Solubilization of Phosphatidylcholine Bilayers by Octyl Glucoside†

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ABSTRACT: The solubilization of large, unilamellar egg phosphatidylcholine vesicles by the nonionic detergent octyl glucoside (OG) was investigated by nuclear magnetic resonance (NMR), fluorescence anisotropy, turbidity, electron microscopy, and centrifugation followed by compositional analysis. The solubilization process is well described by the three-stage model previously proposed for other detergents. In stage I, the OG partitions between the bilayer and aqueous phases with a molar partition coefficient of 59 ± 6. The presence of OG in the bilayers produces a small "fluidizing" effect, as indicated by changes in the NMR and fluorescence anisotropy parameters. A rearrangement that forms large mixed bilayers occurs in the latter part of stage I. Stage II, the conversion of detergent-saturated bilayers into mixed micelles, begins at a ratio of total OG concentration minus the critical micelle concentration to total phosphatidylcholine concentration of approximately 1.5 and continues until this ratio reaches about 3.0. The correction for the critical micelle concentration of the OG is necessary for comparison of experimental results obtained at different lipid concentrations. The mixed bilayer–mixed micelle interconversion is quantified by the centrifugation experiments and by ^{31}P NMR. The agreement between the two methods is excellent. Advantages of the NMR method are discussed. In stage III, which was not studied in detail here, all of the phosphatidylcholine is present as mixed micelles. Evidence is presented that the various structures present in the dispersions are in equilibrium with one another during these experiments.

Biological membranes are complex arrays of proteins and lipids, the study of which frequently requires the use of detergent solubilization (Helenius & Simons, 1975). Moreover, bilayer formation, in the course of reconstitution experiments, usually involves removal of detergent molecules from mixed dispersions by either dialysis, columns, or dilution of the dispersions (Racker, 1979, and references cited therein), a process that is the reverse of membrane solubilization. Although a large number of studies has been published that employed detergent solubilization, very little informaion is available that quantitatively characterizes the solubilization process (Lichtenberg et al., 1979; Yedgar et al., 1974; Dennis & Owens, 1973). The nonionic detergent octyl glucoside (OG)[1] has gained widespread use in solubilization and reconstitution studies (Baron & Thompson, 1975; Stubbs et al., 1976; Kasahara & Hinkle, 1976; Helenius et al., 1977; Stubbs & Litman, 1978a,b; Petri & Wagner, 1979). This detergent is particularly useful for such studies, due to its very high critical micelle concentration (cmc) of about 25 mM (Shinoda et al., 1961), which allows for its easy removal. In addition, OG has the advantage of many nonionic detergents in that it does not denature membrane proteins easily (Stubbs et al., 1976), without the disadvantage of being a polydisperse chemical species as is Trition X-100, for example. Very little is, however, known about the details of the solubilization of phospholipids by this detergent and about the reconstitution of bilayers upon its removal.

In this work, we have studied the solubilization of large, unilamellar egg phosphatidylcholine (PC) vesicles, prepared by detergent dialysis (Stubbs et al., 1976; Petri & Wagner, 1979; Mimms et al., 1981). This model system was chosen because it avoids problems associated with the two more commonly used model systems: unsonicated, large, multilamellar vesicles and sonicated, small, unilamellar vesicles. For multilamellar vesicles, it has been shown that the turbidity of preformed dispersions, with sodium deoxycholate or sodium dodecyl sulfate added, may take up to several days to reach its minimum value (Lichtenberg et al., 1979; D. Lichtenberg, unpublished results). In contrast, the addition of these detergents to sonicated, unilamellar vesicles results in rapid equilibration, which suggests that the penetration of detergents through multilamellar structures is a slow process. The small sonicated vesicles, on the other hand, are suspect as a model system since they are highly curved; many studies (Sheetz & Chan, 1972; Suukuusk et al., 1976; Gaber & Peticolas, 1977; Lichtenberg et al., 1981; Nordlund et al., 1981) have shown that the curvature affects the properties of the vesicles. Biological membranes, with some exceptions, are thought to be better represented by larger vesicular structures.

Materials and Methods

Octyl Glucoside Synthesis. Octyl β-D-glucoside (OG) was prepared from acetobromoglucose and 1-octanol by modification of the method of Noller & Rockwell (1938).

Vesicle Preparation. Egg PC used to prepare OG dialysis vesicles was purchased from Avanti Biochemicals and used without further purification, while that used to prepare sonicated vesicles was purified as previously described (Litman, 1973). The purity of the PC was established by TLC at a 1 μM loading. The fatty acid side chain composition of the two preparations was essentially identical. Small unilamellar vesicles were prepared by sonication and fractionated as described by Barenholz et al. (1977). The OG dialysis vesicles used for most of the solubilization experiments were prepared as follows: lyophilized egg PC was solubilized in a solution of 400 mM OG–50 mM KCl so that the final PC concentration was 60 mM, yielding an OG to PC ratio of about 6.7:1. Samples were generally allowed to incubate 2–3 h prior to dialysis. The PC–detergent solutions were dialyzed at 4 °C

† From the Department of Biochemistry, University of Virginia, School of Medicine, Charlottesville, Virginia 22908. *Received March 15, 1982.* This research was supported by National Science Foundation Grant PCM80-12028-01 and by National Institutes of Health Grants EY00548, GM-14628, and GM-17452. A preliminary account of these results was presented at the Joint Biophysical Society–ASBC meeting, New Orleans, LA, 1980 (Schmidt et al., 1980).

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[1] Abbreviations: PC, phosphatidylcholine; OG, octyl β-D-glucoside; cmc, critical micelle concentration; NMR, nuclear magnetic resonance; DPH, diphenylhexatriene; TLC, thin-layer chromatography.

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against a 100-fold excess of 50 mM KCl. The dialysis medium was changed 4 times at 8–12-h intervals. On the basis of previous reports (Stubbs et al., 1976; Helenius et al., 1977; Petri & Wagner, 1979), one would estimate removal of at least 99.95% of the OG by this dialysis procedure, leaving a residual level of less than 1 OG molecule/300 PC molecules. In order to inhibit oxidation of the PC, the dialysis solution was deoxygenated with nitrogen gas.

Electron Microscopy. Negatively stained vesicle preparations were examined on carbon-coated Formvar films on copper grids in a Siemens Elmiscop IA electron microscope operating at 60 kV and ×3500 or ×7000 magnification. The samples were stained with 1% phosphotungstic acid, pH 7.2, and applied to the grid 3 s before blotting.

Dispersion Preparation. In order to study the solubilization process, PC vesicle samples containing increasing concentrations of OG were prepared. In each experiment, the PC concentration was kept constant. An aliquot of the vesicle preparation was added to a calculated volume of 50 mM KCl. The appropriate volume of a stock solution of OG in 50 mM KCl was than added. The concentration of the OG stock solution was typically 400 mM. The samples were assayed for organic phosphate by the method of Bartlett (1959) and for OG by the Anthrone method (Spiro, 1966).

Centrifugation Experiments. Aliquots of the dispersions were spun (at 4 °C) at 100000*g* for 30 min in a Ty-65 Beckman rotor. For some of the experiments, adapters were used in the rotor, allowing 2-mL volumes to be centrifuged. The clear supernatant was removed and assayed for phosphate and detergent as above.

Turbidity Measurements. The turbidity of the PC–OG samples was determined at 22 °C. A 0.25-cm path-length cell was used in a Gilford Model 240 spectrophotometer at 800 nm for the OG dialysis vesicles and 300 nm for the sonicated vesicles.

Fluorescence Measurements. Samples were prepared as described above. PC–detergent dispersions were labeled with the fluorescent probe diphenylhexatriene (DPH) (Shinitzky & Barenholz, 1974) at a PC/DPH ratio of 484:1 (mol/mol). An aliquot of DPH in tetrahydrofuran was injected into the sample. Scattering blanks were prepared that were identical with the samples except that no DPH was added. The samples and blanks were vortexed and incubated at 40 °C for 30 min to ensure complete incorporation of the probe. The PC concentration was 0.2 mM, a level at which scattering in the parallel mode for the blank was 15% or less than that of the signal from the DPH-labeled sample. The OG concentration was varied from 0 to 50 mM. Fluorescent measurements were obtained with a Perkin-Elmer MPF-3 instrument, at 25 °C, that was modified for polarization measurements. The parallel and perpendicular intensities were recorded. The total intensity (F) and anisotropy (r) were calculated according to the relatonships

$$F = I_{\parallel} + 2I_{\perp} \quad (1)$$

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (2)$$

The temperature was measured with a thermistor immersed in the sample cuvette.

Fluorescence Measurements with OG. Solutions of 0–40 mM OG in 50 mM KCl were prepared. So that the DPH concentration among samples could be accurately controlled, an aliquot of the DPH stock solution was injected into 50 mM KCl, and aliquots of this suspension were added to the OG solutions to yield a final DPH concentration of 2.8×10^{-7} M. Scattering blanks, identical with the samples except that 50 mM KCl was added instead of the DPH suspension, were prepared. The samples were incubated at 40 °C for 30 min, and fluorescent measurements were obtained at 25 °C as described above.

Nuclear Magnetic Resonance. All NMR measurements were done with a JEOL FX60Q Fourier-transform NMR spectrometer operating at 24.15 MHz for ^{31}P, an ambient probe temperature of 24 ± 1 °C, continuous ^{1}H decoupling, and 4K data points in the transformed spectra. The spectra were taken under a variety of conditions, depending on whether or not it was desired to observed the PC molecules in the micellar (narrow line width) or bilayer (broad line width) states and whether qualitative or quantitative integral measurements were to be made. The quantitative wide sweep width conditions were a 5-kHz sweep width, a 0.0205-s acquisition time, a 4.0-s delay between acquisitions, and a 16-μs 90° pulse. Typically 5000 scans and 5-Hz line broadening were used. For the solubilization experiments a 500-Hz sweep width, 2.0-s acquisition time, and 18.0-s delay between acquisitions were employed. The long delay was necessary because the 1.0 M K_3PO_4 used as an external integration reference had a relaxation time of around 3 s. This reference was doped with a small amount of Mn^{2+} in order to broaden the line and reduce the relaxation time. The PC in mixed micelles had a relaxation time of around 1.5 s. For the high concentration (27 mM) solubilization experiments, where good signal to noise spectra could be obtained in 100 scans, interference from the broad (bilayer) component was minimal, and the computer integral could be used. In the low concentration (8 mM) solubilization experiments, 400 scans and 1-Hz line broadening were used. To obtain usable integrals, it was necessary to cut out and weigh the tracing of the narrow micellar component.

The large size and resultant broad line width of the PC vesicles prepared by OG dialysis creates some problems for the measurement of an accurate outside to inside ratio. It was necessary to use a broadening reagent (Mn^{2+}), in relatively high concentration (Mn^{2+}/PC = 0.3), added isosmotically so that the vesicles did not leak. The spectral conditions were the 5-kHz sweep width parameters.

Results

Characterization of OG Dialysis Vesicles. The average diameter of the OG dialysis vesicles was determined by negative-stain electron microscopy to be approximately 1400 Å. A typical mass-weighted size distribution is shown in Figure 1. While the distribution varied somewhat from experiment to experiment, the average diameter remained relatively constant. The ratio of the number of PC molecules in the outer monolayer to the number in the inner monolayer of the vesicles was determined with ^{31}P NMR by the addition of the paramagnetic ion Mn^{2+} to the sample. The ratio was determined to be 1.0 ± 0.2, indicating that the vesicles were predominantly large and unilamellar. When the spectra of the vesicles were rerun, the outside to inside ratio increased slowly, indicating that the vesicles were leaky to Mn^{2+}. The permeability of the vesicles varied among different preparations.

Fluorescence Anisotropy Measurements. The fluorescent probe DPH has been used to monitor the fluidity of the hydrocarbon regions of lipid bilayers and membranes (Shinitzky & Barenholz, 1974, 1978; Stubbs et al., 1976). We have employed the fluorescent properties of DPH to determine the cmc of OG and characterize the solubilization of PC vesicles by OG. DPH was added to samples containing increasing concentrations of OG in 50 mM KCl. The dependence of the

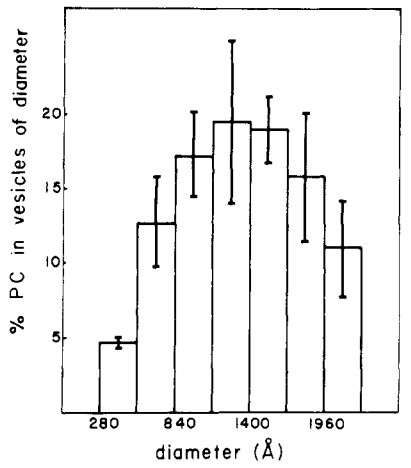

FIGURE 1: Mass-weighted size distribution of PC dialysis vesicles. The weighting procedure assumed that the mass of lipid in a vesicle is proportional to the radius squared. This histogram is the average of three vesicle preparations; 1408, 1580, and 1550 vesicles were counted. The error bars represent the average deviations from the mean weight percents.

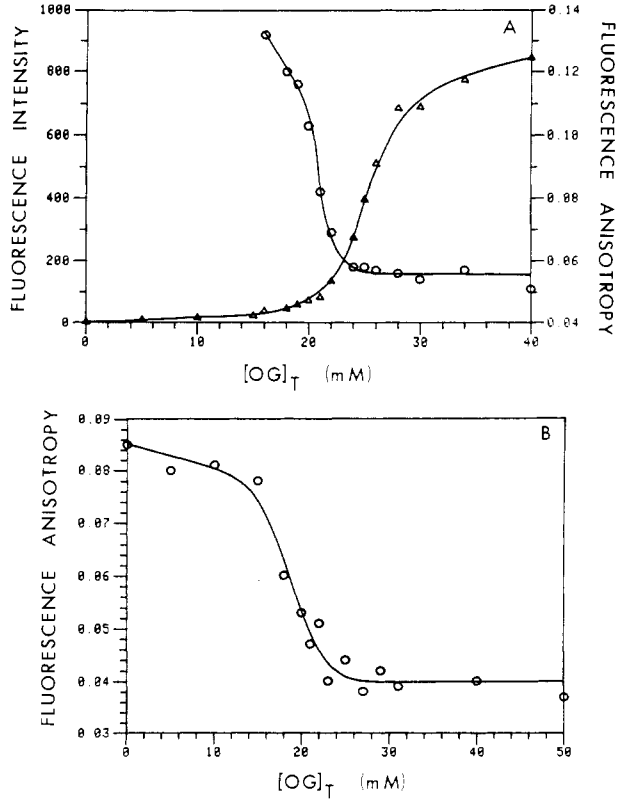

FIGURE 2: (A) Total fluorescence intensity (Δ) and fluorescence anisotropy (O) vs. OG concentration for OG in 50 mM KCl with the fluorescent probe DPH. (B) Fluorescence anisotropy vs. OG concentration for PC dialysis vesicles labeled with DPH. The dispersions were prepared in 50 mM KCl; the PC concentration in (B) was 0.2 mM.

total intensity (F) and anistropy (r) of DPH, as a function of the concentration of OG, are shown in Figure 2A. The fluorescence intensity was near zero until approximately 16 mM, where it began to rise slowly with increasing OG concentration. At 20 mM, the intensity began to rise sharply until 30 mM OG. At higher OG levels the intensity showed a continued but slower rate of increase. The fluorscence anisotropy dropped rapidly from 0.132 at 16 mM OG to 0.058 at 24 mM OG and then remained essentially constant at 0.056 ± 0.003 as the OG concentration was increased further (Figure

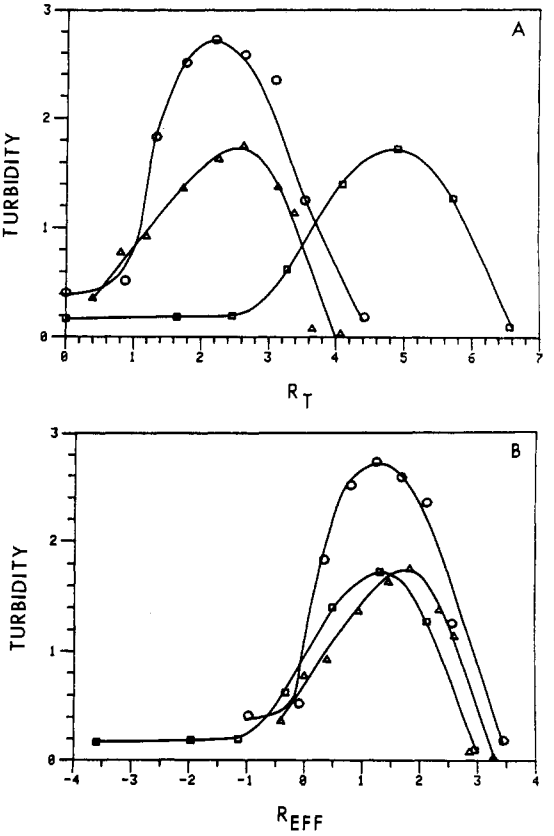

FIGURE 3: (A) Turbidity vs. R_t and (B) turbidity vs. R_{eff} for 22.6 (O) and 6.1 mM PC sonicated vesicles (□) and 27.7 mM PC dialysis vesicles (Δ). Vesicles were prepared in 50 mM KCl; the turbidity was measured at 300 nm for the sonicated vesicles and at 800 nm for the dialysis vesicles.

2A). Reliable anisotropy values could not be obtained below 16 mM OG due to the low observed fluorescence intensities. Since DPH exhibits little or no fluorescence in an aqueous environment, the dramatic increase in the total intensity is indicative of detergent micelle formation. By extrapolation of the total intensity curve, a cmc of 22 mM is obtained for OG in 50 mM KCl. This value is in good agreement with previously reported values (Shinoda et al., 1961; deGrip & Bovee-Geurts, 1979).

Samples 0.2 mM in PC containing from 0 to 50 mM OG in 50 mM KCl were labeled with DPH, Figure 2B. The anisotropy for the pure PC sample was 0.085. As the concentration of OG increased from 0 to 15 mM, a slight reduction in the anisotropy was observed. A sharp drop in anisotropy occurred over the range of 16–22 mM OG. Above 22 mM OG, the anisotropy was independent of OG concentration, having an average value of 0.040 ± 0.004.

Turbidity Measurements. The turbidity of preformed PC vesicle samples was measured as a function of added OG, as a means of monitoring the solubilization process. These measurements are plotted as a function of R_t, the molar ratio of the total OG to total PC concentrations in the sample, in Figure 3A. As increasing amounts of OG were added to preformed vesicles, the turbidity showed an initial increase, passed through a maximum, and then decreased to a minimal value. The R_t value at which the turbidity maximum occurred was found to be dependent on the PC concentration. When the OG to PC ratio was corrected for the OG monomer concentration expected to be in equilibrium with either mixed bilayers or mixed micelles, so as to obtain an effective molar ratio of micellar OG to PC, R_{eff} [for a cmc of 22 mM, $R_{eff} = ([OG]_t - 22)/[PC]$],[2] and the turbidity was plotted as a

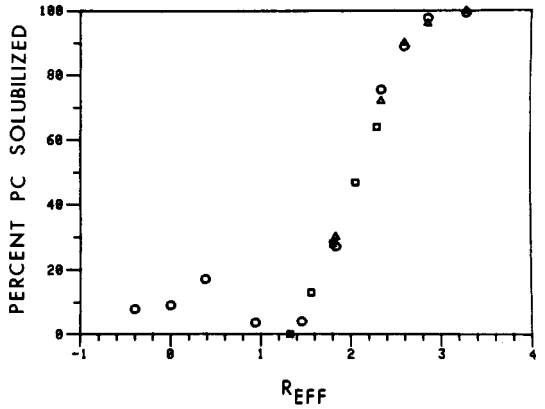

FIGURE 4: R_{eff} vs. percent PC solubilized for 27.7 mM PC dispersions determined by percent PC in supernatant after centrifugation at 100000g for 30 min (○), percent sharp component of NMR signal (△), and percent sharp component of NMR signal of a sample with an R_{eff} = 1.33 tritated stepwise to an R_{eff} = 2.30 with a 470 mM OG stock solution (□).

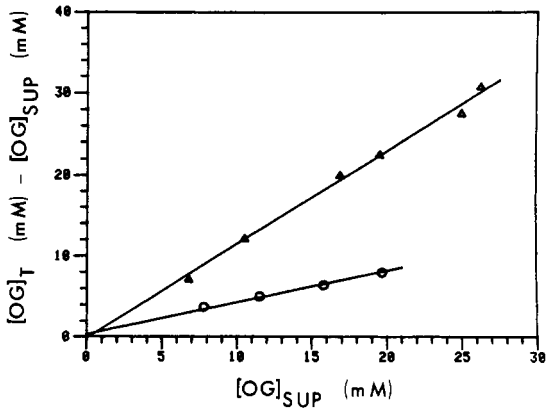

FIGURE 5: Distribution of OG between pellet and supernatant for subsolubilizing levels of OG. $[OG]_t - [OG]_{sup}$ vs. $[OG]_{sup}$ for 9.3 (○) and 23.0 (△) mM PC samples. $[OG]_{sup}$ is the concentration of OG in the supernatant after centrifuging the sample at 100000g for 30 min. $[OG]_t$ is the total OG concentration in the sample.

function of this corrected ratio, the positions of the turbidity maxima for different PC sample concentrations were in close alignment, Figure 3B. This is demonstrated by a shift in the turbidity maximum for 6.1 and 22.6 mM PC sonicated vesicle samples from R_t values of 4.9 and 2.2 to R_{eff} values of 1.3 and 1.2, respectively. The high monomer concentration of OG therefore necessitates a significant correction of the detergent to PC ratio. Larger dialysis vesicles, having a PC concentration of 27.7 mM, showed a turbidity maximum at R_t = 2.6 and an R_{eff} = 1.8, indicating a possible dependence of the solubilization process on vesicle size.

Compositional Characterization of Solubilization Process. Lipid dispersions containing increasing concentrations of OG were prepared as described under Materials and Methods. For characterization of the solubilization process, the concentrations of PC and OG in the dispersions were determined before and after centrifugation at 100000g for 30 min. Under these centrifugation conditions, bilayer vesicles pellet, but mixed micelles of OG and PC remain in the supernatant. The dependence of the percentage of PC in the supernatant on R_{eff} is shown in Figure 4 for a PC concentration of 27.7 mM. Below an R_{eff} value of 1.5, only a small percentage of the PC was found in the supernatant, while above this value, the percentage of PC in the supernatant increased rapidly, finally plateauing at 100% PC solubilization at an R_{eff} of about 3.0. The concentration of OG in the supernatant ($[OG]_{sup}$) was found to increase slowly with increasing R_{eff} up to a value of 1.5. Between R_{eff} values of 1.5 and 3.0, a more rapid, roughly linear increase was observed (data not shown). The distribution of OG between the pellet and supernatant, for R_{eff} values less than 1.5, at PC concentrations of 9.3 and 23.0 mM is shown in Figure 5. It can be seen that these plots are linear, with slopes of 0.40 and 1.15, respectively. A third set of samples with 27.7 mM PC gave a slope of 1.14 (not shown).

NMR Measurements. ^{31}P NMR spectra for samples with fixed OG to PC ratios, prepared as described under Materials and Methods, are shown in Figure 6. The appearance of the

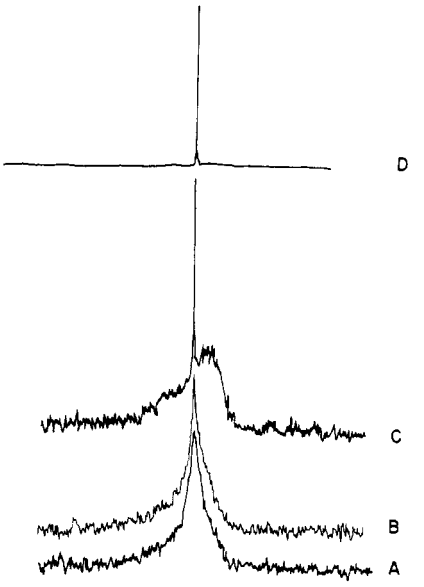

FIGURE 6: ^{31}P NMR spectra of 27.7 mM PC dispersions: (A) no OG; samples with an R_{eff} of (B) 0.23, (C) 1.55, and (D) 2.25.

5-KHz sweep width spectrum for dialysis vesicles without added OG, Figure 6A, reflects the size and heterogeneity of the vesicles; i.e., the spectrum has a broad width at half the total height (about 200 Hz) and is distinctly non-Lorentzian. The larger vesicles in the sample give rise to ^{31}P resonances that are no longer isotropic, producing the assymmetry observed in the spectrum. A ^{31}P NMR spectrum characteristic of PC solubilized into mixed micelles is shown in Figure 6D. The ^{31}P resonance is narrow (line width 3.5 Hz) and Lorentzian. For samples with subsolubilizing OG concentrations, the overall ^{31}P spectral envelope appears to be narrowed, Figure 6B, although this is difficult to quantitate because of the distribution of the line widths and shapes. Starting at an R_{eff} value of about 1.5, the ^{31}P NMR spectrum, Figure 6C, shows the superposition of a narrow peak, typical of mixed micellar PC, and a much broader resonance typical of PC in large mixed bilayers. This indicates that the exchange of PC molecules between the micellar and bilayer states is slow on the ^{31}P NMR time scale (about 5 ms). This is in contrast to results obtained by ^{31}C or ^{1}H NMR (results not shown). The presence of two component spectra in the range of R_{eff} values where solubilization occurs means that the integrated intensity of the sharp component relative to the total integral can be used as a quantititive measure of the amount of solubilized

[2] The results of this study, and others, indicate that the conversion of mixed bilayers into mixed micelles begins near or at the point at which the detergent monomer concentration reaches the cmc. At lower concentrations the cmc has not been reached. The expression for R_{eff} is therefore only approximate in stage I. It is least accurate near zero OG concentration, which accounts for the "negative" R_{eff} values in Figures 3B and 4. We have used this simplified expression because the exact equation, written in terms of the partition coefficient, is cumbersome and discontinuous at the cmc.

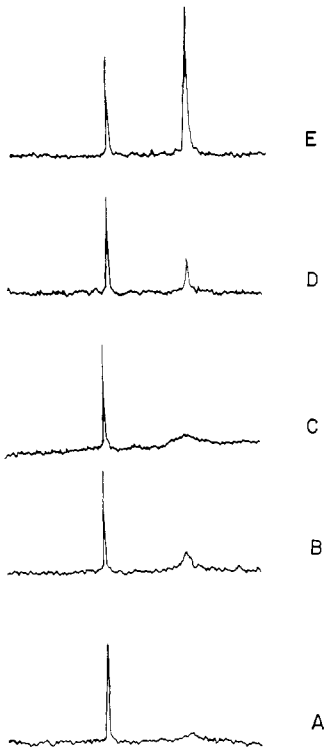

FIGURE 7: High-resolution ^{31}P spectra of 7.8 mM PC dialysis vesicles (A) before and after addition of OG to an R_{eff} of (B) 0.60, (C) 1.05, (D) 1.50, and (E) 2.62. The spectra were recorded in the presence of K_3PO_4, as an external intensity reference.

PC. This was done for an aliquot of the 30% solubilized sample used for the centrifugation experiments described in the previous section, and excellent agreement was obtained between the two methods (spectrum not shown).

Alternatively, the broad, bilayer component of the spectrum can be effectively filtered out by increasing the delay between the end of the pulse and the start of data acquisition, which is done automatically when the spectral sweep width is reduced. The percentage of PC solubilized can then be determined with respect to an external reference standard and a sample in which the PC has been completely solubilized. Representative 500-Hz sweep width spectra illustrating this procedure are shown in Figure 7. The advantage of this procedure is that it requires much less time per spectrum than the whole spectrum procedure described above. When the percent solubilization was determined by the narrow sweep width technique for aliquots of the centrifugation samples described in the previous section, the agreement between the two techniques was excellent, Figure 4. In addition, a sample with R_{eff} = 1.33 was titrated by addition of small amounts of OG from a concentrated stock solution. These data are also shown in Figure 4. For these 27.7 mM PC samples, the computer integral was used to determine the percent solubilization. For lower concentration samples, such as the 7.8 mM PC samples for which spectra are shown in Figure 7, the lower signal to noise ratio and the more obvious presence of the broad ^{31}P component make it necessary to integrate peak areas by cutting and weighing expanded spectra of the narrow peaks. The results from the 27.7 and 7.8 mM samples, and other concentrations in between, agree within experimental error.

Discussion

Previous studies of the interaction of detergents with biological and model membranes have indicated that, as increasing amounts of detergent are added to bilayers, the conversion into mixed micelles occurs in three distinct stages (Helenius & Simons, 1975; Stubbs & Litman, 1978a,b). In stage I, the detergent distributes between the bilayers and the aqueous solution. In stage II, the mixed bilayers convert into mixed micelles until all of the bilayer phase has disappeared. In stage III, the detergent to lipid ratio in the mixed micelles increases and the size of the micelles decreases. We have investigated these processes for the nonionic detergent octyl glucoside, which is widely used in solubilizing biological membranes and reconstituting purified membrane proteins. Large, unilamellar vesicles prepared by detergent dialysis have been used in order to avoid problems associated with highly curved sonicated vesicles or multilamellar dispersions (see introduction).

One important general conclusion is that it is necessary to subtract the concentration of aqueous detergent monomers before experimental results at different lipid concentrations can be compared, as demonstrated in Figure 3A,B. This has been pointed out previously (Shankland, 1970; Helenius & Simons, 1975; Stubbs & Litman, 1978a,b), but is does not yet appear to be widely appreciated. The reason for this is no doubt partially because the necessary correction is small when the lipid concentration is high with respect to the cmc. However, for many membrane protein studies, where low concentrations are often necessary, or for detergents such as OG or sodium cholate with high cmc's, the correction is significant and must be taken into consideration. For example, it has been shown, in the two reconstitution studies where the question has been examined in detail, that the detergent to lipid ratio affects the properties of the resulting glycophorin containing vesicles (Mimms et al., 1981; Ong et al., 1981). It is possible that some of these effects could be better understood in terms of variation in the effective detergent to lipid ratio.

Stage I. Below R_{eff} values of about 1.5, OG distributes between the vesicles and the aqueous solution without solubilizing the PC (Figure 4). This stage of the interaction has not been studied in great detail for other commonly used detergents. It is usually assumed that the situation is analogous to that for anesthetics (Roth & Seeman, 1972), i.e., that the concentration of detergent in the bilayer phase is directly proportional to the concentration in the aqueous phase. Figure 5 indicates that this is indeed the case for OG and PC, so that a partition coefficient of 59 ± 6 mol/L of bilayer phase per mol/L of aqueous phase can be calculated from the slopes given under Results. This coefficient can be compared with the value of 116 ± 16 obtained by Stubbs & Litman (1978a,b) for the partitioning of OG between bovine retinal rod outer segment disk membranes and the aqueous solution. It is possible that the difference between these two coefficients is due to the preferential interaction of OG with rhodopsin. This is indicated by the fact that rhodopsin solubilization occurs more rapidly than disk lipid solubilization (Stubbs & Litman, 1978a,b).

The physical effect of the presence of detergent molecules in the bilayer phase has also not been investigated for commonly used detergents. Again, it is generally assumed that the effect of detergents is similar to that of anesthetics, which have been found to have a "fluidizing" effect on bilayers; that is, the rate and/or amplitude of a motional parameter increases [reviewed by Richards (1978)]. We find that this analogy is valid for OG in PC bilayers, as indicated by the small decrease in DPH fluorescence anisotropy in the region of 0–15 mM OG (Figure 2B), by the decrease in the PC ^{31}P NMR line width (Figure 6, spectrum B), and by decreases in the ^{13}C NMR line widths of both the choline methyl and fatty acid double bond

carbon resonances, which are well resolved from the OG ^{13}C resonances (not shown).

In contrast to the small decreases in DPH fluorescence anisotropy and NMR line widths is the large increase in turbidity that occurs during stage I. The addition of detergent molecules to vesicles would be expected to produce some increase in the amount of light scattered (Fu & Laughlin, 1980), but not the many fold increase observed here. Such an increase is indicative of massive vesicle aggregation and/or fusion. Another large-scale change that occurs during stage I is the broadening of the ^{31}P NMR spectral envelope. This broadening, in contrast to the spectral narrowing seen at low R_{eff} values, becomes detectable at R_{eff} values of about 1.0 and continues until conversion into mixed micelles begins.[3] The new line shape is shown in Figure 6C. The width and the shape of the bilayer part of the envelope are comparable to those observed for unsonicated dispersions of egg PC, which are considerably larger than the original population of dialysis vesicles. It is, however, difficult to unequivocally prove that the ^{31}P NMR spectral changes are not due to vesicle aggregation, since aggregation is difficult to turn on or off. But aggregation as a large-scale broadening agent seems unlikely for a variety of nonconclusive reasons. First, in fusion studies on small sonicated vesicles, aggregation, which normally precedes fusion, has not been shown to have a significant effect on NMR line widths (Liao & Prestegard, 1979, 1980; Koter et al., 1978; Schmidt et al., 1981). Second, the forces inducing aggregation, which are not at all obvious for OG containing vesicles, would have to be strong enough to significantly reduce the tumbling rate of the vesicles. And third, for large unsonicated vesicles, the tumbling of the vesicles is too slow to produce spectral narrowing; i.e., beyond a certain radius (a few thousand angstroms) the spectral width no longer increases and is determined solely by the internal motions of the phosphate group (McLaughlin et al., 1975; Burnell et al., 1980). The larger the vesicles, then, the less sensitive the ^{31}P NMR spectrum to effects that decrease the effective tumbling rate. We therefore conclude that the vesicles are undergoing a size transformation during the latter part of stage I.

Stage II. At an R_{eff} value of about 1.5 the aqueous detergent concentration is approximately equal to the cmc (data not shown), and large changes in the turbidity, in the sedimentation of the PC, in the DPH fluorescence anisotropy, and in the ^{31}P NMR spectra begin to occur. These changes indicate that detergent-saturated mixed bilayers are being converted into small mixed micelles. Since the micelles do not scatter appreciable amounts of light, the turbidity decreases (Figure 3). The micelles also do not sediment at a measureable degree under the conditions used, so solubilized PC appears in the supernatant (Figure 4). In the past, sedimentation experiments have been the only effective method for determining the percent solubilization for biological or model membranes. We have determined that ^{31}P NMR can be used for this purpose due to the slow bilayer–micellar exchange of the PC on the ^{31}P NMR time scale (Figures 6 and 7). The agreement between the two methods is excellent (Figure 4). The NMR method has several advantages, primarily because it does not require the physical separation of the two phases. Titration experiments are thus possible, which eliminates error due to the preparation of multiple samples and reduces the total amount of material and time needed. Possible artifacts such as the sedimentation of micelles or monomers trapped within bilayers, or the incomplete sedimentation of smaller mixed bilayer structures, are also eliminated. The time per NMR spectrum is usually less than or comparable to that needed for a centrifugation experiment, and additional time is saved because chemical analyses are not necessary. In terms of future applications to phospholipid mixtures and biological membranes, it has been shown that the common phospholipids give resolvable ^{31}P resonances in the micellar state (London & Feigenson, 1979), so the need for laborious chromatography to quantitate the solubilization of specific lipids is eliminated.

The DPH fluorescence anisotropy results (Figure 2B) indicate that the motion of this probe in the micellar phase is less restricted than it is when in the bilayer phase. Reduced ^{1}H and ^{13}C NMR line widths in the micellar state have also, in the past, been taken as an indication that Triton–PC mixed micelles are more disordered than PC bilayers (Ribeiro & Dennis, 1975, 1976). We find that this is also the case for OG–PC mixed micelles (data not shown). However, the line width comparisons, are made, by necessity, with small sonicated vesicles, and the lack of quantitative knowledge about micellar size and structure and their effect, and the effect of overall aggregate tumbling, on NMR line widths (Gent & Prestegard, 1974; Schmidt et al., 1977; Lichtenberg & Zilberman, 1979) makes the rigorous interpretation of such results difficult.

The molar ratio of OG to PC in the pelleted PC vesicles throughout most of stage II was 1.29 ± 0.21; this value is in good agreement with the point at which stage II is initiated. Apparently at this ratio, the PC bilayer is saturated with OG, and the further addition of OG leads to disruption of the bilayer with subsequent formation of OG–PC mixed micelles. The linearity of the concentration of OG in the supernatant with increasing R_{eff} during stage II (not shown) indicates that the ratio of OG to PC in the mixed micelles remains constant. The effective OG to PC ratio in the supernatant $[([OG]_{sup} - 0.022)/[PC]_{sup}]$ for the 27.7 mM PC samples in a range of R_{eff} values of 1.84–2.86 was found to be 2.70 ± 0.12. The properties of the micelles would therefore be expected to remain constant during the bilayer to micelle conversion. However, if more than one nondetergent component were present in the bilayer phase, this may not be the case. Such experiments may provide useful information about interactions between membrane components.

Stage III. At R_{eff} values greater than about 3.0, only mixed micelles are present in the solutions. The parameters monitored in this study do not show measurable changes in the region. However, extensive studies on detergent–phospholipid mixed micelles, using other physical techniques (Yedgar et al., 1974; Mazer et al., 1980; Helenius & Simons, 1975; Robson & Dennis, 1978), do demonstrate changes in the structure and properties of mixed micelles as the detergent concentration increases.

In the preceding discussion, it has been implicitly assumed that the dispersions were at equilibrium during the course of the experiments. This is a potential problem in detergent–bilayer studies since multilamellar dispersions may require several days to equilibrate when deoxycholate is added after

[3] The conversion of the ^{31}P NMR spectral envelope, and verification of the R_{eff} values given for it, can be seen in the narrow sweep width spectra of Figure 7. At an R_{eff} value of 0.60 (Figure 7B), a small relatively broad (nonmicellar) peak appears, consistent with the narrowing of the spectral envelope seen in Figure 6B (R_{eff} = 0.23). As OG is added and R_{eff} increases, this peak broadens, as seen in Figure 7C (R_{eff} = 1.05). At an R_{eff} value of 1.4 it is no longer detectable, and the wide (5-kHz) sweep width spectrum (not shown) is similar to the broad part of the spectrum shown in Figure 6C. This indicates that the conversion of the original vesicles into the vesicles characterized by the broad ^{31}P NMR spectral envelope is complete before the conversion into mixed micelles begins.

vesicle preparation (Lichtenberg et al., 1979). However, for the large, unilamellar vesicles used in this study, the NMR spectral parameters were not significantly different from their initial values when remeasured at times up to 24 h after the addition of OG. In addition, experiments were performed in which dispersions in stage II were converted into stage I dispersions by dilution with water, which lowered the R_{eff} value. ^{1}H NMR spectra, completed within 2 min after the addition of water, were not only reproducible with time up to 4 h but also were very similar to spectra of samples with the same R_{eff} made by adding OG to vesicles. These experiments indicate that the dispersions used in this study reach equilibrium rapidly.

In summary, these studies serve to characterize the various stages of solubilization of large, unilamellar PC vesicles by the nonionic detergent octyl glucoside. The importance of using corrected detergent to lipid ratios $(R_{eff})^2$ for comparison of measurements carried out at different lipid concentrations has been demonstrated. In addition, ^{31}P NMR has been shown to be a simple and useful technique for following the conversion of detergent saturated bilayers into mixed micelles.

Acknowledgments

We thank Nancy Salomonsky for expert technical assistance with the electron microscopy studies.

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