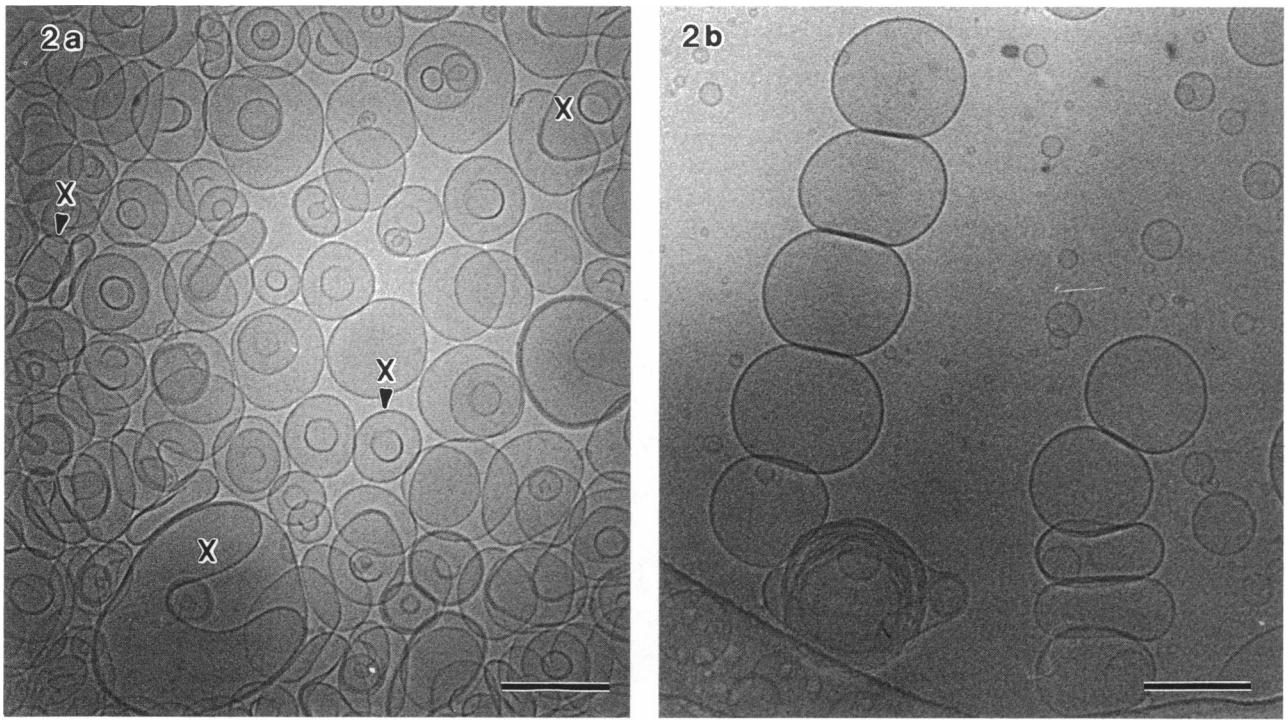

FIGURE 2 (*a*) Vesicles (X) that have invaginations because of specimen evaporation during sample preparation. Similar structures form by mixing vesicles with concentrated salt solutions. Bar = 250 nm. (*b*) Vesicles aligned and squeezed together by liquid flow during sample thinning. Bar = 250 nm.

A second type of distortion may arise from the mechanical stress due to the fluid flows on the grid when the sample is blotted to form the very thin liquid film. An example of aligned vesicles is seen in Fig. 2 *b*; the shear forces of this flow are sufficient to squeeze the vesicles into the wall of the carbon support and into each other. When there is time for these vesicles to redistribute within the liquid film before vitrification, they appear distorted.

Cryo-transmission electron microscopy of OG-PC mixed structures

The TEM samples were aqueous vesicular dispersions of egg PC to which OG was added. Optical density, 90° light scattering or RET was measured simultaneously. An example of 90° scattering for a preparation that was initially dialysis vesicles is shown in Fig. 3. At the high lipid concentrations used, the fluorescence measurements are difficult to interpret since there is both significant absorbance within the sample and aberrant signals due to light scattering, thus, these are not shown. In addition, for large vesicles at high lipid concentrations, the scattering peak just before solubilization is obscured in the initial signal to a lump. However, in at least one example, the turbidity maximum was clearly seen. The arrows in Fig. 3 represent equivalent points for samples shown in the electron micrographs: these were calculated from the Kp and total OG plus egg PC in the sample as described in the Materials and Methods section. The sequence of electron micrographs shown in Figs. 4, 6, and 7 represents the solubilization of egg PC vesicles by incremental addition of octyl glucoside as indicated by the arrows in Fig. 3. The micrographs selected are representative of the images seen from six preparations of lipid and detergent. Because the detergent-lipid mixtures were prepared from 30 min to several weeks before vitrification, the structures observed are relatively stable. Therefore, these should correspond to states reached during slow addition or removal of octyl glucoside from egg PC in an aqueous environment.

Fig. 4, *a–c* were taken of samples containing low levels of octyl glucoside. At the lowest total [OG] examined (7.8 mM OG and 9.8 mM PC) (Fig. 4 *a*), the vesicles

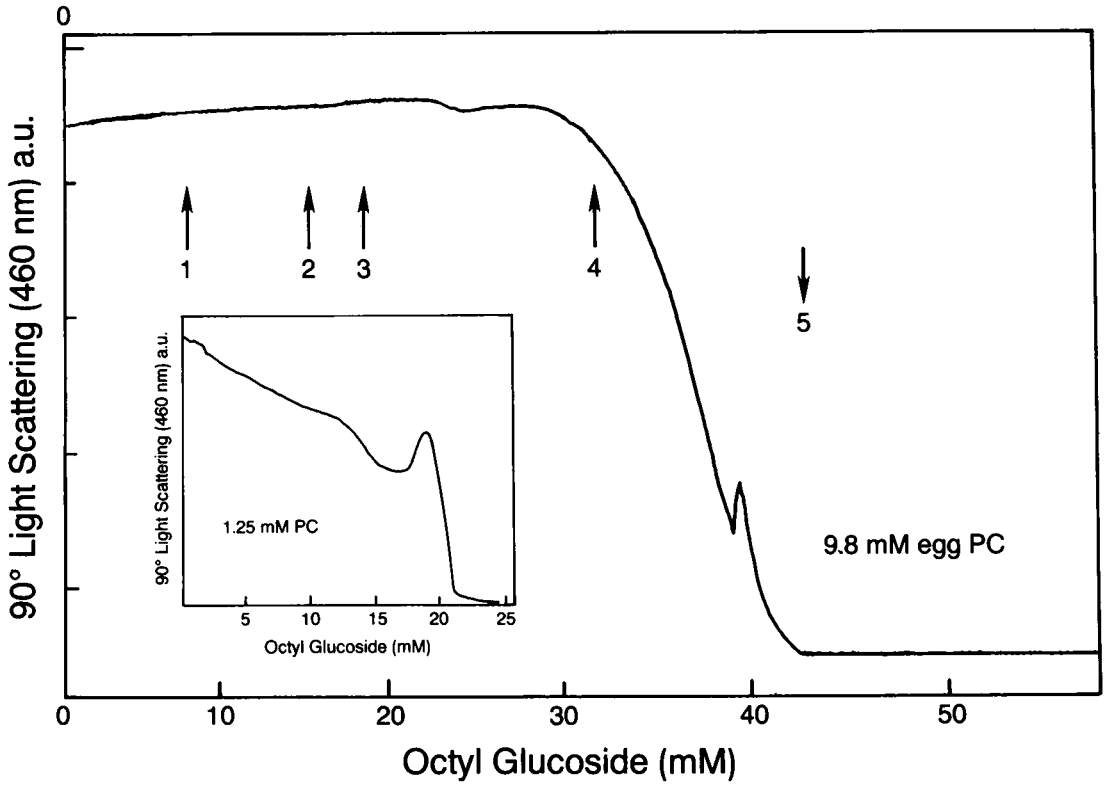

FIGURE 3 Solubilization of egg PC by OG monitored by 90° light scattering as a function of [OG] when the initial [PC] is 9.8 mM. The arrows indicate equivalent samples imaged by cryo-TEM. The insert is a similar trace at a lower total [PC] (1.25 mM) indicates an initial decrease in scattering and a turbidity peak just before the sample becomes clear. At high [PC] the optical signal is compromised due to severe inner filter effects.

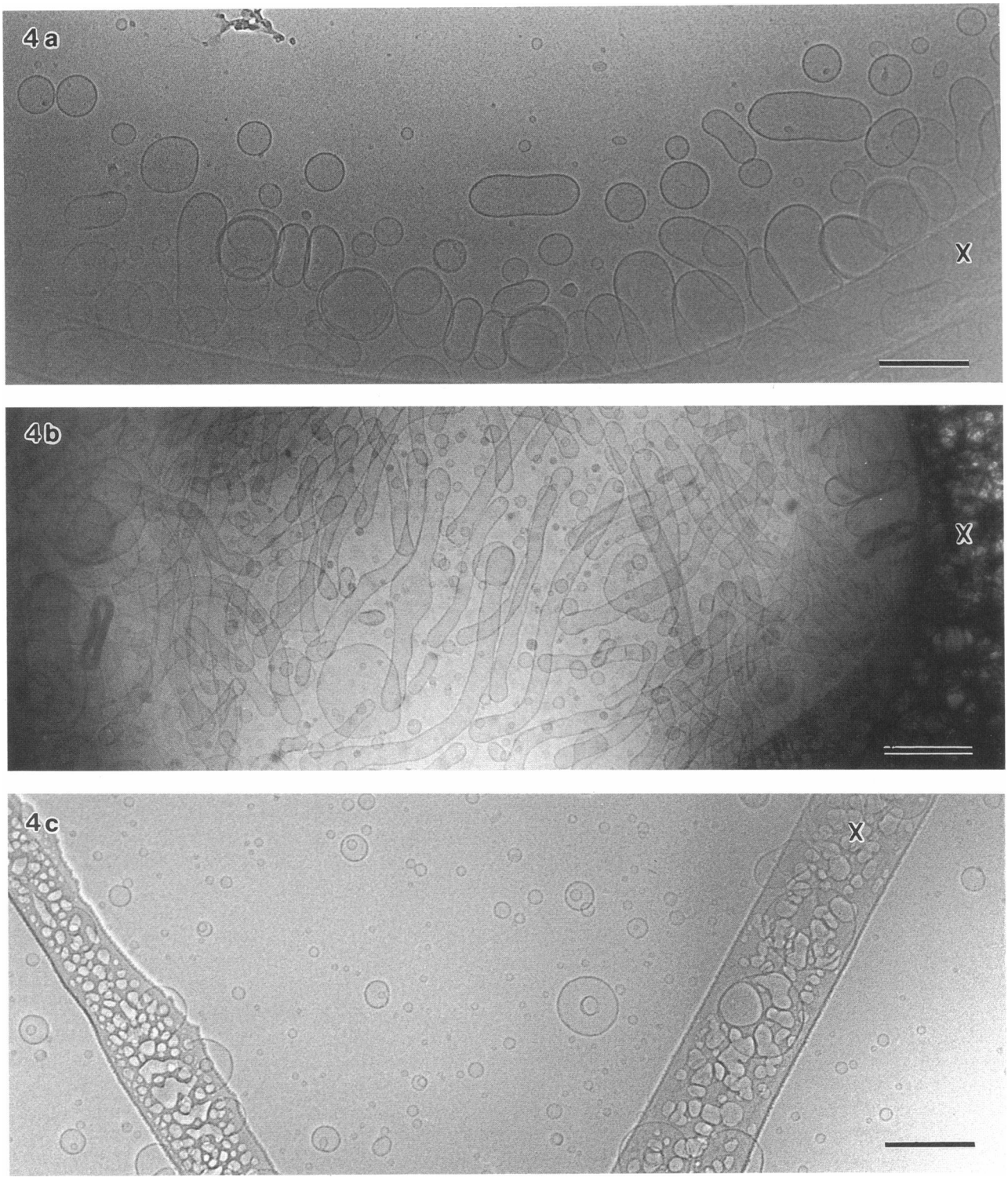

FIGURE 4 Vesicles (DUV) at low levels of OG progressively decrease in radius of curvature. Samples corresponding to (*a*) position 1 in Fig. 3 and the size distribution of Fig. 5 *b* (7.8 mm total OG< 9.8 mm PC, OG:PC in the structures is 0.24), (*b*) position 2 in Fig. 3 (OG:PC in the structures of 0.48), and (*c*) position 2 in Fig. 3 and the size distribution of Fig. 5 *c* (OG:PC in the structures is 0.48). The holey carbon support film can be seen at (X). Bar = 250 nm.

appeared to retain their original morphology. If we assume a K_p of 58 (Jackson et al., 1982; Ollivon et al., 1988) and molecular surface areas of 65 Å^2 and 38 Å^2 for PC and OG respectively (Small, 1986; Ollivon et al., 1988), then the expected size changes may be calculated. If the OG is uniformly distributed, these vesicles should have increased their surface area 14%, which corresponds to a 6.5% increase in average diameter. Instead, these vesicles present a bimodal size distribution (Fig. 5 *b*) showing a shift from a mode of ~160 nm diameter for control vesicles (Fig. 5 *a*) to a population centered on 200 nm diameter and a greater number of vesicles with diameters of 67 nm or smaller. If the vesicle radius is determined by the amount of OG incorporated in the bilayer, then these images suggest that OG is not equally distributed among structures under these conditions. Mixed aggregate populations, where the lipid and detergent are not uniformly mixed, are well known (e.g., Ollivon et al., 1988; Urbaneja et al., 1988).

At concentrations of OG where the average ratio of OG to PC molecules in the structures is ~1:2 and $[OG]_{aq}$ is ~11 mM, the vesicles were either elongated to form tubular shapes (Fig. 4 *b*) or significantly smaller (diameter < 67 nm) than the original vesicles (Figs. 4 *c*, 5 *c*). The radius of the tubular vesicles is similar to that of the small vesicle population; these tubules show some relatively swollen regions. Thus, their appearance suggests they may be intermediate structures between the larger and smaller vesicle populations.

The electron micrographs in Fig. 6, *a* and *b* represent samples where the molecular ratio of OG to PC in the structures, calculated from the K_p, ranges between 0.6 and 1. It is in this region that the vesicles were expected to open, and that the ultimate structure would be the so-called "detergent-saturated" lamellar phase. Open vesicles were clearly and frequently observed over this range of compositions (Figs. 6 and 7) but never in the absence of or at low concentrations of OG. In addition, at the higher range of [OG], long string-like structures presumably cylindrical micelles were seen to coexist with intact and open vesicles. The fraction of these cylindrical micelles appeared to increase with increasing total OG, but the data are not unequivocal on this point. Moreover, the strings seemed to emerge from the vesicles (Fig. 7 *a*): indeed, these pictures indicated that the transition from vesicles to cylindrical micelles occurred by a separation of the lamellar sheet into strings of material. These strings

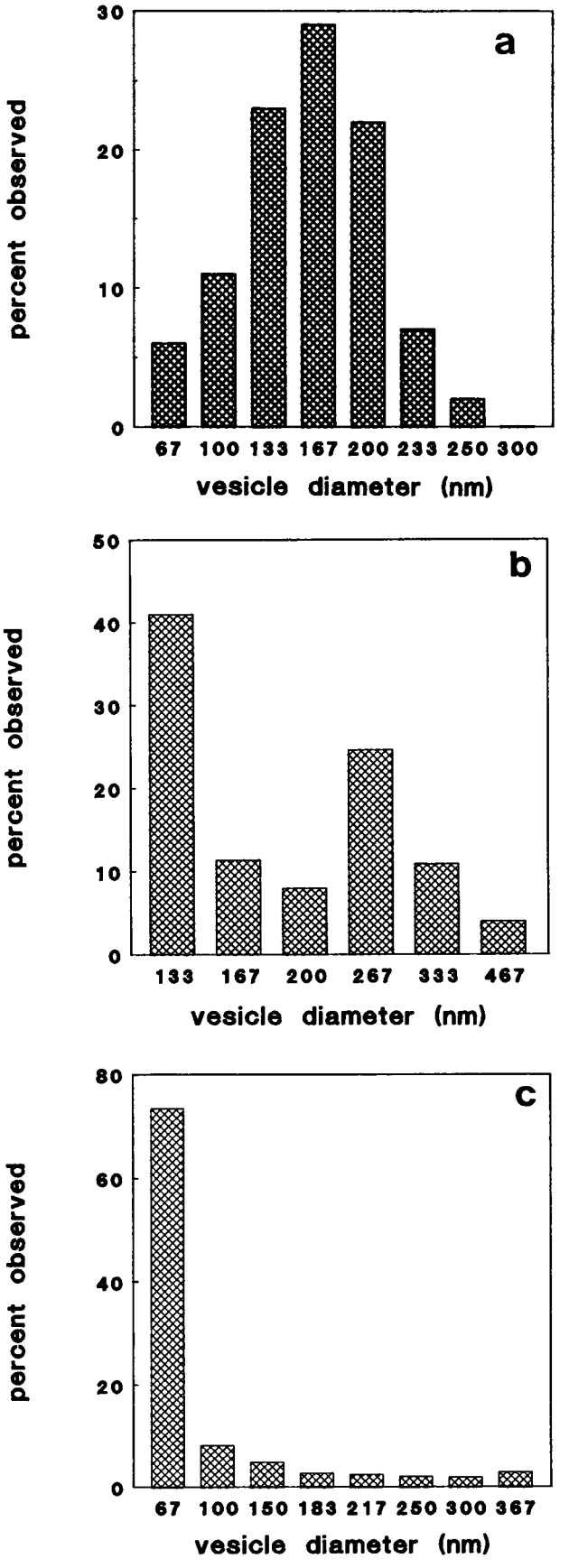

FIGURE 5 Size distributions for egg PC vesicles (*a*) in the absence of OG (DUV) (n = 167), (*b*) with an average OG:PC = 0.24 in structures (n = 119), and (*c*) with an OG:PC = 0.48 (n = 703). "n" refers to the number of vesicles included in the distribution. As [OG] increases, the vesicles size distribution shifts toward smaller vesicles consistent with 90° light scattering (Fig. 3, *insert*).

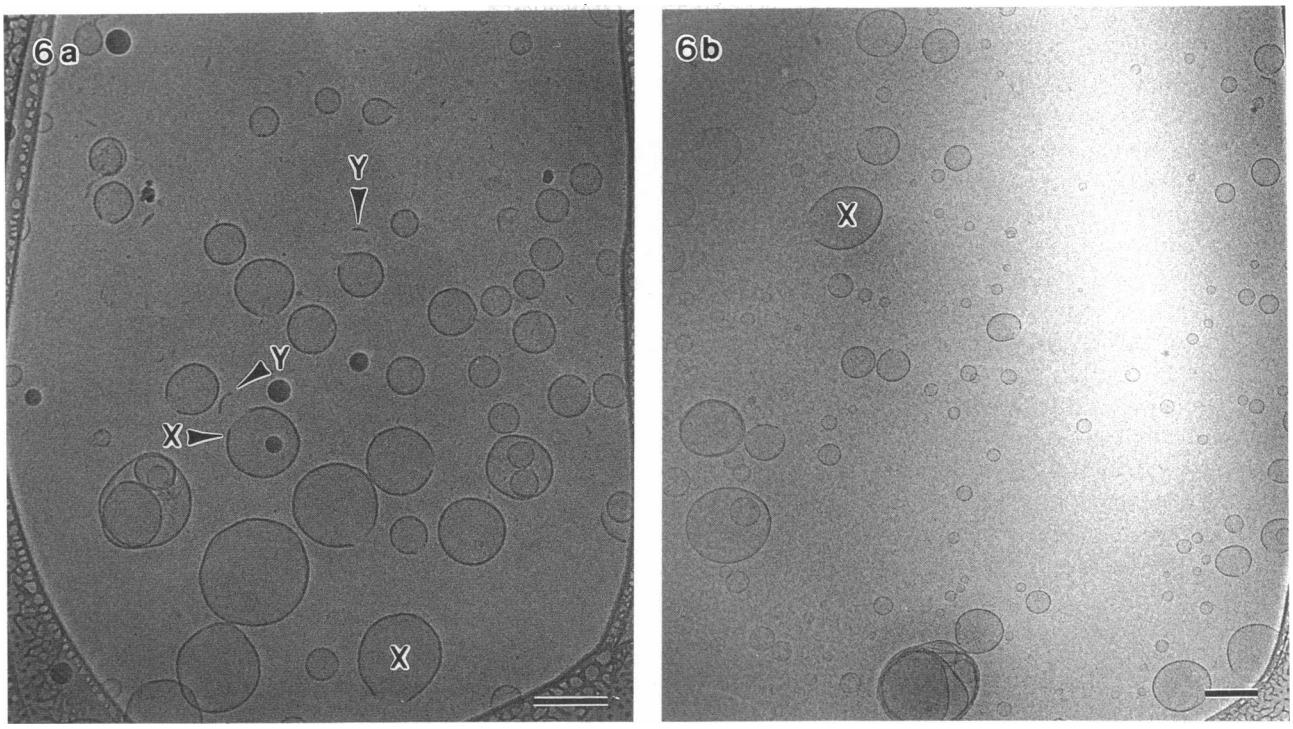

FIGURE 6 (*a* and *b*). Open (X) and closed vesicles and what seems to be pieces of lamellae (Y) found at position 3 in Fig. 3 (OG:PC in the structures is ~0.58). Bar = 250 nm.

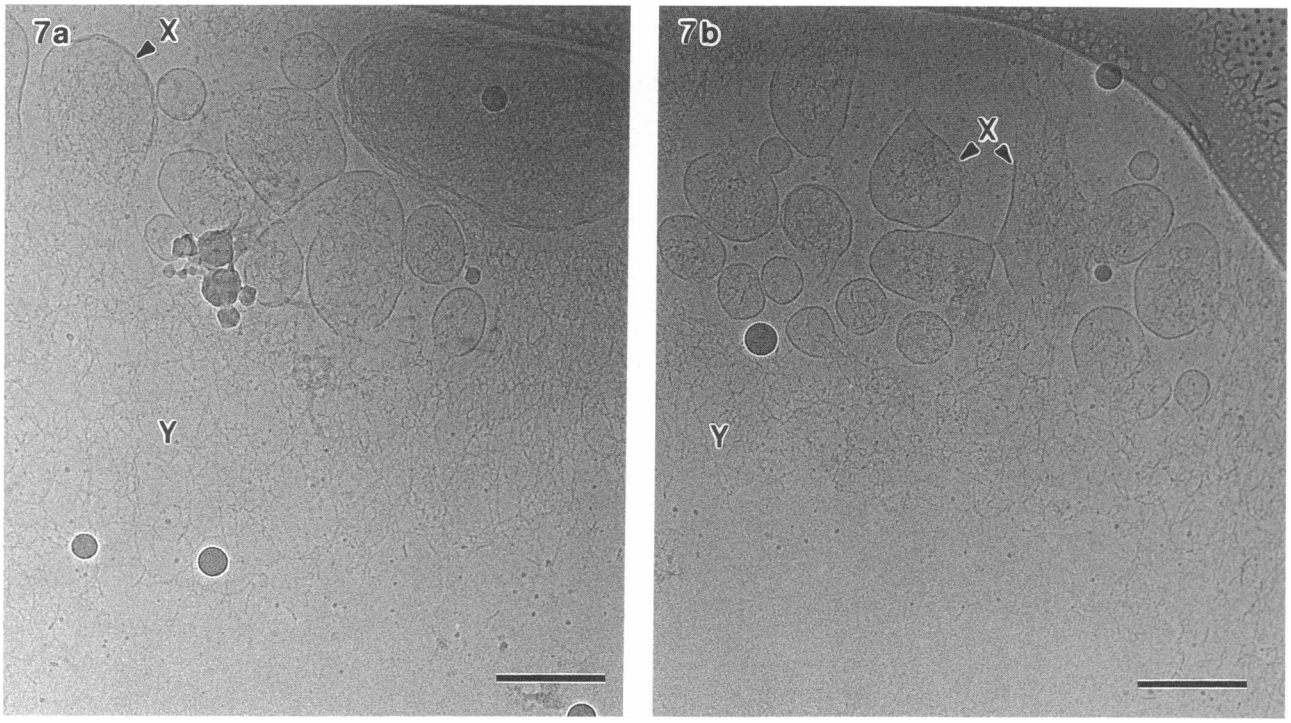

FIGURE 7 (*a* and *b*). Transition structures (X) illustrating the transformation from lamellae to long cylindrical micelles (Y). These images are from position 3 in Fig. 3. Bar = 250 nm.

appeared to be thinner than the vesicle walls, suggesting that they were not strips of lamellar phase. Most probably these are mixed cylindrical micelles of phospholipid and octyl glucoside.

Micrographs corresponding to higher OG to PC ratios are shown in Fig. 8. This region had been considered to be mixed lamellar and micellar lipids with discrete OG:PC ratios in the structures of ~2:1 and 3:1 respectively (e.g., Jackson et al., 1982; Ollivon et al., 1988). Although a few vesicular and lamellar structures were observed, the predominating structures were cylindrical micelles, ~3–4 nm in diameter (Fig. 8 *a*). At the high OG to PC ratios, near those required to cause the mixture to appear optically clear, the number of small spheroidal micelles increased compared with the number of cylindrical micelles. At average molecular ratios of OG:PC in the structures of 3.5:1 and greater, the micellar population is predominately spheroidal (Fig. 8 *b*) and indistinguishable by cryo-TEM from the pure OG micelles seen in Fig. 1 *a*. However, these structures are close to the limits of resolution in this study, and average diameters significantly smaller would not be resolved.

The appearance of open vesicles in the micrographs was associated with instability in the 90° light scattering trace (Fig. 3), but not with an abrupt change in fluorescence energy transfer (Ollivon et al., 1988). The rise in turbidity indicated by both optical density and 90° light scattering correlated with the micrographs containing significant material in the form of cylindrical micelles. At OG concentrations beyond the peak in the turbidity and the sharp break in the RET curve, the material was visualized only in micellar form.

DISCUSSION

Examination of the vesicle-micelle transition by electron microscopy is the one means to discover the actual microstructures that are formed. Because several microscopic species may coexist, inferring these by indirect techniques such as light scattering, is extremely difficult. Indeed, without knowing something about the types of structures in a suspension or solution, it is impossible to properly interpret light scattering data; however, once the structures are known, light scattering, column chromatography, nuclear magnetic resonance and differential centrifugation are all means to determine the distribution of lipid and detergent among the structures known to be present.

Cryo-transmission electron microscopy is suitable as

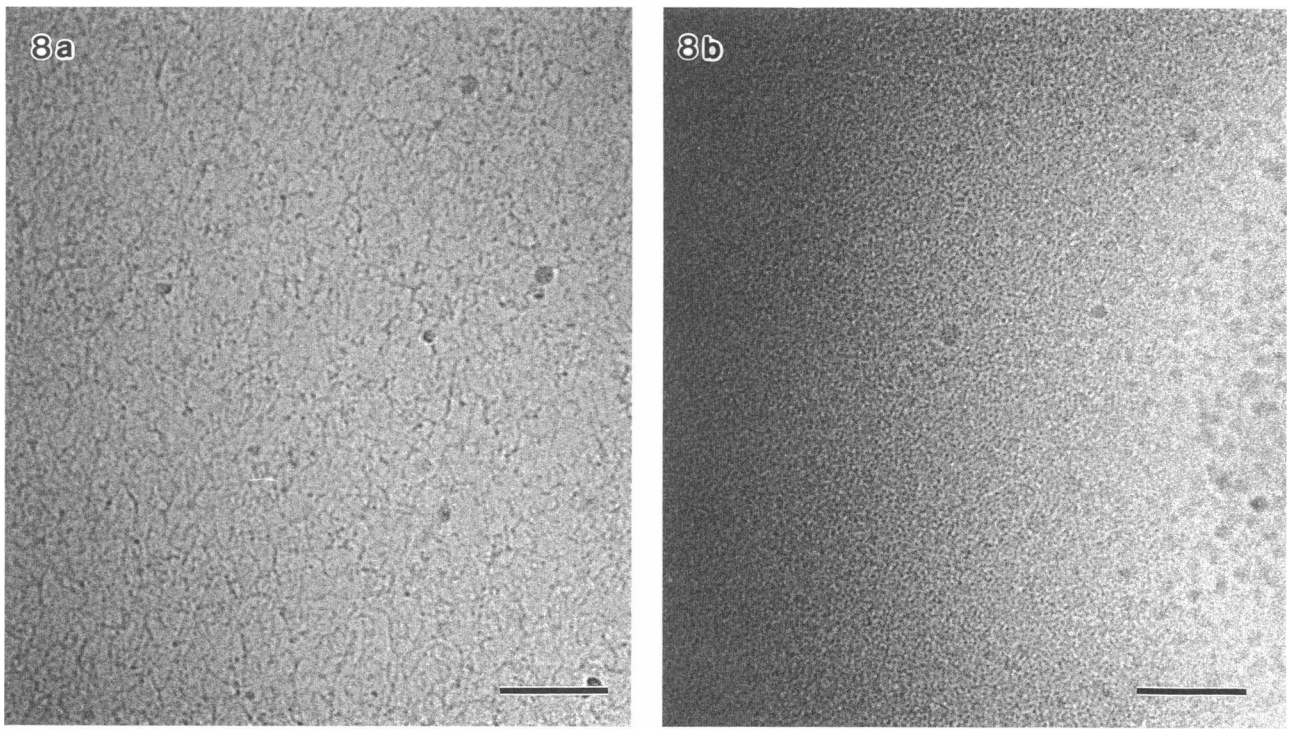

FIGURE 8 (*a*) Cylindrical and spheroidal micelles observed at position 5 in Fig. 3. The average OG:PC in the structures ranges from ~2:1 to 3:1 when both micelles are observed simultaneously. (*b*) Spheroidal micelles observed at position 5 in Fig. 3 and at higher levels of OG, where OG:PC in the structures is >3. Bar = 150 nm.

the direct observation technique for mixed amphiphilic systems because it preserves delicate and transient structures. Because the state of hydration is a critical parameter for the phase behavior of mixed micellar systems, rapidly frozen samples in vitreous ice are more likely to represent the true state of the amphiphilic structures than specimens prepared by other techniques.

Transmission electron microscopy is the natural technique for studying colloidal microstructures, because it provides direct images with resolution on the order of 1 nm. Specimen preparation in the controlled environment vitrification system (CEVS) after vitrification assures specimen compatibility with the instrument, and preservation of microstructure during preparation and observation.

The CEVS allows the sample to be prepared with minimal composition changes and at any desired temperature. Vitrification of the aqueous sample by ultra-rapid cooling reduces the vapor pressure, arrests all supramolecular motion, preserves microstructure and avoids any crystallization-related artifacts. Samples are made thin enough to prevent excessive inelastic scattering of the electron beam.

Mass-thickness contrast (due mainly to density differences) is inherently low in these samples. Contrast is enhanced by defocusing the objective lens of the microscope, leading to so-called phase-contrast effects (arising from phase differences between the scattered and unscattered electrons). The micrographs shown here were recorded with a nominal underfocus of 3.9 μm. This accentuates spatial frequencies corresponding to 4–7 nm, and attenuates spatial frequencies corresponding to smaller real space distances. This is the reason the bilayer of the vesicle appears as a single line, ~5 nm thick, and not as the two lines expected, had the two layers of the bilayer been resolved. This and other factors (electron beam radiation damage, mechanical stability of the specimen holder) prevent us also from resolving the inner structures of the investigated micelles.

The major structures observed in these samples were large spherical vesicles, a population of tubular and smaller spherical vesicles, long cylindrical micelles, and spheroidal micelles. Because the optical density and/or RET was determined for each sample, it was possible to correlate previous optical studies (Ollivon et al., 1988; Eidelman et al., 1988) with the structures observed in the electron micrographs.

The first change in the vesicle structure as octyl glucoside was added to the mixture was a change in the shape and the size distribution of the vesicles (Figs. 4, 5). A similar decrement in average vesicle size for equivalent mixtures of OG and PC was reported by Paternostre et al. (1988) when the vesicles had been prepared by reverse-phase evaporation and examined by freeze fracture electron microscopy. This change occurred with some decrease in the amount of scattered light, a property which is dependent on the size, shape, number, and refractive index of the particles, and no dramatic changes in the energy transfer efficiency, which measures the distance between phospholipid headgroups. (If the initial vesicles are small, e.g., 25 nm diam, then some growth is predicted in the presence of OG.) Very small vesicles of octyl glucoside-egg PC mixtures have been implicated at higher [OG], as the initial event in the formation of vesicles during detergent removal, on the basis of small angle light scattering measurements (Lichtenberg, personal communication). These were considered transitional because they appeared to last on the order of milliseconds to seconds, and relaxed either to mixed micelles or larger vesicles. Because the samples in the present study were equilibrated for periods of minutes to weeks, the small vesicles observed are probably more stable than those described from the light scattering studies. Indeed, the transformation from large to small vesicles may be a very slow process.

Although egg PC alone tends to form increasingly larger vesicles with time, to minimize the radius of curvature, the addition of octyl glucoside to the system changes the optimal curvature due to changes in the headgroup area relative to the effective acyl chain area. OG incorporation would affect optimal packing of these two amphiphiles to decrease the radius of the outer monolayer. The inner monolayer would resist this increase, and the OG to PC ratios in the two monolayers would be expected to be different (high in the outer monolayer and low in the inner monolayer) to accommodate smaller vesicles. At a similar position in the phase diagram, cholate and egg PC mixtures form structures that grow from very small vesicles to larger ones over a period of hours in the absence of any change in composition of the mixture (Almog et al., 1986).

Vesicle opening is a step in the vesicular-micellar transition that had been suggested to occur when the average OG to PC ratio in the mixed structures was a little <1. Discrete opening of vesicles rather than dissolution was inferred from the observation that entrapped large molecules are released from vesicle-detergent mixtures where all the lipid appeared by RET to be in the lamellar phase, and where no changes in the macroscopic behavior of the mixture were observed (Ollivon et al., 1988). The point of leakage could also be explained by transient detergent-stabilized pores of increasing diameter at higher detergent concentrations analogous to data reported for cholate-egg PC mixtures (Schubert et al., 1986). It is very clear from Fig. 6, *a* and *b* that the lamellar phase may exist, at least transiently, as open vesicles. The dynamic appearance of these images could suggest that the openings are caused by stresses during

specimen preparation. We feel this is very unlikely because vesicle distortion (suggestive of forces) is rare at low surfactant concentrations and open vesicles are regularly seen only in the composition range where open vesicles are predicted from solute entrapment studies cited above. Moreover, the dynamic appearance may indeed indicate that these are metastable rapidly fluctuating detergent-stabilized openings. Opened vesicles have also been seen by cryo-TEM for mixtures of Triton X-100 and phosphatidylcholine (Edwards et al., 1989). It is important to note that most of the preparations that exhibited opened vesicles also had cylindrical micelles, either in the same region of the grid, or in another region. These open lamellar sheets present very little bilayer edge and retain the vesicle curvature unlike the discs reported for egg PC and members of the cholate family of detergents. For example, Fromherz and Rüppel (1985) have inferred from negative stain electron micrographs (certainly an artifact-ridden specimen preparation technique, as shown by Talmon, 1983) that taurochenodesoxycholate with egg PC (0.8 mM and 2.5 mM, respectively) form small disks of lamellar phase lipid that are presumed to be stabilized by the detergent partitioning on the edges (e.g., Mazer et al., 1980).

At somewhat higher surfactant concentrations, the mixtures of PC with OG, Triton X-100, or bile salts tend to exhibit an increase in optical density (e.g., Jackson et al., 1982; Goni et al., 1986; Almog et al., 1986; Ollivon et al., 1988; Paternostre et al., 1988) that is particularly dramatic if the initial vesicle preparation is small unilamillar vesicles prepared by sonication. This jump in turbidity has been postulated to be due to the vesicles becoming larger and/or multilamellar (Jackson et al., 1982; Almog et al., 1986; Paternostre et al., 1988; Urbaneja et al., 1988). Here, the rise in turbidity is correlated with the formation of cylindrical micelles; the micelles are first observed just before the rise in light scattering, and are the predominating structure observed at the peak turbidity. Cylindrical micelles identified by neutron scattering have recently been proposed as a stage before spheroidal micelles in mixtures of glycocholate and egg PC (Hjelm et al., 1988). The cylindrical mixed OG-PC micelles closely resemble the cylindrical micelles imaged in the aqueous cetyltrimethylammonium bromide-NaBr system (Vinson, 1988).

Several possible artifacts should be considered before the appearance of cylindrical micelles is accepted as the source of the peak in the turbidity signal. One possible explanation is that the structures responsible for the rise in turbidity were excluded from the electron micrographs. This is a real possibility. The very thin liquid specimen films needed for cryo-TEM are <0.5 μm thick. Thicker specimens are opaque to the electron beam and any structures trapped in these would be obscured. In addition, large particles are excluded from the liquid film during blotting. Larger structures contributing to the rise in turbidity may be systematically excluded if these are so large that they precipitate from the aqueous suspension.

A more troubling possible explanation for our seeing only cylindrical micelles in a region where other data are consistent with lamellar phase would be that, somehow, during specimen preparation the state of the mixed system changed. Although the phase behavior is quite temperature sensitive, the temperature in the CEVS is maintained to ± 0.1°C and temperature gradients within the CEVS do not exceed 1°C. The rate of cooling is estimated to be 10^5K/s, so it is unlikely that transitions expected to take milliseconds or longer could occur during temperature transients. If the small water molecules did not rearrange upon cooling to form the thermodynamically favored hexagonal ice, then the much larger amphiphile molecules certainly did not change their original state of aggregation. Moreover, cooling the sample would tend to push it from micellar to lamellar phase (Miguel et al., 1989) so temperature effects seem extremely unlikely. The composition of the solution on the grid is presumed to be that in larger volumes observed in the cuvette. We did however, consider the drastic difference in surface area-to-volume ratio on the grid after blotting and what effect that might have on the bulk concentration of a surfactant such as octyl glucoside. Simple calculation indicates that [OG] in the bulk phase would decrease to meet surface requirements when the droplet is blotted. A decrease in $[OG]_{aq}$ would tend to push the phase diagram toward vesicle formation and away from mixed micelles. Finally, if there was significant evaporation from the grids before rapid cooling, the OG to PC ratio in the structures would change; yet in very few of the vesicular (no OG) samples were there structures observed consistent with evaporative water loss. Moreover, the appearance of cylindrical micelles and the turbidity peak coincide in every preparation examined making it unlikely that evaporation (which happens only sporadically) and subsequent increase in [OG] is the cause of the cylindrical micelles.

The spheroidal micelles are seen almost exclusively at levels of OG sufficient to make the solution optically clear. Previous studies (Eidelman et al., 1988) indicate that these micelles change both composition and size with increasing amounts of detergent until only 1 PC molecule is solubilized in a surfactant micelle. Those data could be fit by assuming the micelle is an oblate ellipsoid with major and minor axes 4.7 and 1.2 nm near the point of solubilization, and becomes progressively smaller at higher OG concentrations until the two axes are nearly identical at 1–2 molecules of PC per micelle (Eidelman et al., 1988). Cryo-TEM images of the spherical micelles of pure OG and for these micelles, which contain a maximum amount of PC, are 2 to 5 nm in diam, values which

could fit the calculated dimensions but do not permit discriminating between the largest and smallest predicted structures.

CONCLUSIONS

The direct images obtained by cryo-transmission electron microscopy clearly show that the progress of solubilization of egg PC vesicles by the surfactant octyl glucoside begins by transforming these to vesicles with a lower radius of curvature, that these vesicles then open with narrow and wide pores before collapsing to long cylindrical micelles that coexist with small spheroidal micelles, which are the dominant structure at high detergent concentrations. The technique used for the fixation of these samples, rapid cooling that leads to vitrified specimens from a temperature-and humidity-controlled environment, ensures that the observed structures are unperturbed. The present study corroborated much of the previous information on the behavior of OG-PC mixed systems. In addition, the previous poorly understood turbidity maximum was shown to correlate with cylindrical micelle formation. The speculated existence of large openings in vesicle bilayers was confirmed, and the optimal vesicle size was shown to decrease instead of increase as previously thought. Thus, in conjunction with other experimental techniques, cryo-TEM is an important tool for studying the behavior of aqueous dispersions and solutions of amphiphile mixtures.

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