States of Aggregation and Phase Transformations in Mixtures of Phosphatidylcholine and Octyl Glucoside†

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ABSTRACT: The result of mixing varying concentrations of the nonionic detergent octyl glucoside (OG) with small unilamellar vesicles (SUV) of egg phosphatidylcholine (PC) made by sonication depends on the ratio between OG and PC in the mixed aggregates. When this molar ratio (R_e) is lower than 1.4, the detergent partitions between the PC vesicles and the aqueous medium with a partition coefficient of $K = 0.033$ mM^{-1}. As a consequence of introduction of OG into the bilayers, the vesicles grow in size. The resultant vesicles have a mean diameter that is an increasing function of R_e and is independent of the total PC concentration. Experiments in which the vesicles were loaded with high molecular weight dextran prior to being exposed to OG suggest that the mechanism responsible for the size growth involves lipid transfer rather than fusion. Mixtures with R_e values within the range of 1.4–3.2 separate into two macroscopic phases: The lower phase is clear but very viscous. It contains constant OG and PC concentrations and is characterized by an R_e value of 3.2, independent of the composition of the whole dispersion. The upper phase contains vesicles of varying concentrations of OG and PC, but a constant R_e of 1.4. When the saturating level of 1.4 OG molecules per PC molecule is approached, the concentration of OG monomers in the aqueous medium reaches the value of 16.6 ± 0.3 mM, which is the apparent cmc of OG in the lipid-containing medium. OG–PC mixed micelles contain at least 3.2 OG molecules per PC molecule. The mixed micelles present at R_e = 3.2 apparently have the shape of oblate ellipsoids with a minor axis of about 2 nm and two major axes of about 25 nm. The surface area of the mixed micelles at this point is just sufficient for them to undergo conversion into the smallest possible spherical vesicles of a radius of 12 nm. At R_e values above 3.2, the major axis of the mixed micelles becomes smaller as R_e increases, while at values of R_e below 3.2 the micelles would have been expected to grow very rapidly with decreasing R_e. This may explain the partial vesicle closure occurring below R_e = 3.2.

One of the most commonly used surfactants in membrane solubilization and reconstitution experiments (Racker, 1985) is the nonionic detergent octyl glucoside (OG),[1] which is believed to be a "mild" detergent with respect to its denaturing effect on proteins. Previous studies on the solubilization of phospholipid bilayers by this detergent demonstrated the following: (1) At subsolubilizing concentrations, OG partitions between the lipid bilayers and the aqueous medium with a partition coefficient of about 60 (Jackson et al., 1982; Lichtenberg, 1985; Ollivon et al., 1988). (2) This may alter the size of the vesicles, a phenomenon that depends on the initial vesicle size. As long as the detergent to lipid ratio in the mixed bilayer (R_e) is lower than 1.0, OG causes very small size changes in small unilamellar vesicles (SUV) and even less pronounced changes in the size of larger vesicles (LUV), due to membrane expansion (Albert, 1978; Jackson et al., 1982). (3) Within the range of R_e values of 1.0 to about 2.0, detergent addition to SUV caused an increase of turbidity to values that were an increasing function of R_e (Paternostre et al., 1988; Ollivon et al., 1988). This has been interpreted to mean that within this range of R_e values the lipid is contained in discoidal lamellar sheets (Ollivon et al., 1988) or else in large vesicles of increasing sizes (Jackson et al., 1982). (4) Within the range of R_e = 2.0–3.0, two types of aggregates, namely, micelles and larger aggregates, coexist. The latter are either mixed OG–PC disks (Ollivon et al., 1988), or mixed bilayer vesicles (Jackson et al., 1982). (5) At R_e values above 3.0, all the lipid in the system is contained in PC–OG mixed micelles, whose size is a decreasing function of R_e (Eidelman et al., 1988).

In spite of the systematic nature of these studies, several structural and mechanistic issues remain unresolved. Some of these issues are addressed in this paper. Specifically, the state of aggregation in mixtures obtained by adding varying

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[1] Abbreviations: PC, egg phosphatidylcholine; OG, octyl β-D-glucoside; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; OD, optical density; E, molar optical density (OD/M); R_e, effective ratio, molar ratio of OG/PC in the mixed aggregates (vesicles and micelles); R_e^{SAT}, R_e value at which the vesicles are saturated with OG; R_e^{SOL}, R_e value at which the solubilization of the lipid is completed; K, distribution coefficient of OG between PC vesicles and the aqueous medium; QLS, quasi-elastic light scattering; NMR, nuclear magnetic resonance; R_h, mean hydrodynamic radius of vesicles or micelles; FITC, fluorescein isothiocyanate; Con A, concanavalin A.

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OG concentrations to PC SUV is studied as a function of added OG. The previously described partition equilibrium theory (Schurtenberger et al., 1985; Lichtenberg, 1985) is refined and tested experimentally. In addition, the mechanistic aspects of the OG-induced vesicle size growth are investigated. New information is also provided on the structure of OG–PC aggregates which exist within the range of OG/PC ratios where vesicles and micelles coexist. These investigations shed some light on the mechanism of vesicle formation upon removal of OG from OG–PC mixed micelles. The data presented here may be used to understand the role of various factors in determining the effect of the experimental details of reconstitution experiments on the size of the resultant vesicles.

Materials and Methods

Materials. Octyl β-D-glucoside (OG) was synthesized from acetobromoglucose and 1-octanol by modification of the method of Noller and Rockwell (1938). The final purified product exhibited only one spot by thin-layer chromatography when developed twice in a solvent system of ethyl acetate–methanol (4:1 v/v) and visualized by spraying with 5% $K_2Cr_2O_7$ and heating in an oven, as described by Jackson et al. (1982). High-purity egg phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) and fluorescein isothiocyanate–dextran (FITC–dextran, MW 70000, Sigma) were used without further purification. Concanavalin A (Con A)–Sepharose was a product of Pharmacia.

Preparation of Small Unilamellar Vesicles (SUV). SUV were prepared in 135 mM NaCl, pH 7.4, by ultrasonic irradiation and fractionated as described by Barenholz et al. (1977). Vesicles and micelles of different compositions were prepared by dilution of SUV with saline solution (135 mM NaCl) containing different concentrations of OG.

Determination of OG to PC Ratio in Lipid Bilayers (R_e). Equilibrated PC–OG mixed vesicular dispersions (containing subsolubilizing OG concentrations) were analyzed for PC (Stewart, 1980) and OG (Spiro, 1966). The dispersions were then spun at 140000*g* at 20 °C for 4 h to remove the vesicles. The supernatants of all the mixed dispersions were tested again for PC and OG. All the samples were assayed in quadruplicate. No PC was detected in any of the supernatants. R_e was calculated according to

$$R_e = (D_T - D_W)/L$$

where D_T and L are the OG and PC concentrations before centrifugation and D_W is the OG concentration in the supernatant.

Entrapment and Retention of Dextran in Unilamellar Vesicles. Vesicles preloaded with FITC–dextran were prepared by the extrusion method as previously described (Almog & Lichtenberg, 1988). This procedure resulted in the formation of vesicles with a mean hydrodynamic radius of 34 nm and a polydispersity of 15% as measured by QLS. Aliquots of the vesicular preparation were diluted 1:1 with 135 mM NaCl solution containing varying concentrations (0–45.9 mM) of OG. Subsequently, the samples were equilibrated at room temperature for 1 h and passed through 10 × 20 mm Con A–Sepharose columns to remove nonentrapped dextran. The vesicles, emerging in the void volume, were solubilized by excess detergent, and FITC–dextran retained in them was determined by a fluorometer (excitation 490 nm; emission 520 nm). The fluorescence intensities were normalized for the PC concentrations measured in the eluants.

Quasi-Elastic Light Scattering (QLS) Measurements. Mean hydrodynamic radius ($\bar{R}_h$) and polydispersity of mixed PC–OG vesicles were measured as described by Somjen and Gilat (1983) with a Nicomp Model HN-5/90 helium–neon ion laser scattering spectrometer (λ = 638.2 nm) equipped with a computing autocorrelator (Model 6864). Measurements of PC–OG mixed micelles were performed on a Malvern 4700 QLS apparatus equipped with a helium–neon laser (Spectra Physics; 35 mW; λ = 638.2 nm) and a 64-channel autocorrelator. All the measurements were taken at 20 ± 0.1 °C and a scattering angle of 90°.

Turbidity Measurements. The kinetics of OG–PC interactions was monitored by continuous turbidity measurements at 520 nm, with a Brinkmann PC-801 probe colorimeter equipped with an LKB dual-channel pen recorder. Turbidity of equilibrated PC–OG vesicular dispersions was measured usually at 520 nm on a Shimadzu UV 160 double-beam spectrophotometer.

Viscosity Measurements. Relative viscosities were determined by measuring the flow times through a capillary viscosimeter (Cannon Instruments) at room temperature, as described by Yedgar et al. (1982).

Nuclear Magnetic Resonance (NMR) Measurements. The NMR measurements were performed as described in our previous work (Almog et al., 1986) on a Bruker AM 360-WH Fourier-transformation spectrometer.

Low-Angle X-ray Diffraction Measurements. Low-angle X-ray diffraction experiments were performed on a Phillips sealed-tube fine-focus generator operated at 40 kV and 34 mA producing copper radiation. Monochromatization was provided by a nickel filter and one Franks mirror, and the beam was collimated to 4-mm height and 350-μm width in the plane of the specimen. The specimen to detector distance was 460 mm. The diffraction pattern was recorded by a linear position-sensitive electronic detector of the delay line type (Reich et al., 1982). Exposure times were 1–2 h. During acquisition, the data were histogrammed and stored in a Z-80-based microprocessor unit. Following completion of the experiment the data were transferred to and processed by an IBM 3081 computer.

Results and Analyses

(1) Partition of OG between Egg PC Bilayers and Aqueous Solutions: Theoretical Considerations and Experimental Evaluation. In our recent analysis (Lichtenberg, 1985) of the equilibrium partition model proposed by Schurtenberger et al. (1985), we have shown that for ideal mixing of lipid (at a concentration L) and detergent (at a concentration D_T), in dilute aqueous media, the distribution of detergent between lipid bilayers and aqueous media obeys a partition coefficient K, given (in mM^{-1}) by

$$K = D_b/[(L + D_b)D_w] \quad (1)$$

where D_b is the concentration of detergent that resides in the membrane and D_w is the detergent concentration in the aqueous phase. For $L \gg D_b$, the definition of K, as given by Schurtenberger et al. (1985), applies:

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

where R_e is the ratio of detergent to phospholipid in the vesicle bilayer; $R_e = D_b/L$. Under any other conditions, eq 1 has to be employed to define K; this yields

$$1/K = (LD_w + D_bD_w)/D_b = D_w(L/D_b + 1) \quad (3)$$

Hence

$$L/D_b = (1/K)(1/D_w) - 1 \quad (4)$$

i.e., a linear dependence is expected between L/D_b and $1/D_w$; this line should have a slope of $1/K$, intersect with the L/D_b

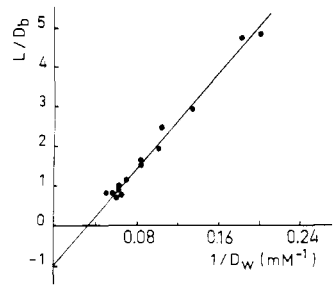

FIGURE 1: Determination of the equilibrium constant describing the partitioning of OG between PC bilayers and aqueous media. Following equilibration of mixtures of PC SUV (3–12 mM) and OG (5–34 mM), the dispersions were ultracentrifuged. The concentrations of PC and OG were determined prior to and following the centrifugation. No PC was detected in the supernatants. L/D_b (the ratio between PC and OG in the bilayers) was plotted as a function of the reciprocal of D_w (OG concentration in the supernatant). The line describing this dependence is linear ($r^2 = 0.98$) and follows the equation $L/D_b = 30/[(1/D_w) - 0.97]$.

axis at −1, and intersect with the $1/D_w$ axis at K.

To test the validity of this model, small unilamellar PC vesicles (SUV) were mixed with varying (subsolubilizing) concentrations of OG (D_T). The resultant OG-containing vesicles were then ultracentrifuged. No PC was detected in the supernatants. The OG concentration in the supernatants (D_w) was determined, and the concentration of detergent that resided in the lipid bilayers (D_b) was calculated[2] ($D_b = D_T - D_w$). The results of experiments in which we have measured D_b and D_w carried out at different PC and OG concentrations (5–15 mM PC; 5–35 mM OG) were plotted in terms of the dependence of L/D_b on $1/D_w$ (Figure 1). A straight line was obtained ($r^2 = 0.98$), which was independent of L and intersected with the L/D_w axis at −0.97 ± 0.11. Both the linearity of this dependence and the proximity of the intercept to −1 support our model (eq 4). The slope of this dependence yields a value of $1/K = 30 \pm 1$ mM ($K = 0.033 \pm 0.001$ mM^{-1} = 33 ± 1 M^{-1}),[3] a conclusion that was then further tested as described below.

Another form of eq 1 is

$$1/K = (LD_w/D_b) + D_w = D_w(1/R_e + 1) \quad (5)$$

D_w can be expressed in terms of R_e defined by $R_e = (D_T - D_w)/L = D_b/L$ and the total lipid (L) and detergent (D_T) concentrations:

$$D_w = D_T - D_b = D_T - LR_e \quad (6)$$

Hence

$$1/K = (D_T - LR_e)(1/R_e + 1) \quad (7)$$

$$1/K = D_T(1 + 1/R_e) - L(1 + R_e) = (D_T/R_e)(R_e + 1) - L(R_e + 1)$$

i.e.

$$1/[K(R_e + 1)] = D_T/R_e - L$$

Thus

$$D_T = R_e\{L + 1/[K(R_e + 1)]\} \quad (8)$$

This means that the total detergent concentration, D_T, required for obtaining any effective ratio R_e has a linear dependence on L. R_e is given by the slope of the line, which should intercept with the lipid concentration axis at $-1/[K(R_e + 1)]$.

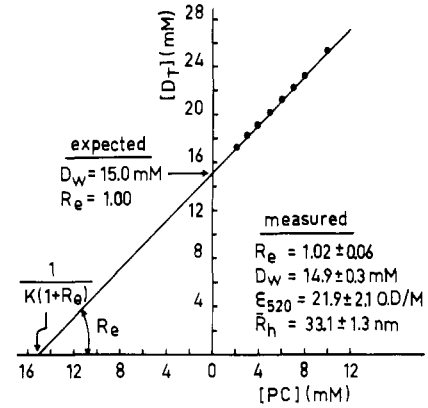

FIGURE 2: Compositions of OG-containing vesicles made to have an R_e value of 1.00. PC vesicles containing varying PC concentrations were mixed with OG at concentrations (D_T) that were chosen to yield $R_e = 1.00$ according to eq 8. Following equilibration, the molar absorbance, as well as the hydrodynamic radii, in all the dispersions was constant, within experimental error, as indicated in the figure. Subsequently, the dispersions were ultracentrifuged and the supernatants analyzed for PC and OG. None of the supernatants had any detectable [PC], while they all had a constant [OG] (D_w). All of the vesicles had the same OG/PC ratio (R_e), as indicated in the figure.

To further test our model, we have added OG to small unilamellar vesicles of various PC concentration, using OG concentrations that were chosen to yield, according to eq 8, a constant R_e value of 1.00. Subsequently, the dispersions were centrifuged, and the PC and OG concentrations in the supernatants (D_w) were determined. No PC was detected in the supernatants. Equation 5 predicts that D_w is a function of K and R_e and that, for values of $1/K = 30$ mM (see above) and $R_e = 1.00$, D_w is calculated to be 15.0 mM. The OG concentration in the seven dispersions whose composition is described in Figure 2 was 14.9 ± 0.3 mM, in a good agreement with the expected value. Furthermore, the R_e value of all the sedimented vesicles as measured independently following ultracentrifugation [$R_e = (D_T - D_w)/L$] was 1.02 ± 0.06, in accord with the expected value of 1.00. Noticeably, all the dispersions contained vesicles of similar sizes ($\bar{R}_h = 33.1 \pm 1.3$ nm), as measured by QLS, suggesting that the size of the vesicles is merely a function of their composition. This conclusion is also supported by additional data presented below.

(2) Structure of OG–PC Mixed Aggregates as a Function of Their Composition. Addition of concentrated OG solutions t[o] small unilamellar egg PC vesicles (PC SUV) results in turbidity changes. We have followed these changes continuously for mixtures made at a variety of OG and PC concentrations. Figure 3 presents the apparent equilibrium turbidity of samples made of 11.6 mM PC and 0–60 mM OG. This figure indicates the following:

(i) OG concentrations of up to 32 mM cause an increase of turbidity. This increase of turbidity occurred in a few seconds (see below) with no further turbidity increase in the following 70 h. The initial turbidity increase is also accompanied by an increase of the mean hydrodynamic radius of the vesicles as studied by QLS (see below). Membrane expansion can be only partially responsible for this size increase since dilution of the sample (thus decreasing the OG content of the vesicles) reduces the mean hydrodynamic radius only slightly (data not shown). Within this range of OG concentrations, no PC was detected in the supernatants after ultracentrifugation, indicating that in this range the dispersions were essentially vesicular.

[2] Under our experimental conditions, the largest estimate of water-soluble OG monomers entrapped within the vesicles was no more than 1.0% of the extravesicular OG monomers. Thus, counting this soluble OG as being membrane associated introduces only a minor error in our distribution measurements.

[3] The value of K obtained as a point estimate when $L/D_b = 0$ was found to be 32 M^{-1}, which is not significantly different from the value of K as estimated from the slope. As the amount of information in K as estimated from the slope is much greater than that in its estimate from a single point of the function, we have chosen to use the former value.

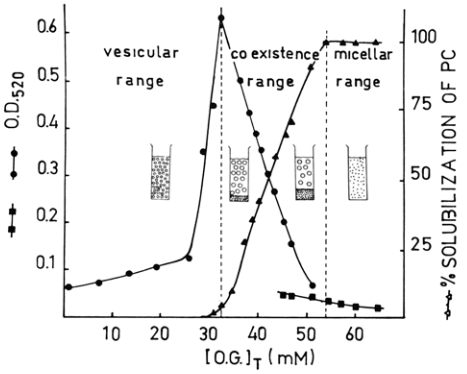

FIGURE 3: Dependence of the turbidity (circles and squares) and percent PC solubilization (triangles) on the total OG concentration in mixtures made of 11.6 mM PC SUV and varying OG concentrations (0–65 mM). These mixtures were equilibrated for 5 h. At OG concentrations within the range of 35–54 mM, a macroscopic phase separation occurred as indicated in the figure. The samples were centrifuged at 2000*g* for 20 min. Thus, the turbidities of the vesicular (circles) and of the micellar (squares) fractions, each separately, as well as the PC concentrations were measured. Subsequently, the dispersions were centrifuged at 140000*g* for 3 h, and the supernatants were analyzed for PC and OG concentrations. Percent solubilization refers to percent of the total PC contained in the lower phase of the first centrifugation plus the percent of the total PC contained in the supernatants of the second centrifugation.

(ii) OG concentrations of 32–54 mM resulted in the appearance of "oil like" droplets accompanied by a dramatic increase of the turbidity (not shown). In a few hours a spontaneous macroscopic phase separation occurred, as previously described by Ollivon et al. (1988) and further investigated in this study (see below). Complete phase separation was achieved by centrifugation at 2000*g* for 20 min, yielding a lower viscous but clear phase and an upper turbid phase whose turbidity is a decreasing function of [OG]. Upon ultracentrifugation of the upper phase, the supernatants were free of PC, indicating that the PC in this phase is contained in vesicles. As the OG concentration increases, less PC is detected in the vesicular fraction and more PC is solubilized and appears in the supernatant.

(iii) OG at 54 mM or more yielded transparent solutions, in which all PC appeared in the supernatants, indicating complete solubilization of the vesicles.

A similar pattern was observed for various PC concentrations: up to a certain OG concentration that we denote D_T^{SAT} (Lichtenberg et al., 1983), no PC became solubilized, and the size of the aggregates, as studied by QLS, was an increasing function of [OG]; at higher [OG], phase separation was observed, and only at yet higher [OG], which we denote D_T^{SOL}, one transparent phase was observed, consistent with complete solubilization of the PC vesicles. The dependencies of D_T^{SAT} and D_T^{SOL} on the total PC concentration are plotted in Figure 4. The detergent level at which solubilization begins is given by $D_T^{SAT} = 16.6 + 1.4[PC]$; solubilization is complete when $D_T^{SOL} = 16.6 + 3.2[PC]$. In other words, solubilization begins at $R_e = 1.4$ and ends at $R_e = 3.2$. Throughout the range of $R_e = 1.4$–3.2, the concentration of OG in the water remains constant (16.6 mM).

According to the terms defined by us previously (Lichtenberg, 1985), the phase boundary occurring at $R_e = 1.4$ is denoted R_e^{SAT}, the boundary at $R_e = 3.2$ is denoted R_e^{SOL}, and the apparent cmc of OG in the PC-containing media is 16.6 mM. Similar results were obtained by other investigators, using different techniques. Ollivon et al. (1988) reported values of 2.1 ± 0.6, 3.0 ± 0.5, and 15.4 ± 0.4 for R_e^{SAT}, R_e^{SOL}, and the apparent cmc, respectively, while the values given by

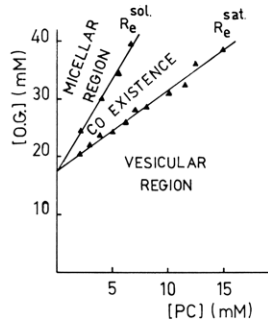

FIGURE 4: Phase diagram describing the phases that occur in PC–OG mixtures. The maximal OG concentration that can be added to PC SUV prior to the occurrence of a macroscopic phase separation ($[OG]^{SAT}$) and the OG concentration required for complete solubilization ($[OG]^{SOL}$) are described as a function of [PC] (see text for details).

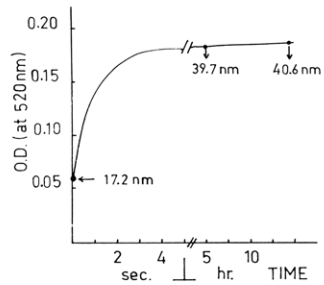

FIGURE 5: Time course of vesicle size growth upon addition of OG (22 mM) to PC SUV (5 mM). The turbidity was monitored continuously. The mean hydrodynamic radius, as measured by QLS 0, 5, and 15 h after mixing the vesicles with the OG solution, are indicated in the figure.

Paternostre et al. (1988) are closer to ours ($R_e^{SAT} = 1.3$; $R_e^{SOL} = 3.6$; cmc = 17 mM).

To further improve our understanding of PC–OG interaction, we extended the studies and characterized the structure of the vesicles that exist at R_e values below 1.4, the OG-induced size growth that occurs within this range, the composition of the two phases occurring at R_e values between 1.4 and 3.2, the structure of the aggregates present in these two phases, and the structure of the mixed micelles obtained at OG concentrations higher than those required for complete solubilization.

(*a*) *Vesicular Range, Characterization of the Vesicles, and OG-Induced Size Growth Process.* Addition of subsolubilizing OG concentrations to PC SUV results in rapid size growth, occurring on a time scale of several seconds with not much change in vesicle size over a time scale of hours, as examplified in Figure 5. The apparent equilibrium size of the resultant vesicles is plotted in Figure 6 as a function of R_e, as defined in eq 8 for four different concentrations of PC. Apparently, the size of the vesicles is independent of the lipid concentration,[4] supporting our conclusion that the size of the vesicles is merely a function of their composition (R_e). This is similar to the behavior observed for the vesicles made by addition of bile salts to small unilamellar vesicles (Schurtenberger et al.,

[4] For the purpose of statistical comparison of $\bar{R}_h$ versus R_e curves (Figure 6), the curve correspondent to each of the PC concentrations was fitted, according to Batschelet (1971), to $\bar{R}_h = A/(1 + Be^{-ACE_e})$. The postfitting procedure was done on an HP-85 microcomputer using the iterative least-squares nonlinear regression analysis of Nichols and Peck (1971). The fitted regression parameters (*A*, *B*, and C) were compared by examination of the mean values, standard errors, and percentage coefficient variation. There were not significant differences betwen the fitted regression parameters of the four curves.

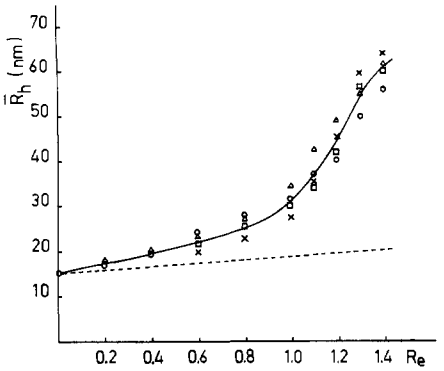

FIGURE 6: Dependence of the mean hydrodynamic radius (R_h) of equilibrated OG-containing PC vesicles on the OG/PC molar ratio in the bilayers (R_e). PC vesicles (R_h = 15 nm) of different PC concentrations [(Δ) 2.5, (○) 5, (□) 7.5, and (×) 10 mM] were mixed with OG solutions (final concentrations 0–31.5 mM) to yield mixed vesicles of different R_e values (0–1.4). Following equilibration, the hydrodynamic radii were measured by QLS. For any given OG and PC concentrations, R_e was calculated by the use of eq 8. The broken line indicates the calculated vesicle size following OG insertion into the PC bilayer. This calculation is based on the assumptions that the surface area occupied by a PC molecule is 72 Å^2 and the surface area occupied by an OG molecules is 38 Å^2. On the basis of these assumptions, the ratio between the surface of an OG-containing vesicles and a vesicle containing (the same number of) PC molecules is given by $(\bar{R}_{h,OG-PC}/\bar{R}_{h,PC})^2 = (1 + 38R_e/72)$. The broken line represents calculations based on this relationship.

1984, 1985, 1986; Almog et al., 1986; Almog & Lichtenberg, 1988).

Noticeably, the changes in size cannot be fully accounted for by membrane expansion at R_e values above 1.0. The same phenomenon has been described by Ollivon et al. (1988), who found that the change in vesicle size, as studied by gel exclusion chromatography, can be accounted for by membrane expansion due to detergent incorporation into the lipid bilayers only at R_e values below R_e = 1.0. Below this ratio, the membranes remained impermeable to entrapped inulin (Ollivon et al., 1988). At higher R_e values these authors suggested the existence of open lamellar disks, whose size is an increasing function of R_e. An alternative interpretation (Jackson et al., 1982) is that within this range (R_e = 1.0–1.4) vesicles of increasing sizes exist in the dispersion.

To differentiate between these two possibilities, three series of experiments were carried out. First, the molar turbidity (turbidity per mole of lipid) of 10 different mixtures of R_e values within the range of 0–1.4 was measured. The turbidity, as measured at 520 nm (E_{520}) was found to increase linearly (r^2 = 0.980) with the vesicle hydrodynamic radius ($\bar{R}_h$, in nanometers), as measured by QLS, according to the empirical relationship $E_{520} = 1.03\bar{R}_h - 13.6$. This result accords ($p < 0.01$) with the expression derived by Nir et al. (1983) for vesicular systems on the basis of Kerker's (1969) equation for the intensity of light scattered by single hollow spheres. The close agreement obtained throughout the whole range of R_e = 0–1.4 (not just 0–1.0) indicates that all the systems, including those of R_e = 1.0–1.4, are predominantly, if not exclusively, vesicular. Second, if the aggregates present in mixtures with R_e values between 1.0 and 1.4 were discoidal, then dilution of the mixture to R_e values below 1.0 (where the steady-state structure is vesicular) should have led to a disks → vesicles transformation. This should have resulted in a decrease of the hydrodynamic radius. Experimentally, dilution of the mixtures with R_e = 1.0–1.4 to R_e < 1.0 did not affect the hydrodynamic radii, as measured by QLS, indicating the existence of vesicles within this range. This conclusion is strongly supported by an experiment in which we have added

Table I: Retention of Dextran (MW = 70000) within Vesicles in the Course of OG-Induced Vesicle Size Growth[a]

R_e[b]	$\bar{R}_h$ (nm)[c]	% maintained	
		calcd[d]	exptl[e]
0.59	35	94.0	91.2
0.78	39	76.0	83.0
0.99	41	68.8	73.4
1.31	55	36.5	29.1

[a] Vesicles of a mean hydrodynamic radius of 34 nm, made by the extrusion method, were used in all the experiments. [b] R_e is calculated from [PC] and [OG] according to eq 8. [c] The final vesicle size ($\bar{R}_h$) was determined by QLS. [d] The calculated percent of dextran maintained in the vesicles is based upon the following considerations: A liquid-transfer mechanism by which n_i small vesicles form n_f larger vesicles implies that n_f small vesicles grow at the expense of $n_i - n_f$ vesicles, which disintegrate altogether. Assuming that the surface area per PC molecule is maintained constant, mass conservation requires that $n_f r_f^2 = n_i r_i^2$, where r_i and r_f are the initial and final mean radii of the vesicles, respectively. If only the n_f vesicles which grow into larger ones maintained the dextran entrapped within them, it follows that the percent of dextran maintained within vesicles be given by % maintained at $100(n_f/n_i) = 100\ (r_i/r_f)^2$. Accordingly, a size growth based on this mechanism can be expected to yield vesicles that retain $100(34/\bar{R}_h)^2$% of the entrapped dextran. [e] The experimental percent of dextran maintained in the vesicles was measured as described under Materials and Methods.

OG to PC vesicles preloaded with labeled dextran of a molecular weight of 70000. This treatment cause only partial release of the entrapped dextran (Table I), contrary to the complete release that would have been expected if the vesicles were converted into discoidal (nonvesicular) aggregates. This shows that the particles present in mixtures of R_e = 1.0–1.4 are indeed mainly vesicles.

The data of Table I are also indicative of the mechanism involved in the vesicle size growth. This process could have occurred through fusion of SUV, in which case all the entrapped dextran could have been expected to be retained within vesicles. By contrast, transformation of the vesicles into larger ones through lamellar disks should have resulted in a complete loss of entrapped dextran. Alternatively, a lipid-transfer mechanism could have been involved, in which case a partial, calculatable retention of the entrapped macromolecules could have been expected (Table I, footnote *d*). In fact, addition of OG to an R_e value of 1.31 resulted in an increase in the mean radius of the vesicles from 34 to 55 nm. This size increase was accompanied by a loss of 70.9% of the entrapped dextran as compared to a calculated value of 63.5%, expected for a vesicle size growth via lipid transfer (Table I). The less-pronounced size growth processes, observed upon addition of lower OG concentrations (to R_e values of 0.59–0.99), result in maintenance of higher fractions of the entrapped dextran (Table I), which agrees reasonably well with the values expected for a lipid-transfer mechanism of vesicle size growth. Thus, it appears that the particles of R_e values of 1.0–1.4 are vesicles, formed by OG-induced vesicle size growth occurring through a lipid-transfer mechanism.

This conclusion is consistent with our kinetic studies: we found that for PC concentrations within the range of 2.5–10 mM the half-life of the size growth is dependent on R_e (Figure 7) but independent of PC concentration (for R_e = 0.8 and 1.4, $t_{1/2}$ = 0.44 ± 0.06 and 1.02 ± 0.06 s, respectively), as expected for a lipid-transfer mechanism (Lawaczeck, 1978; see Discussion).

(*b*) *Range of Coexistence of Mixed Micelles and Vesicles.* Dispersions made by mixing PC SUV and OG solutions with $1.4 < R_e < 3.2$ separated into two macroscopic phases. The time scale of this macroscopic phase separation ranged between a few seconds and several hours, for higher and lower con-

Table II: Analysis of PC–OG Mixed Systems with Fixed [PC] (=11.6 mM) and Varying $[OG]_T$ (40.3–48.7 mM) in the Range of Macroscopic Phase Separation[a]

	upper phase					lower phase			
		OG							
$[OG]_T$	[PC]	$[OG]_T$	$[OG]_W$	$[OG]_b$	R_e	[PC]	[OG]	R_e[b]	% of total vol
40.3	7.3	27.0	17.0	10.0	1.37	40.5	140.3	3.06	13
41.2	6.8	26.4	16.6	9.8	1.44	37.2	145.7	3.47	15
42.2	6.3	25.2	16.4	8.8	1.39	35.9	129.2	3.14	18
43.1	5.9	25.1	16.1	9.0	1.52	36.8	140.4	3.38	20
45.0	4.4	22.8	16.7	6.1	1.38	38.6	143.7	3.29	21
48.7	2.7	20.4	16.6	3.8	1.41	35.9	136.9	3.35	24
mean ± SD			16.6 ± 0.3		1.42 ± 0.05	37.5 ± 1.6	139.4 ± 5.3	3.28 ± 0.14	

[a] Concentrations are in millimolar. [b] In the lower, mixed micellar phase, $R_e = ([OG] - 16.6)/[PC]$ (Lichtenberg, 1985).

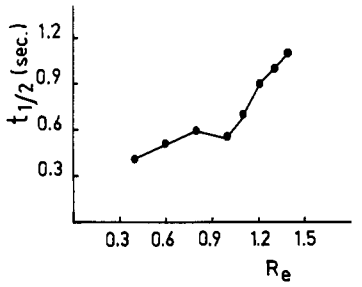

FIGURE 7: Dependence of the half-life of SUV size growth on R_e at a constant PC concentration. Aliquots of SUV (10 mM PC) were mixed at time zero with equal volumes of different OG solutions chosen to yield varying R_e values as calculated according to eq 8. The resultant time-dependent turbidity increase was monitored continuously. $t_{1/2}$ refers to the time required for the turbidity to increase halfway between the initial and the steady-state values.

centrations, respectively. However, separation occurred in all the mixtures, even when the PC concentration was as low as 1 mM. This is not necessarily inconsistent with the finding of Ollivon et al. (1988), who reported a similar separation of phases only at PC concentrations of 10 mM and more, since in the latter studies the dispersions were observed for shorter periods of time.

To analyze the two macroscopic phases, we have first centrifuged the dispersions for 20 min at 2000*g*, to facilitate the separation into two phases according to their densities: a clear viscous lower phase (high density) and an upper turbid phase (low density). The latter phase was then ultracentrifuged (at 140000*g*), resulting in clear supernatants that contained no PC. Increasing the OG concentration within this range of OG/PC ratios resulted in a monotonic increase in the relative volume of the lower viscous phase (Table II). Nevertheless the concentrations of both OG and PC found in this phase did not depend on the total OG concentration. In fact, the concentrations of both PC and OG were high, almost constant (37.5 ± 1.6 mM PC; 139.4 ± 5.3 mM OG), and corresponded to an effective ratio of 3.28 ± 0.14. The PC and OG concentrations in the upper turbid phase decreased with increasing the total OG concentration. However, throughout the whole range of $1.4 < R_e < 3.2$, the upper phase contained vesicles of a constant size and composition ($R_h = 60 \pm 3$ nm; $R_e = 1.42 \pm 0.05$). These results are similar to those of Ollivon et al. (1988), who found that in a mixture of 21.1 mM PC and 61.4 mM OG the upper phase contained 16.6 mM PC and 38.2 mM OG while the lower phase contained 35.6 mM PC and 130 mM OG.

We propose that within the whole range of coexistence of two phases the system contains a mixture of two populations: vesicles whose composition and size are similar to those of the vesicles present at an R_e of 1.4 and mixed micelles of an R_e of 3.2. The micelles form a distinct macroscopic phase, whose specific density is 1.085 g/mL, which is consistent with the finding that in solutions made in D_2O (density = 1.1007) this phase floats to the top of the D_2O vesicle-containing solution. The viscosity of this phase (as measured after separation from the upper phase) is 1440-fold higher than that of water. When kept separately, the two phases were stable in terms of their viscosities for at least several weeks. Furthermore, no phase "reseparation" occurred.

To further investigate the state of lipid aggregation within the "condensed phase", we have separated this phase and studied its ^1H NMR spectrum and small-angle X-ray scattering. All the NMR resonances were only slightly broader than those observed in the spectrum of a one-phase mixed micellar solution of an $R_e = 3.2$. As an example, the choline head group signal of the condensed phase had a line width of 11.6 Hz as compared to 7.2 Hz observed in the less-concentrated solution of $R_e = 3.2$ (not shown). This broadening can be due to the increased viscosity, as indicted by the parallel broadening of the HOD signal from 2.6 to 5.3 Hz. Yet, the line widths of the PC resonances in the condensed phase are too narrow to be consistent with a high degree of motional restriction. The small-angle X-ray scattering profile of the condensed phase is also very similar to that of a mixed micellar dispersion of $R_e = 3.2$ (not shown): it reveals two relatively broad but distinct peaks at 45 and 18 Å, an intensity minimum at 22 Å, and no sharp discrete peaks indicative of a higher order of organization. On the basis of these results, we propose that the lipids in the condensed phase are contained in OG–PC mixed micelles of $R_e = 3.2$, which are similar to the mixed micelles present in a solution of an overall effective ratio of 3.2.

(*c*) *Mixed Micellar Range.* As previously proposed for many lipid–detergent mixed micelles [e.g., Yedgar et al. (1974)], OG–PC mixed micelles also appear to have the geometry of oblate ellipsoids (Eidleman et al., 1988). Our light scattering studies are quite consistent with this proposal as the experimental points in Figure 8, which represent these results, accord reasonably well with the theoretical dependence of R_h on R_e, calculated as follows: First, we have adopted the general model of Eidelman et al. (1988) based on the assumptions that all the polar head groups face the aqueous medium, that no hydrophobic surface is exposed to water, that the hydrophobic core is liquid-like in nature, and that the micelles have the shape of oblate ellipsoids. Next, we have described the volume and the surface area of the ellipsoidal mixed micelles each in two ways, namely, by its short (d) and long (r) axis and by the volumes and surface areas of the PC and OG molecules (V_{PC}, V_{OG}, S_{PC}, and S_{OG}, respectively). Each micelle with n molecules of PC contains nR_e molecules of OG. The volume of each micelle is given by

$$V = 4\pi r^2 d/3 = n(V_{PC} + R_e V_{OG}) \quad (9)$$

and the surface area is

$$S = 2\pi r^2 + (\pi d^2/\epsilon) \ln [(1 + \epsilon)/(1 - \epsilon)] = S_{PC} + R_e S_{OG} \quad (10)$$

where $\epsilon = [1 - (d/r)^2]^{1/2}$. Hence

$$(V_{PC} + R_e V_{OG})/(S_{PC} + R_e S_{OG}) = (4r^2 d/3)/[2r^2 + (\pi d^2/e) \ln [(1 + \epsilon)/(1 - \epsilon)]]$$

Some information on the thickness of the ellipsoidal (nearly discoidal) mixed micelles of $R_e = 3.2$ can be deduced from the small-angle X-ray scattering data. The similarity of the scattering profile to that obtained from single phospholipid bilayers (Blaurock, 1982) suggests that the electron-density distribution along the minor axis of the ellipsoid may be "bilayer-like", i.e., an inter head group distance of approximately 38 Å (Blaurock, 1982).

The solid line in Figure 8 is the result of calculations based on eq 11 assuming (1) that the short axis has a constant value of $d = 19$ Å (see above), (2) that the molecular volumes are those used by Eidelman et al. (1988), namely, $V_{PC} = 1620$ Å^3 and $V_{OG} = 243$ Å^3, and (3) that the molecular surface areas are as previously observed, i.e., $S_{OG} = 38$ Å^2 (Ollivon et al., 1988) and $S_{PC} = 72$ Å^2 (Cornell et al., 1980). The calculations based on these assumptions gave a discrete value of the long axis (r) for any given R_e from which R_h was derived according to the dependent of $\bar{R}_h$ on d and r (Mazer et al., 1980). The close proximity of the experimental data points to this line ($r = 0.98$) supports the validity of our structural model. The results obtained by Eidelman et al. (1988) for OG–PC mixed micelles of R_e values above 10 (a range not studied in the this work) also appear to be consistent with our data on the larger micelles obtained at $R_e < 5.0$.

Discussion

Composition and Physical Properties of OG–PC Vesicles. Upon addition of subsolubilizing concentrations of OG to PC small unilamellar vesicles, the detergent apparently distributes between the vesicles and the aqueous medium according to a single partition coefficient K, defined by eq 1. At R_e values within the range of 0.21–1.35, the value of K is 0.033 mM^{-1}, in good agreement with previously reported values.[5] As long as no solubilization occurs ($R_e < 1.4$), the actual ratio within the bilayers, R_e, is a function of [PC], [OG], and K:

$$R_e = D_T/\{L + 1/[K(R_e + 1)]\} \quad (11)$$

[5] A similar value of K was reported very recently by Ueno (1989) for systems with R_e values higher than 0.3. Interestingly, at lower R_e values, a higher K value was obtained ($K = 75$ M^{-1}; Ueno, 1989), in accordance with the results of Schubert et al. (1986), who reported that the distribution of cholate between PC vesicles and aqueous media decreases upon increasing R_e. Previously (Jackson et al., 1982; Ollivon et al., 1988), the partition of OG between PC bilayers and the aqueous medium has been described in terms of a unitless partition coefficient $\bar{K}$, defined by $\bar{K} = (D_b/V_b)/(D_w/V_w) = (D_b/D_w)/(V_b/V_w)$. V_b/V_w is the ratio between the volume of the lipid bilayer and the volume of the aqueous media; i.e., $V_b/V_w = LM\bar{V} \times 10^{-6}$, where L, M, and $\bar{V}$ are the concentration, molecular weight, and specific volume of PC, respectively. From the partition coefficient given in this paper (eq 1 in mM^{-1} units), it follows that $D_b/D_w = K(L + D_b)$. Hence, $\bar{K} = K(L + D_b)/(LM\bar{V} \times 10^{-6})$. For $D_b \ll L$, $\bar{K} = K/(M\bar{V} \times 10^{-6})$, and in terms of $\bar{K}$, a partition coefficient $K = 1/30$ mM^{-1} means that $\bar{K} = 45$. When D_b is not much smaller than L (high R_e values), $V_b/V_w = (LM_L\bar{V}_L + D_bM_D\bar{V}_D)10^{-6}$ and $\bar{K} = K(L + D_b)/[(LM_L\bar{V}_L + D_bM_D\bar{V}_D)10^{-6}]$. This means that $\bar{K}$ depends on R_e. Thus, description of the partition in terms of this constant is only valid at low detergent concentrations in the bilayer where it can be explicitly correlated with the constant K, derived from the basic assumption that the chemical potential of the detergent in bilayer equals the potential of the detergent in water (Lichtenberg, 1985).

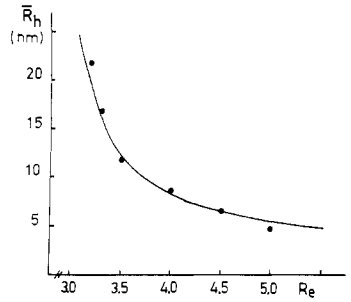

FIGURE 8: Dependence of the hydrodynamic radius (R_h) of OG–PC mixed micelles on their OG/PC ratio (R_e). The data points are as obtained by QLS (Malvern) for mixtures of PC and OG of various concentrations. For each such mixture, R_e was calculated from the equation $R_e = (D_T - 16.6)/L$ (see text). The solid line describes the dependence of $\bar{R}_h$ on R_e calculated on the basis of theoretical considerations, based on the assumption that the mixed micelles have the shape of oblate ellipsoids with a fixed short axis of $d = 19$ Å and a varying long axis (r) which was calculated for each R_e as described in the text. These calculations yielded a theoretical r value for each R_e. R_h was then computed for each R_e, from the equation given by Mazer et al. (1980) for the dependence of R_h on r and d.

As a consequence of introduction of OG into the vesicle bilayers, the membranes expand and become leaky to solutes (Mimms et al., 1981; Ollivon et al., 1988; Ueno, 1989). A similar effect has been previously observed upon introduction of cholate into egg PC vesicles. Increasing the cholate content of the vesicles resulted in leakage of sugars of increasingly higher molecular weights (Schubert et al., 1986). Similarly, OG-containing vesicles of an R_e value above 1.0 retained very little inulin of a molecular weight at about 5000 (Ollivon et al., 1988). In contrast, the leakage of entrapped high molecular weight dextran is very slow even at $R_e = 1.31$.

Size of OG–PC Vesicles. Addition of OG to PC vesicles to yield R_e values of 1.0–1.4 induces rapid vesicle size growth; the size of the resultant vesicles is an increasing function of R_e. Detergent-induced size growth of small phospholipid vesicles appears to be a general phenomenon (Hertz & Barenholz, 1977; Schurtenberger et al., 1985; Enoch & Strittmatter, 1979; Lichtenberg et al., 1984; Almog et al., 1986; Almog & Lichtenberg, 1988; Paternostre et al., 1988; Ollivon et al., 1988). The dependence of $\bar{R}_h$ on R_e in cholate-induced size growth experiments was independent of the presence of calcium, although calcium changes the rate and the mechanism involved in the vesicle size growth (Almog et al., 1986; Almog & Lichtenberg, 1988). This similarity of sizes indicates that the steady-state size distribution reported for cholate-containing vesicles represents a state of equilibrium.

Our results on the OG–PC systems are qualitatively similar to those observed for the PC–bile salt systems. However, we do not have any direct evidence to conclude that the (R_e dependent, concentration independent) size of the vesicles represents equilibrium. Equilibration is possible since lipid exchange between OG-containing vesicles is rapid (Ollivon et al., 1988), not only at $R_e > 1.0$, where detergent-induced size growth is observed, but even in vesicles with $R_e < 1.0$, where an OG-induced lipid exchange between vesicles occurs without a net transfer of lipids. Thus, a lipid-transfer mechanism *could* have potentially led to vesicle size growth even when $R_e < 1.0$. The lack of such a process under these conditions may serve as an indirect evidence that the ultimate size of the vesicles may be thermodynamically controlled, over the whole range of R_e values, similar to the results in PC–cholate systems.

In contrast, when vesicles of a given size were produced upon addition of OG to SUV, extraction of OG from the vesicles (through dilution or any other detergent removal technique; Lichtenberg & Barenholz, 1988) had only a minor effect on

the vesicle size, as studied either by QLS or by molar turbidity (not shown). This may indicate that, in the absence of an efficient mechanism for size decrease, vesicle size is kinetically "trapped". This result is similar to that obtained for PC–bile salt systems (Schurtenberger et al., 1984, 1985; Almog et al., 1986), where the production of vesicles that lasted unaltered for at least 2 months following detergent removal has been utilized to produce vesicles of adjustable sizes (Schurtenberger et al., 1984, 1985). This does not rule out the possibility that over much longer time scales the vesicles may decrease in size. In fact, in a recent work, Paternostre et al. (1988) have shown that addition of subsolubilizing concentrations (R_e = 1.0) of OG to large unilamellar PC vesicles results in a decrease of the size of part of the vesicles. We have never observed such a phenomenon. This apparent contradiction could have resulted from the difference in the way OG was added to the performed vesicles. While we mixed equal volumes of vesicle dispersions and OG solutions, Paternostre et al. (1988) have added (stepwise) small volumes of "concentrated stock solutions" of OG to the preformed vesicles. Possibly, this resulted in a high local OG concentration, which led to solubilization of vesicles in those volume elements to which the OG was first added. The mixed micelles thus formed subsequently underwent revesiculation, and since the R_e value of the mixture was relatively low, small vesicles were formed.

The dependent of size on R_e observed for the OG–PC vesicles is qualitatively similar to that reported by Schurtenberger et al. (1985) for vesicles made by dilution of mixed micelles of PC and glycocholate. These authors tried to explain the observed dependence of R_h on R_e in terms of geometrical considerations based on the model of Israelachvili et al. (1980). This model predicts that the "critical" radius of curvature (R_c) of a phospholipid bilayer made of one lipid be equal to

$$R_c = \frac{l_c}{6} \frac{[3(4P - 1)]^{1/2} + 3}{1 - P}$$

where l_c is the length of the fully extended lipid chain and P is the packing parameter defined by $P = (V/l_c)a_o$, where V is the volume of the lipid molecule and a_o is the critical surface this molecule occupies at the water–lipid interface.

For a bilayer made of two components of the same "length" l_c, R_c was predicted to be given by a similar expression in which the packing parameter P is a weighted average of the P values of the individual lipids weighted by the relative abundance in the membrane. For R_c to become larger upon addition of a detergent to a phospholipid bilayer, the packing parameter of the detergent has to be larger than that of the phospholipid.

In fact, Schurtenberger et al. (1985) observed a reasonable agreement between the radii of the vesicles obtained in their dilution experiments (in a 0.9% NaCl solution at room temperature) and the radii predicted on the basis of Israelachvili's model, only when the volumes of the bile salt molecules, thus the packing parameters, were assumed to be larger than the values based on direct measurements (V = 3449 Å^3; P = 1.44). This inconsistency may be explained in terms of the difference between the volume as measured directly and the volume that the bile salt molecule occupied within a membrane. Nevertheless, one cannot expect the packing parameter of the micelle-forming amphiphile OG to be higher than that of PC. Therefore, the dependence of $\bar{R}_h$ of R_e observed here cannot be explained in terms of Israelachvili's model.

An alternative approach can be based on detergent-induced changes in the elasticity of the lipid bilayer. To qualitatively explain the detergent-induced size growth in terms of this approach, the elasticity of the lipid bilayer has to decrease as a result of detergent insertion. Such a decrease in elasticity may occur if OG-rich domains are segregated within the bilayer, in which the packing of OG and PC molecules is similar to the packing of these molecules in the disk-like large mixed micelles. For example, if the detergent is organized within the bilayer in such a fashion that the vesicle assumes a polygonal shape, its elasticity will be reduced, and further detergent addition will result in its rupture. If such a shape change occurs, the elasticity of the bilayer can be expected to be insufficient to allow for the preservation of a small radius of curvature. Vesicle size growth will then have to ocur to allow for the existence of detergent-rich domains within the vesicles. Such an effect can be expected only under conditions where phase separation occurs in the plane of the membrane. This may require a critical level of OG in the bilayer, which may correspond to R_e = 1.0, above which $\bar{R}_h$ increase markedly upon increasing R_e.

Mechanism of OG-Induced Vesicle Size Growth. Three different mechanisms were considered for the transformation of SUV into larger vesicles upon addition of OG: (1) transformation through lamellar disks, which we rule out on the basis of the dextran retention experiments described in Table I; (2) a lipid-transfer mechanism; (3) OG-induced vesicle–vesicle fusion.

The independence of $t_{1/2}$ of the overall size growth process on PC concentration is consistent with a lipid-transfer mechanism (Lawaczeck, 1978). Nevertheless, it is not inconsistent with a two-step fusion mechanism (aggregation and fusion) since the lipid concentrations studied here may be sufficiently high for the fusion rate rather than the aggregation rate to be limiting (Nir et al., 1983). The results of the dextran retention experiments (Table I) can serve as more direct evidence for the involvement of a lipid-transfer mechanism. More specifically, the agreement between the experimental retention and the retention predicted on the basis of the assumption that the process occurs via lipid transfer strongly supports the latter assumption. For a fusion mechanism to be consistent with these data, we must assume that the progressively graded leakage of dextran with additional OG is a result of a formation of transient detergent-stabilized pores through which large (70 000 daltons) dextran molecules can "leak" to an extent which is (incidentally) similar to that predicted for the alternative lipid-transfer mechanism.

It is of interest to note that in our previous study on cholate-induced size growth of SUV on calcium-containing medium (Almog & Lichtenberg, 1988) we have observed, under conditions similar to those described in this paper, that (1) at moderate calcium concentrations $t_{1/2}$ of the size growth process decreased with increasing [PC] and (2) the leakage of high molecular weight dextan during size growth was much smaller than the leakage predicted for a lipid-transfer mechanism. On the basis of these data, we have concluded that, in the presence of calcium, cholate induces fusion of the SUV into larger vesicles.

The OG-induced size growth is different with respect to both its kinetics and its dextran retention. In view of these differences, the results of the present paper, taken collectively, indicate that size growth probably occurs through lipid transfer although the alternative fusion mechanism cannot be ruled out.

In terms of a lipid-transfer mechanism, the apparent decrease of the size growth process (increasing $t_{1/2}$), observed upon increasing R_e (Figure 7), may reflect the need for more lipid-transfer steps required at higher R_e for the formation of larger vesicles. Alternatively, it may be caused by a decrease in the rate of PC transfer due to the increase in vesicle size,

similar to the curvature effect on the exchange of cholesterol between vesicles (Thomas & Poznansky, 1988).

Saturation of PC Vesicles by OG. Transformation of OG–PC vesicles into mixed micellar structures begins when the OG concentration reaches the level of [OG] = 16.6 + 1.4[PC] mM. This empirical correlation may reflect "saturation" of the membrane at R_e = 1.4 (i.e., a membrane composed of n molecules of PC can accommodate up to 1.4n OG molecules without being transformed into mixed micelles). Alternatively, solubilization may begin when the detergent concentration in the aqueous medium exceeds 16.6 mM, which can then be regarded as being the level of the cmc of OG in the presence of PC. In the latter case OG micelles will form, which will begin solubilizing PC molecules. The definition of K, as given by eq 1, is valid only as long as D_w < cmc. When D_w = cmc

$$K = D_b/[\text{cmc}(L + D_b)] \quad (12)$$

Hence

$$K \times \text{cmc} = D_b/(L + D_b) = R_e/(1 + R_e) \quad (13)$$

i.e.

$$R_e = K \times \text{cmc}/(1 - K \times \text{cmc}) \quad (14)$$

In the OG–PC mixtures, K = 0.033 mM^{-1}, and the highest level of OG monomers found in a vesicle-containing mixture was 16.6 mM. If the latter value is the cmc of this detergent in lipid-containing media, then the above mechanism of solubilization requires that, according to eq 14, R_e^{SAT} = 1.21. In fact, this value of R_e is quite close to the observed value of R_e^{SAT}, which is consistent with a mechanism of solubilization involving the formation of detergent micelles as the first step of solubilization. This does not rule out the alternative mechanism of membrane saturation by detergent. In fact, both mechanisms may contribute to solubilization, and their relative contribution may vary from one detergent–lipid system to another. Furthermore, in practical terms, when detergent molecules are added to a system in which D_w = cmc, its partitioning into membranes is likely to be higher than that obtained at lower values of D_w. Under these conditions, when a detergent is added, most of it partitions into the bilayer as has been reported for Triton X-100 (Goni et al., 1986). Consequently, a saturating (critical) level of OG in the membrane bilayer is approached.

Complete Solubilization of PC Vesicles and PC–OG Mixed Micelles. Complete solubilization of PC by OG occurs when the total OG concentration (D_T in mM) is equal to D_T = 16.6 + 3.2[PC]. Our interpretation of this finding is that PC and OG form pure mixed micellar structures only when the OG/PC effective ratio is higher than 3.2. At the point where $R_e = R_e^{SOL}$ = 3.2, monomeric OG concentration is 16.6, which may be considered the cmc of OG in lipid-containing media (see above). At this point the mixed micelles have the shape of an oblate ellipsoid of a major axis of about 24 nm. At higher R_e values the major axis decreases upon increasing R_e while at slightly lower R_e values it is expected, theoretically, to increase dramatically when R_e decreases (Figure 8). Noticeably, the surface area of an oblate ellipsoid of a major axis of 24 nm (which exists at R_e = 3.2) is about equal to that of small unilamellar vesicles of 12-nm radius. We propose that vesicle formation at R_e < 3.2 is a consequence of the steep dependence of the radius on R_e. This hypothesis has been previously made by Lasic (1982) and Fromhertz (1985) for PC–bile salt mixtures, on the basis of their model by taking into account the bending elasticity of the PC bilayer. If it is general, it may be concluded that complete solubilization occurs only when R_e approaches a level R_e^{SOL}, above which micelles, if formed, cannot reclose into vesicles. For a relatively small major axis (with a major axis/minor axis ratio smaller than about 10), micelles are formed. Above this ratio, micelles will be transformed into vesicles.

Coexistence of OG–PC Mixed Micelles and Vesicles. In the coexistence range, R_e = 1.4–3.2, macroscopic separation of OG–PC mixed micelles (R_e = 3.2) from PC–OG vesicles (R = 1.4) occurs. This may be seen as an example of the type of phase separation observed in binary mixtures of macromolecules, such as high molecular weight dextran and poly(ethylene glycol) (Albertsson, 1971). For very large molecules, a small advantage in pair interaction energy of one of the two species for its own kind is multiplied manyfold due to the large molecular weight. This results in macroscopic separation of the two species. Droplet formation of one phase within the other may occur. In the case of the PC–OG systems geometric factors may be responsible for a tighter packing of the mixed micelles than of the vesicles. This may result in a difference in density which in turn explains the finding that the mixed micelles form the bottom layer. The large viscosity of this layer is probably due to the pronounced anisotropy of the micellar particles. In this dense solution, the PC–OG mixed micelles may interact with each other or may even be loosely packed in the form of stacks of micelles. However, the relatively narrow NMR resonances and the lack of any evidence for a high degree of organization in the small-angle X-ray scattering suggest that if the micelles are packed within such a stack, they are not severely immobilized by this packing.

While further work is certainly required for characterization of the exact state of aggregation in the condensed micellar phase, the mere existence of such a phase appears to be general and should be taken into account even when such a state of aggregation is not apparent. We believe that the large increase in turbidity reported by Ollivon et al. (1988) and Paternostre et al. (1988) under certain conditions might have been due to formation of droplets of such a condensed phase. Only at higher PC concentrations did these authors report macroscopic phase separation. Noticeably, charged impurities (e.g., cholate; Paternostre et al., 1988) can interfere with the formation or coalescence of droplets of such a condensed phase. This can be attributed to decreased micellar aggregation due to the electrostatic repulsion between the charged micelles.

Macroscopic phase separation may have a small effect on the state of aggregation of mixed micelles formed upon solubilization of lamellar lipid as the equilibration of mixed vesicles appears to be rapid. Since this study is relevant for membrane solubilization (for protein purification) as well as for reconstitution of proteoliposomes, it is important to note that proteins distribute between two aqueous phases according to their hydrophobicity and charge, and their preference for one phase over the other may be very large. Such an uneven distribution of proteins between two different aqueous phases has been extensively used for separation and purification of various proteins (Albertsson, 1971). The phase separation observed in the OG–PC mixture at R_e values of 1.4–3.2 is therefore likely to have a pronounced effect on the reconstitution of vesicles from mixed micelles, upon detergent removal. It may be, at least partly, responsible for the heterogeneity of reconstituted membranes: membrane proteins may partition unevenly between vesicles and condensed micellar phases. As a consequence, the first vesicles to be formed upon detergent removal from lipid–protein–detergent mixed micelles may contain different levels of proteins from vesicles formed at later stages of detergent removal.

It should be noted that the results of the present study appear to invalidate our previous hypothesis regarding the range of coexistence. Previously, we have proposed (Lichtenberg et al., 1983) that if solubilization starts at a critical detergent/lipid effective ratio R_e^{SAT} and is completed at another critical effective ratio R_e^{SOL}, it means that when any given vesicle reaches some intermediary ratio R_e^{crit}, it will be transformed into mixed micelles. Thus, the range of coexistence was interpreted as being due to a normal distribution of detergent molecules among vesicles. We now think that R_e^{SAT} is the highest detergent/phospholipid ratio that can exist in a vesicle; at higher effective ratios, the vesicles disintegrate into mixed micelles. On the other hand, R_e^{SOL} is the lowest detergent/lipid ratio required to keep the lipid and detergent in the form of mixed micelles. Thus, within the range of coexistence, micelles of a composition given by R_e^{SOL} coexist with vesicles whose detergent/lipid ratio equals R_e^{SAT} (Table II). Similar results were obtained in PC–cholate mixtures (S. Almog, unpublished results). This is consistent with the interpretation of our previous ^{31}P NMR studies of PC–OG mixtures (Jackson et al., 1982).

Concluding Remarks

On the basis of our studies, it appears that mixtures of OG and PC are essentially vesicular at all effective detergent/lipid ratios below $R_e = 1.4$. The composition of the vesicles is a function of the lipid and detergent concentrations and the partition coefficient ($K = 0.033\ mM^{-1}$), which describes the partition of OG between lipid bilayers and the aqueous medium. Increasing R_e results in a monotonic increase in the size of the vesicles and in leakage of solutes of increasingly higher molecular weights. Vesicles of $R_e = 1.0$–1.4 are permeable to inulin (Ollivon et al., 1988) but not to high molecular weight dextran. These vesicles may even undergo transient changes into disks, yet reclose rapidly, such that their steady-state spherical geometry is maintained. The highest OG/PC ratio in a vesicle is 1.4. The lowest OG/PC ratio in a mixed micelle is 3.2. Within the range of $R_e = 1.4$–3.2, vesicles and micelles coexist. These mixtures, however, separate into two macroscopic phases.

Removal of OG from PC–OG mixed micellar systems results in spontaneous formation of PC vesicles (Mimms et al., 1981; Ollivon et al., 1988). The detailed mechanism by which this process occurs is not clear at the present time. However, the OG-induced size growth of the vesicles described above implies that if OG–PC mixed micelles transform initially into small vesicles upon removal of OG, the resultant vesicles are likely to grow in size, depending on the rate of detergent removal. Such a size growth mechanism was observed for reconsitution of PC vesicles from PC–cholate mixed micelles (Almog et al., 1986) although cholate-induced (postvesiculation) size growth is much slower. This may explain the fact that reconstitution procedures based on cholate removal yield vesicles whose size varies over much a larger range (30–150 nm) than that of vesicles made through removal of OG from OG–PC mixed micelles (Crommelin, 1984). However, other factors may also be involved in determining the size of reconstituted vesicles, the most important of which is the behavior of OG–PC mixtures within the range of OG/PC concentrations where mixed micelles and vesicles coexist. The formation of a separate condensed phase of aggregated micelles within this range is very likely to play a role in reconstitution experiments. More work will, however, be required to determine this effect.

Registry No. OG, 29836-26-8.

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Study of the Structure of *N*-Acyldipalmitoylphosphatidylethanolamines in Aqueous Dispersion by Infrared and Raman Spectroscopies†

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ABSTRACT: The effect of the headgroup chain length on the structure and on the thermotropic behavior of *N*-acyldipalmitoylphosphatidylethanolamines (*N*-acyl-DPPEs) has been studied by infrared and Raman spectroscopies. The results show that the *N*-acyl-DPPEs can be divided in two classes depending on the *N*-acyl chain length. When the *N*-acyl chain contains 10 carbon atoms or more, it penetrates into the bilayer while it remains at the level of the glycerol backbone for shorter *N*-acyl chains. For both classes of *N*-acyl-DPPEs, the rotation of the lipid chains in the liquid-crystalline phase is hindered by the presence of the *N*-acyl group. In addition, the disruption of the hydrogen bonds between the amino and phosphate groups by N-acylation of the amino group results in an increase of the hydration of the phosphate group compared to that in DPPE. The hydration occurred at both the phosphate and amide group levels; the phosphate group is more hydrated for phospholipids with long *N*-acyl chains while in the case of short-chain derivatives both the phosphate and amide groups are hydrated. This higher degree of hydration coupled with the immobilization of the lipid molecule may contribute to the bilayer stabilizer role of *N*-acyl-PEs since hydration is an important factor in bilayer stability.

The *N*-acylphosphatidylethanolamines are a minor lipid component of seeds (Bomstein, 1965; Dawson et al., 1969; Hargin & Morrison, 1980), microorganisms (Hazlewood & Dawson, 1975; Clarke et al., 1976), and some vertebrate tissues (Matsumoto & Miwa, 1973; Gray, 1976; Somerharju & Renkonen, 1979; Epps et al., 1980; Natarajan et al., 1985, 1986). It has been shown that *N*-acyl-PEs[1] are authentic components of membranes (Matsumoto & Miwa, 1973) and not a product made by transacylation during extraction of lipids as suggested by Wren and Merryfield (1965). *N*-Acyl-PEs are mainly produced as a result of a degenerative change or cell injury. In wheat grain, for example, they are synthesized during dehydration of the endosperm in which *N*-acylethanolamine phospholipids account for more than 50% of the total phospholipids of dehydrated tissue (Hargin & Morrison, 1980). The precise role of these lipids is still unknown although it has recently been reported that they might be the precursor of *N*-acylethanolamine, a derivative produced by phospholipase D activity, which has potential protective effects against cell injury (Epps et al., 1982; Parinandi & Schmid, 1988). However, *N*-acetylethanolamine phospholids are normally present in some tissues (Natarajan et al., 1985) and during some period of the life cycle of microorganisms

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[1] Abbreviations: DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; *N*-acyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-*N*-acylethanolamine; *N*-oleoyl-DOPE, 1,2-dioleoyl-*sn*-glycero-3-phospho-*N*-oleoylethanolamine; *N*-C4-DPPE or *N*-butyryl-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-butyrylethanolamine; *N*-C6-DPPE or *N*-caproyl-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-hexanoylethanolamine; *N*-C8-DPPE or *N*-capryl-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-octanoylethanolamine; *N*-C10-DPPE or *N*-capryl-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-decanoylethanolamine; *N*-C12-DPPE or *N*-lauryl-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-dodecanoylethanolamine; *N*-C16-DPPE or *N*-palmitoyl-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-hexadecanoylethanolamine; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance.

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