Mechanisms of Membrane Protein Insertion into Liposomes during Reconstitution Procedures Involving the Use of Detergents. 1. Solubilization of Large Unilamellar Liposomes (Prepared by Reverse-Phase Evaporation) by Triton X-100, Octyl Glucoside, and Sodium Cholate†

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ABSTRACT: The mechanisms governing the solubilization by Triton X-100, octyl glucoside, and sodium cholate of large unilamellar liposomes prepared by reverse-phase evaporation were investigated. The solubilization process is described by the three-stage model previously proposed for these detergents [Lichtenberg, D., Robson, R. J., & Dennis, E. A. (1983) *Biochim. Biophys. Acta 737*, 285-304]. In stage I, detergent monomers are incorporated into the phospholipid bilayers until they saturate the liposomes. At that point, i.e., stage II, mixed phospholipid–detergent micelles begin to form. By stage III, the lamellar to micellar transition is complete and all the phospholipids are present as mixed micelles. The turbidity of liposome preparations was systematically measured as a function of the amount of detergent added for a wide range of phospholipid concentrations (from 0.25 to 20 mM phospholipid). The results allowed a quantitative determination of R^{Sat}, the effective detergent to lipid molar ratios in the saturated liposomes, which were 0.64, 1.3, and 0.30 for Triton X-100, octyl glucoside, and sodium cholate, respectively. The corresponding ratios in the mixed micelles, R^{Sol}, were 2.5, 3.8, and 0.9 mol of detergent/mol of phospholipid. The monomer concentrations of the three detergents in the aqueous phase were also determined at the lamellar to micellar transitions (0.18, 17, and 2.8 mM, respectively). These transitions were also investigated by ^{31}P NMR spectroscopy, and complete agreement was found with turbidity measurements. Freeze-fracture electron microscopy and permeability studies in the sublytic range of detergent concentrations indicated that during stage I of solubilization detergent partitioning between the aqueous phase and the lipid bilayer greatly affects the basic permeability of the liposomes without significantly changing the morphology of the preparations. A rough approximation of the partition coefficients was derived from the turbidity and permeability data (K = 3.5, 0.09, and 0.11 mM^{-1} for Triton X-100, octyl glucoside, and sodium cholate, respectively). It is concluded that when performed systematically, turbidity measurements constitute a very convenient and powerful technique for the quantitative study of the liposome solubilization process by detergents.

Reconstitution of integral membrane proteins into unilamellar phospholipid vesicles to form functioning membranes provides a powerful tool in membrane research. Although many techniques have been used to reconstitute biologically active proteoliposomes, one of the most useful methods involves the use of detergents [for reviews, see Racker (1979) and Eytan (1982)]. Purified membrane proteins are first solubilized with detergents in the presence of phospholipids to give an isotropic solution of mixed phospholipid–protein–detergent micelles. Then the detergent concentration is lowered by dialysis, gel filtration, dilution or adsorption (Møller et al., 1986). As this concentration decreases to a critical level, the proteins spontaneously associate with the lipids to form proteoliposomes. Protein insertion occurs by one of two mechanisms (Eytan, 1982): (1) The protein simply participates in the membrane formation process, which corresponds to the phase transition from an isotropic micellar solution to a lamellar phase, or (2) liposomes are first formed by partial detergent removal and only after further removal is the protein inserted into the preformed liposomes. As pointed out by Eytan (1982), the mechanism by which protein is incorporated seems to be a key factor in determining the final orientation of proteins in the reconstituted proteoliposomes. Although there are indications that the rate of detergent removal (Eytan, 1982) as well as the state of aggregation of the protein under study (Helenius et al., 1981) may be involved, very little is known about the factors determining the mechanisms of protein incorporation into proteoliposomes. The results presented in this and the accompanying paper constitute an initial attempt to improve understanding of how proteins are inserted into liposomes during detergent-mediated reconstitution procedures. The protocol employed is based on the idea that such reconstitution procedures represent the reverse of membrane–detergent solubilization (Helenius & Simons, 1975): Accordingly, detergents are first added to preformed liposomes through all the range of detergent addition that causes the transformation of lamellar structures into mixed micelles. The protein, which has to be reconstituted, is then added, and its incorporation can be suitably studied in each step of the lamellar to micellar transition.

In this paper we report our findings for the solubilization by different detergents of large unilamellar liposomes prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978; Rigaud et al., 1983a,b). This model system was chosen because it avoids problems associated with highly curved sonicated vesicles (Gaber & Peticolas, 1977; Lichtenberg et al., 1981). We have analyzed the effects on physical properties of these large liposomes of the three most commonly used detergents in solubilization and reconstitution studies (Triton X-100, sodium cholate, and octyl glucoside). The methods

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used include turbidity measurements, magnetic resonance spectroscopy, freeze-fracture electron microscopy, and permeability determinations. The results of these studies are related to the three-stage model describing the interactions of detergents with biological and model membranes and analyzed in light of recent reviews on phospholipid solubilization by detergents (Lichtenberg et al., 1983; Lichtenberg, 1985). For a given concentration of liposomes, three stages in the solubilization process are apparent, depending on the nature and the concentration of the detergent: (I) partitioning of nonmicellar detergent between the aqueous medium and the lipid bilayer; (II) gradual solubilization of lipid, resulting in the coexistence of mixed micelles of lipid and detergent and liposomes saturated with detergent; (III) complete solubilization of lipids, which are now present as mixed micelles.

In the following paper we show how these changes in the physical properties of liposomes affect the incorporation of the prototypic transmembrane protein, bacteriorhodopsin.

Materials and Methods

Materials. Phosphatidylcholine (EPC)[1] was extracted from egg yolk according to the method of Singleton et al. (1965). Phosphatidic acid was prepared from the former as described by Allgyer and Wells (1979).

Triton X-100 was purchased from Merck and *n*-octyl β-D-glucopyranoside (octyl glucoside) from Sigma. Cholic acid (Prolabo) was recrystallized as described by Kagawa and Racker (1971). [*phenyl*-^{3}H]Triton X-100 (1.3 mCi/mg) was obtained from New England Nuclear. Pyranine and 6-carboxyfluorescein came from Eastman Kodak and 9-aminoacridine from Sigma. Bacteriorhodopsin was isolated from *Halobacterium halobium* according to the method of Oesterhelt and Stoeckenius (1974).

Preparation of Vesicles. Large unilamellar vesicles of defined size were prepared by reverse-phase evaporation as described previously (Rigaud et al., 1983a,b) using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio 9:1). Buffers used were 20 mM Pipes–KOH or 20 mM KH_2PO_4·KOH, pH 7.2, supplemented with 110 mM K_2SO_4. The vesicle suspension was sequentially extruded through 0.4 and 0.2 μm polycarbonate membranes (Nucleopore) to obtain a uniform size distribution (Szoka et al., 1980; Rigaud et al., 1983a,b). The final phospholipid concentration was about 16 mg/mL (i.e., 20 mM). Small unilamellar vesicles were prepared by sonicating the large vesicles for 30 min under argon at 4 °C. Multilamellar vesicles were prepared by drying the phospholipids (16 mg of phospholipid) under high vacuum, hydrating with 1 mL of the appropriate buffer, and vortex mixing for 5 min. Liposomes were used within 2.3 days without significant phospholipid oxidation and gave similar results.

Turbidity Measurements. The turbidity of phospholipid vesicle suspensions as a function of the detergent concentration was measured between 460 and 700 nm with a Beckmann (Acta III) spectrophotometer. The vesicles were suspended at concentrations ranging from 1.25 to 20 mM phospholipid. The detergent concentration was raised in increments, and absorbance was continuously recorded until a steady-state level was reached. For lower phospholipid vesicle concentrations (below 1.25 mM phospholipid), light-scattering changes were also monitored with a Perkin-Elmer MPF 44A spectrofluorometer set at 460 nm in both excitation and emission monochromators in order to measure the relative changes in light at 90°.

^{31}P NMR Spectroscopy. NMR measurements were performed with a Bruker MSL 300 Fourier transform spectrometer operating at 121.4 MHz for ^{31}P with continuous broadband ^1H decoupling and a probe temperature set at 25 °C. To quantitate the amount of phospholipid solubilized, we used 90° pulses, an acquisition time of 0.684 s, and a 15-s interval between acquisitions (because of the long relaxation time of the ^{31}P nucleus); 200 scans and 1-Hz line broadening were used.

The measurements were performed with vesicle suspensions (12.5 mM phospholipid) in 20 mM Pipes and 110 mM K_2SO_4, pH 7.2, in the presence of 20% D_2O (in order to lock the spectrometer on the D_2O signal), 0.05 mM EDTA, and 6 mM inorganic phosphate (to provide an internal reference).

Freeze-Fracture Electron Microscopy. For freeze-fracture electron microscopy, the samples were cryoprotected with 30% glycerol (w/w) and rapidly frozen in liquid propane by using conventional Balzer's gold planchettes. The fracturing was performed at −150 °C with a nitrogen-cooled knife. The replication of the fractured surfaces was performed by using platinum/carbon shadowing. The replicas were observed in a Philips 301 electron microscope.

Release of Carboxyfluorescein. Vesicles were prepared as described above in a buffer supplemented with 10 mM carboxyfluorescein. After extrusion through calibrated polycarbonate membranes, vesicles were freed of unencapsulated material by two passages through small Sephadex G-25 columns (PD 10; Pharmacia). Small aliquots of the vesicle suspension were added to a quartz cuvette filled with buffered solutions containing different detergent concentrations. The cuvette was preequilibrated and stirred in a Perkin-Elmer MPF 44 fluorometer (λ_{ex} 492 nm; λ_{em} 512 nm). The initial rates of fluorescence changes (i.e., carboxyfluorescein release) were then measured.

Light-Induced Proton Movements in Reconstituted Bacteriorhodopsin Liposomes. Liposomes containing bacteriorhodopsin were prepared by reverse-phase evaporation as described previously (Rigaud et al., 1983b). Actinic illumination of the resulting reconstituted proteoliposomes induced a net proton uptake (Seigneuret & Rigaud, 1986b) that can be assayed by taking the changes in 9-aminoacridine fluorescence as the index of transmembrane pH gradient generation. Excitation was performed at 400 nm and emission recorded at 455 nm. Illumination of bacteriorhodopsin was performed with an Intralux 6000 xenon lamp equipped with a heat filter and a high-pass filter (550 nm < λ < 800 nm) through a flexible light guide whose aperture was positioned above the quartz cuvette.

Results

Turbidity and Ultrastructural Studies. The turbidity of preformed vesicle samples prepared by reverse-phase evaporation was measured as a function of the detergents added as a means of visualizing the solubilization process. Figure 1A depicts the changes in the turbidity of aqueous suspensions containing a fixed concentration of EPC/EPA liposomes (1 mg of phospholipid/mL i.e., 1.25 mM) and various concentrations of Triton X-100 (trace a), octyl glucoside (trace b), or sodium cholate (trace c). In this figure, turbidity was recorded as the optical density at 500 nm, although very similar

[1] Abbreviations: EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; OG, octyl β-D-glucoside; TX 100, Triton X-100; Pipes, 1,4-piperazinediethanesulfonic acid; cmc, critical micelle concentration; 9AA, 9-aminoacridine; 6CF, 6-carboxyfluorescein; NMR, nuclear magnetic resonance; R_{eff}, effective molar ratio of detergent to phospholipid; R^{Sol}, R_{eff} at complete solubilization; R^{Sat}, R_{eff} at the onset of the lamellar/micelle transition; EDTA, ethylenediaminetetraacetic acid.

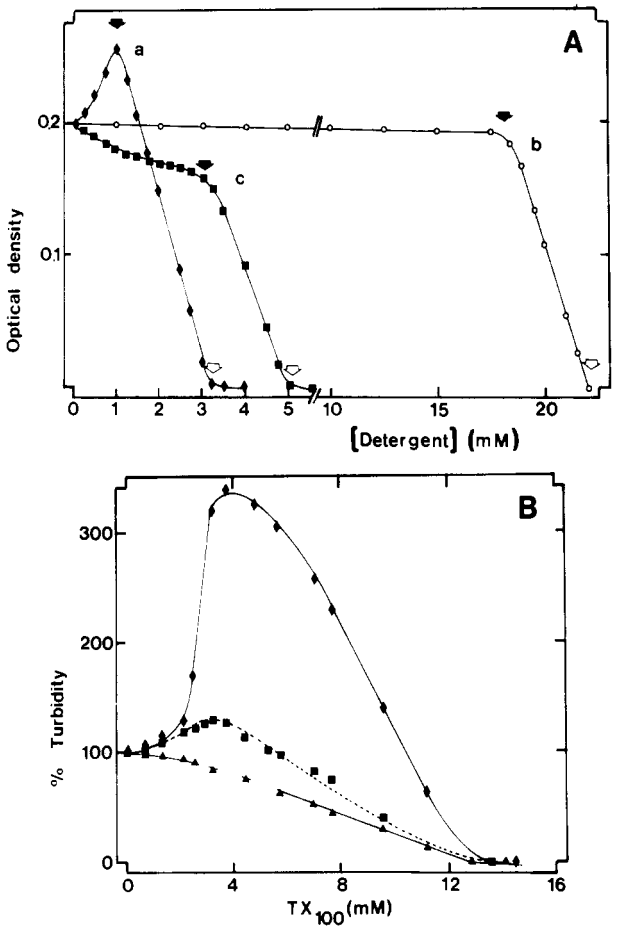

FIGURE 1: Changes in turbidity of liposome suspensions upon addition of various detergents. (A) Liposomes prepared by reverse-phase evaporation were resuspended at a concentration of 1.25 mM in a spectrophotometer cell. Then detergents from concentrated stock solutions were added stepwise to the liposome suspension under constant stirring at room temperature. The turbidity was measured at 500 nm after detergent equilibration. (Trace a, ◆) Triton X-100; (trace b, ○) octyl glucoside; (trace c, ■) sodium cholate. Black and white arrows denote the threshold detergent concentrations for the onset and total liposome solubilization, respectively. (B) Percent changes in turbidity of liposomes prepared by reverse-phase evaporation (■), sonicated liposomes (◆), and multilamellar liposomes (▲) in the presence of various Triton X-100 concentrations. Total phospholipid concentration in all samples 5 mM (100%, turbidity in the absence of detergent).

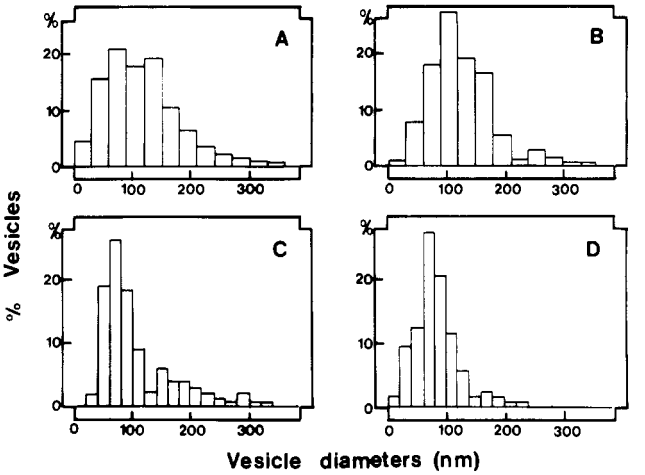

FIGURE 2: Size distribution histograms of liposomes treated by saturating detergent concentrations. Liposomes prepared by reverse-phase evaporation (10 mM phospholipid) were treated with 6.5 mM Triton X-100, 30 mM octyl glucoside, or 5.5 mM sodium cholate. After detergent elimination, the samples were analyzed by freeze-fracture electron microscopy as described under Materials and Methods. Size distribution histograms of untreated liposomes (A) and liposomes treated by saturating concentrations of TX 100 (B), cholate (C), or octyl glucoside (D). Bars are 30 nm in (A) and (B) and 20 nm in (C) and (D).

results were recorded between 400 and 700 nm (data not shown). Previous studies of the interactions of detergents with biological and model membranes (Jackson et al., 1982; Lichtenberg, 1985; Goni et al., 1986) indicated that the complex curves in Figure 1A can be interpreted in terms of a "three-stage" model. Stage I involves partitioning of nonmicellar detergent between the aqueous medium and the lipid bilayer. During this stage (up to the detergent concentrations denoted by black arrows) detergent incorporation into the bilayers induced turbidity changes whose behaviors depended upon the nature of the detergent: for up to 1 mM TX 100, turbidity increased; for up to 3.2 mM sodium cholate, it diminished slightly, and for up to 18 mM octyl glucoside, it did not change significantly. Stage II is marked by a gradual solubilization of lipid, resulting in coexistence of lipid–detergent mixed micelles and lipid bilayers saturated with detergent. For all three detergents the disruption of large liposomes with subsequent formation of mixed micelles of much lower molecular weight greatly reduced turbidity. Stage III is characterized by complete solubilization of lipids. The liposomes were completely converted into mixed micelles when the suspensions became optically transparent. At this point (denoted by white arrows in Figure 1A) excess detergent had no effect on the turbidity of the suspensions. Total solubilization was obtained for TX 100, OG, and cholate concentrations of 3.3, 22, and 5 mM, respectively.

For comparison, the changes in turbidity of multilamellar suspensions and sonicated liposomes caused by the addition of Triton X-100 are shown in Figure 1B. Although the detergent concentrations at which the three phospholipid solutions were completely clear are identical, the behavior of turbidity in stage I was extremely dependent on the mode of preparation of the liposomes. Thus, in stage I, the turbidity of large liposomes increased 1.3-fold as the TX 100 concentration rose and that of sonicated liposomes 3–4-fold; on the other hand, the turbidity of the multilamellar liposomes diminished. Very similar results were obtained (data not shown) when TX 100 was replaced by octyl glucoside or sodium cholate, with in particular a considerable increase in the turbidity of sonicated liposomes at subsolubilizing detergent concentrations. It has been clearly shown in sonicated liposomes (Alonso et al., 1981, 1982; Enoch & Strittmatter, 1979) that this great change in turbidity was correlated with massive vesicle aggregation and/or fusion. We therefore tested these findings in our large liposomes by freeze-fracture electron microscopy. Liposomes prepared by reverse-phase evaporation consisted of large unilamellar vesicles with less than 2% multilamellar structures (Gulik-Krzywicki et al., 1987). Figure 2A, showing a size distribution histogram of one such preparation, indicates a relatively broad size distribution yielding an average diameter of 150 nm.[2] The morphology and the size distribution of the liposomes did not change significantly after treatment with

[2] Since the cleavage plane during the fracture was expected to occur randomly through the liposomes, the resulting frequency distribution would be shifted to smaller profiles compared to the diameter of whole liposomes. However, we estimate that the correction for this artifact must be relatively small because of the good agreement between diameters determined by freeze-fracture, dynamic light scattering or calculated from the entrapped aqueous space (Rigaud et al., 1983; Szoka et al., 1980). The data presented therefore assume that the cleavage plane around each liposome is located close to the equatorial position.

subsolubilizing TX 100 or cholate concentrations (parts B and C of Figure 2). Only preparations treated with subsolubilizing octyl glucoside concentrations (Figure 2D) form a slightly smaller, more uniform population of liposomes whose mean diameter was about 100 nm (fewer than 10% of the liposomes had a diameter above 150 nm). These morphological observations rule out the possibility that, in the present large liposomes, changes in turbidity observed in stage I were due to massive changes in their size or organization unlike what was reported for sonicated liposomes. Consequently, the changes that occurred in light scattering after the addition of subsolubilizing detergent concentration may conceivably be caused by slight changes in vesicle size or liposome aggregation not detected by electron microscopy or by changes in the refractive index of water and/or the membranes after detergent incorporation (Yoshikawa et al., 1983).

Since in our study detergents were added externally to preformed liposomes, it was essential to establish whether the detergents were completely equilibrated in our bilayer preparations. Changes in turbidity induced when TX 100, OG, or cholate was externally added to preformed liposomes preparations were relatively fast: It takes at least 2–3 min to reach equilibrium, and turbidities were not significantly altered after 4–5 h. These experiments, in agreement with the observations of Jackson et al. (1983) for octyl glucoside, indicate that, after detergent addition, the suspensions of the large unilamellar vesicles rapidly reach equilibrium as opposed to the behavior of multilamellar liposomes (Lichtenberg et al., 19799; Goni et al., 1986).

Effect of the Phospholipid Concentration. Characterization of the Solubilization Process. The data available on the solubilization of phospholipids by detergents have been analyzed through two important parameters, R^{Sat} and R^{Sol}. According to the nomenclature adopted by Lichtenberg and collaborators (Lichtenberg et al., 1983; Lichtenberg, 1985) R^{Sat} and R^{Sol} respectively correspond to the effective detergent to phospholipid molar ratios in detergent-saturated liposomes and in mixed micelles. One approach to the derivation of these critical ratios is based on the study of the dependence of the critical detergent concentrations at which the lamellar to micellar transformation begins and is complete on phospholipid concentrations. This is why the turbidity measurements were conducted at various phospholipid concentrations. The results with TX 100, OG, and cholate are shown in Figures 3A, 4, and 5, respectively, for phospholipid concentrations ranging from 1.25 to 10 mM phospholipid. One of the most striking features of the curves obtained was observed with octyl glucoside: With this detergent a very abrupt burst in turbidity occurred during stage II, i.e., during the lamellar to micellar transformation. This new peak of turbidity was clearly observed for phospholipid concentrations above 2 mM, and its width increased with phospholipid concentration. With Triton X-100, a similar increase in turbidity during stage II was also observed but at phospholipid concentrations above 10 mM (see Figure 7A). With sodium cholate no increase was observed, even at 20 mM phospholipid, the highest concentration studied. The origin and the nature of this peak is being studied separately (Paternostre and Rigaud, unpublished results). It seems likely that this sharp change in turbidity represents an aggregate state of the kind described by Fu and Laughlin (1980). According to these authors, this state might be reached by micellar adsorption at a liposome surface: It must resemble a variety of colloidal phenomena in which two hydrophilic particles interact, leading to aggregation especially at high phospholipid concentration. Besides the influence of phos-

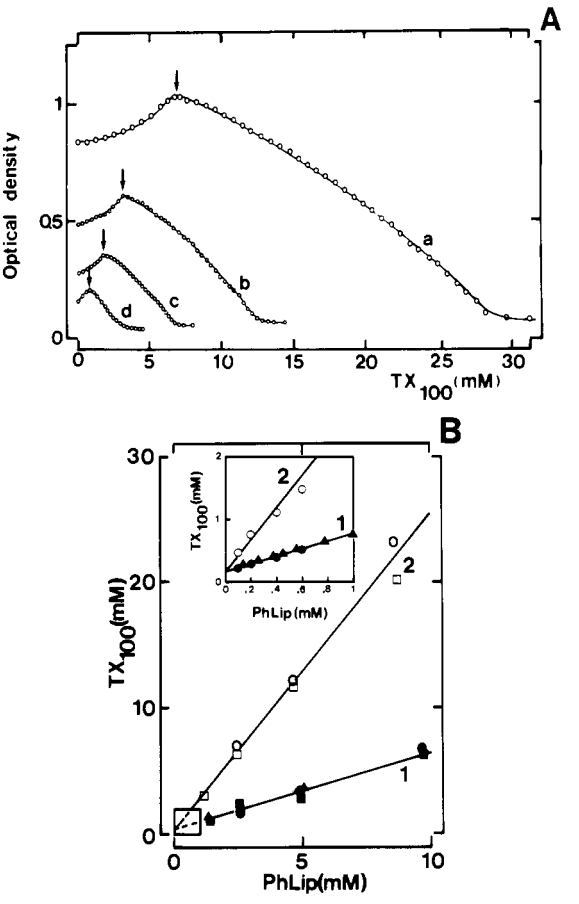

FIGURE 3: Triton X-100 to phospholipid relationships describing the solubilization process of liposomes prepared by reverse-phase evaporation. (A) Turbidity changes induced by stepwise addition of TX 100 to liposome suspensions containing 10 (a), 5 (b), 2.5 (c), or 1.25 mM (d) phospholipid. Arrows indicate the onset of solubilization at each phospholipid concentration. (B) Total TX 100 concentrations corresponding to the onset (filled symbols, trace 1) and complete liposome solubilization (open symbols, trace 2) as a function of the total phospholipid concentrations. Such plots are derived from data in (A) and other similar experiments taking into account the dilution factor due to the detergent addition. (Inset) Phospholipid concentration range up to 1 mM (light-scattering measurements). Different symbols refer to different experiments.

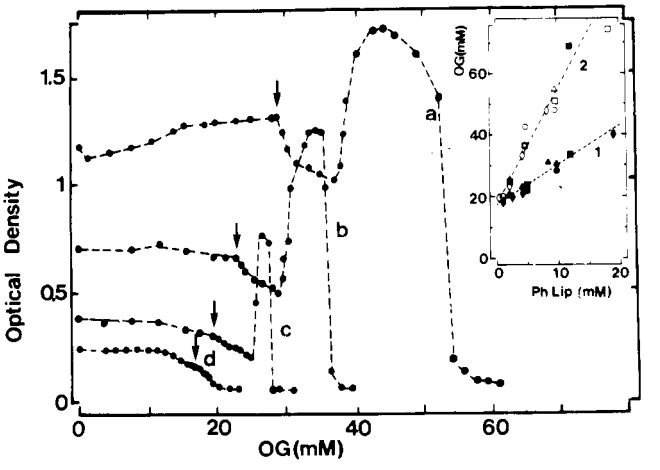

FIGURE 4: Octyl glucoside to phospholipid relationships describing the solubilization process of liposomes prepared by reverse-phase evaporation. Turbidity changes induced by stepwise addition of OG to liposome suspensions containing 10 (a), 5 (b), 2.5 (c), or 1.25 mM (d) phospholipid. Arrows indicate the onset of solubilization at each phospholipid concentration. Note the presence of a large turbidity drop during the solubilization process. (Inset) Total OG concentration corresponding to the onset (filled symbols, trace 1) and complete liposome solubilization (open symbols, trace 2) as a function of the total phospholipid concentrations.

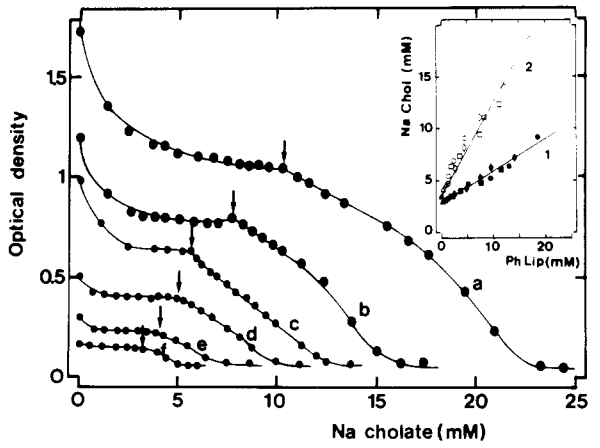

FIGURE 5: Sodium cholate to phospholipid relationships describing the solubilization process of liposomes prepared by reverse-phase evaporation. (A) Turbidity changes induced by stepwise addition of cholate to liposome suspensions containing 20 (a), 12.5 (b), 10 (c), 5 (d), 2.5 (e), or 1.25 mM (f) phospholipid. Arrows indicate the onset of solubilization at each phospholipid concentration. (Inset) Total cholate concentration corresponding to the onset (filled symbols, trace 1) and complete liposome solubilization (open symbols, trace 2) as a function of the total phospholipid concentrations.

pholipid concentrations, the purity of the lipids from which the liposomes are prepared has been shown to be important for the existence of this aggregation state that is abolished by the presence of lysoderivatives, oxidized phospholipids, or traces of other detergents (data not shown).

In order to calculate the critical effective R^{Sat} and R^{Sol} ratios from Figures 3A, 4, and 5, the detergent concentrations corresponding to the onset of solubilization (curves 1) and to total solubilization (curves 2) were plotted versus the total phospholipid concentrations for TX 100, OG, and cholate (Figure 3B and insets of Figures 4 and 5). Striking linear relationships were obtained with the three detergents. The equation describing curves 1

$$[\text{detergent}] = D_w + R^{Sat}[\text{phospholipid}]$$

in which the brackets refer to the total concentration expressed in millimoles per liter, gives the detergent concentration needed to saturate phospholipid membranes in any given liposome sample. From the slopes of curves 1 in Figure 3B and in the insets of Figures 4 and 5, the saturation levels of detergents bound to the membrane (R^{Sat}) were found to be 0.64, 1.3, and 0.30 mol of detergent/mol of phospholipid for TX 100, OG, and cholate, respectively. Extrapolations of curves 1 to zero phospholipid concentrations gave D_w values of 0.18, 17, and 2.8 mM for the respective detergents, which may be interpreted to be the aqueous monomer detergent concentrations in equilibrium with the saturated liposomes. Similarly, the slopes of curves 2 may be interpreted as the molar detergent to phospholipid ratios, R^{Sol}, in the mixed micelles. The values of R^{Sol} were 2.5, 3.6, and 0.9 mol of detergent/mol of phospholipid for TX 100, OG, and cholate, respectively. Again, extrapolation of curves 2 to zero phospholipid concentrations (0.18, 17, and 3.7 mM for the respective detergents) gave the aqueous detergent concentrations in equilibrium with the saturated micelles.[3]

NMR Measurements. Since as explained above the changes in turbidity of liposome suspensions upon detergent addition

[3] Similar turbidity measurements were performed with dodecyl octa(oxyethylene) ether ($C_{12}E_8$) and gave values of 0.7 and 2.6 mol of detergent/mol of phospholipid for R^{Sat} and R^{Sol}, respectively, D_w being equal to 0.2 mM (data not shown).

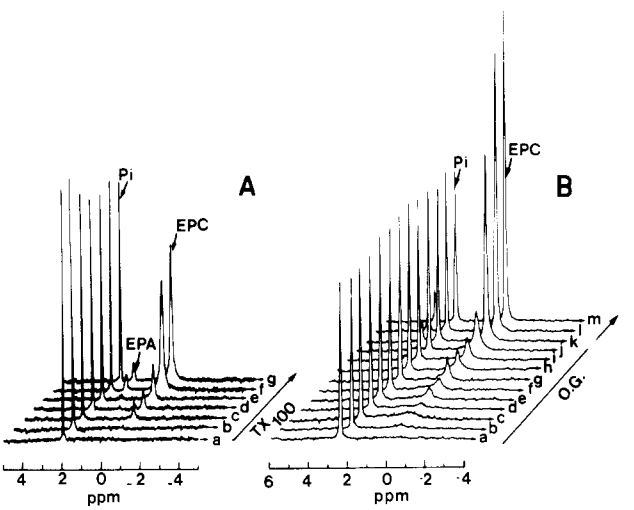

FIGURE 6: ^{31}P NMR spectra of liposomes in the presence of different amounts of Triton X-100 and octyl glucoside. (A) ^{31}P spectra of liposomes prepared by reverse-phase evaporation treated by different TX 100 concentrations: (a) 0, (b) 8, (c) 9, (d) 10.5, (e) 15.9, (f) 53, and (g) 100 mM TX 100. Liposome concentration in all samples was 12.5 mM. Inorganic phosphate (P_i) was included as a reference, and the ppm was given by the resonance of H_3PO_4. (B) ^{31}P NMR spectra of liposomes prepared by reverse-phase evaporation treated by different OG concentrations: (a) 0, (b) 10, (c) 13, (d) 20, (e) 32.5, (f) 38.5, (g) 42.5, (h) 45, (i) 50, (j) 61, (k) 72.5, (l) 95, and (m) 118 mM octyl glucoside.

might be caused by many factors other than liposome–micelle transitions, we used ^{31}P NMR to document the solubilization process. This method was recently used to follow the solubilization of phospholipids in biological and membrane models (Roux & Champeil, 1984; Jackson et al., 1982; Goni et al., 1986; Bayerl et al., 1986). In particular, it allows a clear distinction to be made between phospholipids in micelles and in vesicles (Dennis & Plückthun, 1984; London & Feigenson, 1979).

Figure 6A shows a few representative spectra when TX 100 is added stepwise to a suspension of EPC/EPA liposomes at 12.5 mM phospholipid. Inorganic phosphate (P_i) is also included together with the liposomes in order to provide an internal reference. In the absence of detergent, only the narrow resonance signal of P_i is visible since the broad ^{31}P resonance of the phospholipid head groups is not visible under the conditions of these scans (3000-Hz sweep width). The 30-kHz sweep width spectrum for liposomes prepared by reverse-phase evaporation, with a broad width of about 2000 Hz at half its total height, reflects the large size of the liposomes under study (data not shown). In the presence of TX 100, no significant changes appeared in the ^{31}P NMR spectra up to a concentration of 8 mM. Above this concentration, the inclusion of detergent in the lipid suspension resulted in the appearance of two narrow (15-Hz width) and symmetrical peaks, characteristic of phospholipids in mixed micelles. These two new peaks, with chemical shifts from external H_3PO_4 of −0.4 and 1.4, corresponded respectively to phosphatidylcholine and phosphatidic acid molecules in the micelles. The percentage of solubilized phospholipid was estimated by comparing the integral of the micellar phosphatidylcholine ^{31}P signal (area of the peak) to the integral of the P_i signal and was plotted (Figure 7A) as a function of added Triton X-100. The solubilization started at about 8.5 mM detergent (R^{Sat} = 0.64) and was virtually complete at about 33 mM detergent (R^{Sol} = 2.5), in perfect agreement with the values estimated from the turbidity measurements reported in the same figure (see also previous results). The amounts of phospholipid solubilized

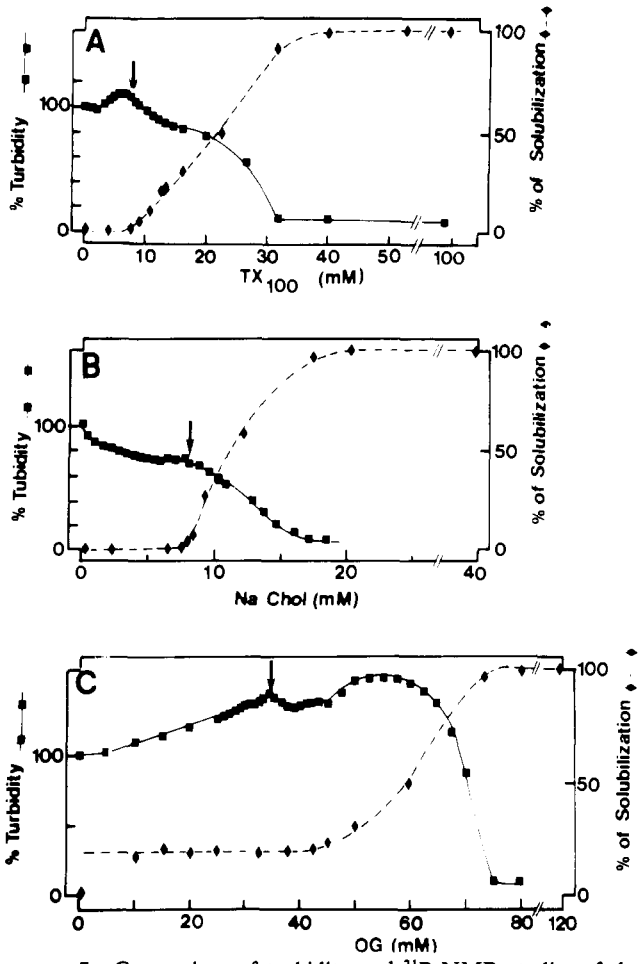

FIGURE 7: Comparison of turbidity and ^{31}P NMR studies of the solubilization process of liposomes by Triton X-100, octyl glucoside, and cholate. (◆) Solubilized phospholipid was estimated by comparing the integral of the micellar phospholipid ^{31}P signal (area of the EPC peak) to the integral of the P_i signal and expressed as percent of maximal values. (■) Turbidity changes of the same liposome suspensions as a function of added detergents (100% turbidity in the absence of detergent). Liposome concentrations in all samples was 12.5 mM. Black arrows indicate the onsets of solubilization. (A) Triton X-100 (same experimental conditions as in Figure 6A). (B) Sodium cholate. (C) Octyl glucoside (same experimental conditions as in Figure 6B).

upon cholate addition deduced from ^{31}P NMR spectra are plotted in Figure 7B versus the detergent concentration, together with the changes in turbidity measured under the same conditions. Again, the most striking feature of these results is the perfect agreement between the two techniques used to determine both the onset of phospholipid solubilization (corresponding to an R^{Sat} of 0.34) and the threshold value at which all the bilayers are converted into mixed micelles (R^{Sol} of 0.9).

The situation appeared more complicated when liposomes were treated with octyl glucoside. Indeed, when this detergent was added at very low concentrations (10 and 13 mM), a narrow peak indicative of isotropic phospholipid motion appeared in the ^{31}P NMR spectrum (Figure 6B, traces b and c). It is important to note that no phospholipid solubilization, i.e., formation of mixed micelles, was expected at these OG concentrations either from our turbidity measurements or from other studies (Jackson et al., 1982). This narrow ^{31}P NMR peak, which is superimposed on the broad anisotropic signal, is most probably due to the formation of smaller vesicles upon addition of OG to phospholipid suspensions, an interpretation corroborated by our electron microscopic observations reported in Figure 2. The same spectra were obtained when unperturbed liposomes were sized through nucleopores of 0.05 μm, which yielded liposome size distributions similar to those obtained in the presence of small amounts of OG (data not shown).

The appearance of this superimposed isotropic peak (about 30-Hz width) at subsolubilizing detergent concentrations makes it difficult to determine precisely the onset of phospholipid solubilization by octyl glucoside, i.e., the appearance of the first mixed micelles. However, the behavior of the integral intensity of the sharp component relative to the integral of the P_i signal as a function of the OG concentrations (Figure 7C) indicated that solubilization started at detergent concentrations between 37.5 and 40 mM, compared to 35 mM as determined by the turbidity changes performed under the same conditions. On the other hand, NMR measurements indicated that solubilization was complete at 75 mM OG, a value similar to that required to obtain an optically transparent suspension.

Besides showing a very good agreement with turbidity measurements corresponding to the threshold values for the onset and total bilayer solubilization, our ^{31}P NMR measurements bring some information about the structure of the mixed micelles. The bandwidths of the isotropic signals observed at R^{Sat} when solubilization was complete (22.5, 16, and 6 Hz for TX 100, OG, and cholate, respectively) reflect the large difference in the size of the micelles of these three detergents. Further, although the solubilized phospholipid peaks had a constant integral above R^{Sat}, they became still narrower when the concentration was further raised. In the presence of an excess of detergent, the bandwidths became, respectively, 15, 6, and 5.5 Hz for the three detergents: This enhanced resolution might be related to the general reduction in the size of the phospholipid–detergent micelles (Roux & Champeil, 1984; Dennis, 1974).

Permeability Studies. It is generally admitted that at subsolubilizing concentrations detergents intercalate into the membrane, where they cause changes in its physical properties (Jackson et al., 1982; Schubert et al., 1986). An obvious consequence of such perturbations might be alterations in membrane permeability. We therefore investigated the abilities of the different detergents to modify the basic permeability of large liposomes.

We began by measuring the rate at which entrapped 6-carboxyfluorescein (6CF) leaked out of the liposomes upon detergent addition. High concentrations of the dye were trapped inside the liposomes: Under these conditions, the fluorescence of the dye was almost completely quenched. Upon leakage the dye was diluted and became highly fluorescent (Weinstein et al., 1976). The initial rates of 6CF fluorescence intensity increases were taken as an index of the changes in liposome permeability.

Next, we investigated the effects of the different detergents upon an active proton influx. To this end, liposomes containing a light-driven proton pump, bacteriorhodopsin, were reconstituted by reverse-phase evaporation. These proteoliposomes have essentially the same characteristics as pure liposomes (Rigaud et al., 1983; Seigneuret & Rigaud, 1986a,b). Upon their illumination, a transmembrane pH gradient (ΔpH, acidic inside) was generated whose amplitude was determined by the balance between active proton pumping and counteracting leakage process due to passive ion permeability: Increasing the passive permeability of the membrane was expected to reduce the amplitude of the steady-state ΔpH. Changes in 9-aminoacridine fluorescence intensity were monitored as an index of the light-induced transmembrane ΔpH generation

Table I: Determination of the Partition Coefficients (K) of Different Detergents between Liposomes and Aqueous Medium[a]

	Triton X-100			octyl glucoside			sodium cholate		
linear relationship describing	R_{eff} (mol/mol)	D_w (mM)	K (mM^{-1})	R_{eff} (mol/mol)	D_w (mM)	K (mM^{-1})	R_{eff} (mol/mol)	D_w (mM)	K (mM^{-1})
onset of solubilization	0.64	0.18	3.55	1.3	17	0.076	0.3	2.8	0.107
total inhibition of light-induced ΔpH	0.230	0.064	3.59	1.05	11	0.095	0.34	3	0.11
half-inhibition of light-induced ΔpH	0.160	0.046	3.47						
max rate of 6CF release	0.118	0.032	3.68						
half-max rate of 6CF release	0.055	0.016	3.43	1	9.1	0.11	0.11	0.86	0.13

[a] The effective detergent to phospholipid molar ratios [R_{eff} = [det]bilayer/[phospholipid] (mol/mol)] and the aqueous concentration of the monomeric detergent [D_w (mM)] were deduced respectively from the slope and the intercept at zero lipid concentration of the linear relationships relating the total detergent concentrations at which different degrees of permeability or onsets of solubilization occurred to the total phospholipid concentration (for experimental conditions, see legends in Figure 8). $K = R_{eff}/D_w$ = [det]bilayer/D_w[phospholipid] (mM^{-1}).

(Schuldiner et al., 1972). Typical 9AA responses of reconstituted bacteriorhodopsin proteoliposomes treated with various amounts of TX 100 are shown in the inset of Figure 8A. Because of the fluorescence quenching caused by the accumulation of the probe upon internal acidification, illumination of the vesicle suspension led to a decrease in 9AA fluorescence until a steady-state was obtained, whose amplitude ($\Delta F/F\%$) was dependent upon the detergent concentration. When the light was turned off, the fluorescence returned to its original level.

The decrease in the light-dependent proton-uptake capacity of bacteriorhodopsin proteoliposomes and the increase in the leak rate of 6-carboxyfluorescein with increasing TX 100 concentrations are illustrated in Figure 8A. A striking feature of the findings reported in this figure was that the leak rate of 6CF leveled off and the transmembrane ΔpH was abolished at TX 100 concentrations well below those necessary to saturate the liposomes with this detergent, as indicated by the turbidimetric changes measured under the same conditions.

At subsolubilizing detergent concentrations it is generally admitted (Jackson et al., 1982; Lichtenberg, 1985) that an equilibrium partition of detergent between the bilayer and the aqueous medium governs the incorporation of the detergent into the bilayers. For a mixture containing L mM phospholipid and D_T mM detergent (D_w in the aqueous medium and D_B in the bilayer) and assuming an equilibrium distribution of the detergent between the bilayer and the aqueous medium, a partition coefficient can be defined as

$$K = (D_B/L)/D_w$$

Since from the definition of the effective detergent to phospholipid ratio $R_{eff} = D_B/L$, it follows that $K = R_{eff}/D_w$. In order to determine K, a series of systematic experiments was conducted by varying the phospholipid concentration from 0.1 to 1 mM and studying the effects of increasing amounts of TX 100 on the release of 6CF and on the amplitude of the light-induced ΔpH in bacteriorhodopsin liposomes. The results are reported in Figure 8B: The subsolubilizing detergent concentrations (D_T) at which light-induced H^+ uptakes were either totally or half inhibited as well as the critical detergent concentrations at which 6CF was released at half or maximal rates were linearly related to the phospholipid concentration. Such linear dependencies can be described by the equation

$$D_T = D_w + R_{eff}[L]$$

Thus, K can be deduced either from the ratios of the slopes (R_{eff}) to the intercepts at zero lipid concentrations (D_w) of each straight lines (see Table I) or from the extrapolated intercepts of these curves with the lipid concentration axis. Findings reported in Table I as well as the constant intercept at −0.25 to −0.3 mM in Figure 8B indicated that a single partition coefficient, K = 3.5 mM^{-1}, could describe the distribution of

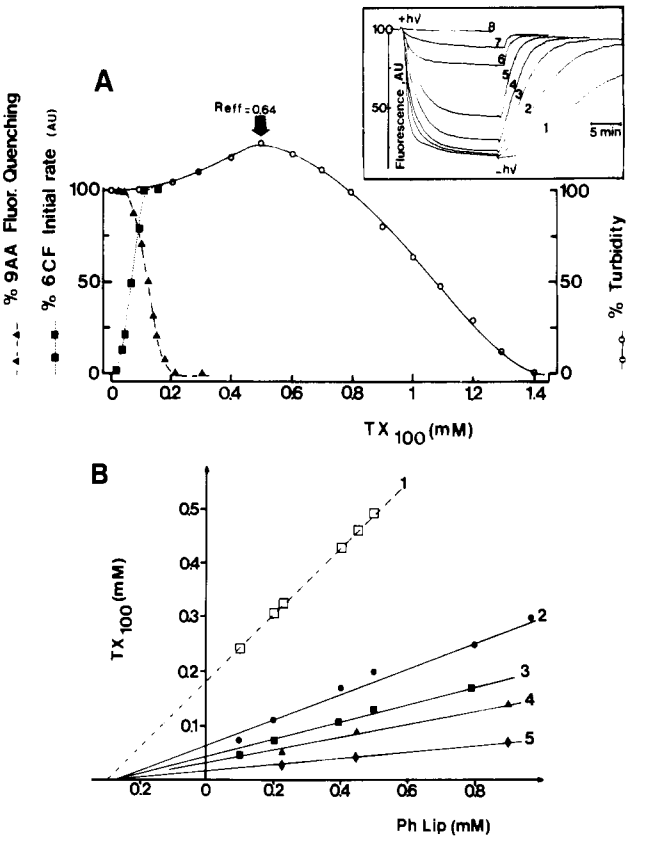

FIGURE 8: Permeability changes induced by subsolubilizing Triton X-100 concentrations. (A) (■) Rate of 6-carboxyfluorescein release from liposomes as a function of increasing amounts of TX 100 (100%, rate of 6CF release at the highest detergent concentration). (▲) Effect of TX 100 addition on the light-induced fluorescence quenching of 9-aminoacridine by bacteriorhodopsin-containing proteoliposomes (100%, 9AA fluorescence quenching in the absence of detergent). (Inset) Changes in 9AA fluorescence intensity upon illumination of bacteriorhodopsin liposomes treated by different amounts of TX 100: (1) 0, (2) 0.05, (3) 0.065, (4) 0.075, (5) 0.1, (6) 0.15; (7) 0.18, and (8) 0.22 mM Triton X-100. (○) Turbidity changes of the same liposome suspension as a function of added detergent. Total phospholipid concentration in all samples 0.5 mM. (B) Total TX 100 concentrations giving (a) total (●) and half inhibition (■) of the light-induced changes in 9AA fluorescence intensity of bacteriorhodopsin liposomes and (b) maximal (▲) and half-maximal (◆) rates of 6CF release as a function of total phospholipid concentrations. The dashed line (−□−) represents the TX 100 to phospholipid relationship describing the onset of liposome solubilization.

TX 100 between the liposomes and the aqueous medium. Interestingly, the partition coefficient was found not much altered over a wide range of detergent bound to the bilayers: Indeed similar K values were obtained for R_{eff} values between 0.64 and 0.055.

Similar linear relationships between the detergent concentrations at which changes in permeability occurred and

phospholipid concentrations were obtained under octyl glucoside or cholate treatment. The corresponding values of R_{eff}, D, and K for each detergent are summarized in Table I. It is worth noting that in the presence of TX 100 or OG changes in the basic permeability of the liposomes occurred at concentrations well below those necessary to saturate the bilayers, whereas for sodium cholate these changes occurred at concentrations only slightly below those necessary for such saturations. Finally, it is stressed that in the case of octyl glucoside the distribution coefficients appeared to vary slightly but significantly when R_{eff} values varied only from 1.3 to 1. Such an observation might indicate that at high detergent concentration bound to the membrane determination of K could be only approximate.

Conclusions

The results presented in this paper concern the interaction of detergents with large unilamellar liposomes prepared by reverse-phase evaporation. The detergents studied, Triton X-100, octyl glucoside, and sodium cholate, are those most commonly used in membrane solubilization, protein isolation, and reconstitution (Helenius & Simons, 1975; Racker, 1979). Furthermore, they offer a wide range of critical micellar concentrations and allow comparisons between ionic and nonionic detergents. In addition, large unilamellar liposomes provide an excellent model for the study of cell membrane solubilization and are more suitable for this purpose than small sonicated or large unsonicated multilamellar liposomes. Besides the heterogeneity of the preparations, the main problem connected with multilamellar phospholipid dispersions is the long time of turbidity equilibration upon detergent additions, lasting from 24 h to several days (Goni et al., 1986; Lichtenberg et al., 1979). In contrast, we showed here that the changes in turbidity observed upon detergent addition to large unilamellar liposomes are complete within a few minutes, in agreement with the observations of Jackson et al. (1983) for octyl glucoside. On the other hand, the small sonicated vesicles, due to their high curvature, exhibited a complex behavior in the presence of detergents. It has been reported that when TX 100, OG, and sodium cholate interact with sonicated liposomes at subsolubilizing concentrations, they induce important changes in the morphology and the size of the liposomes (Alonso et al., 1982; Enoch & Strittmatter, 1979). The detergents act as "wedges", disrupting the vesicle structure and thus releasing it from the lateral strain imposed by the high curvature of these small liposomes. These very unstable open vesicles display a natural tendency to join together and form larger vesicles. Our electron microscopy observations indicate that this is not the case for the large unilamellar liposomes used in this study since their morphological appearance and size distribution are not significantly affected by subsolubilizing detergent concentrations (see Figure 2).

In summary, the experimental data reported here are in line with the mechanisms proposed for detergent solubilization of other biological and model membranes, i.e., the transformation of lamellar structures into mixed micelles, in three distinct stages [for reviews, see Helenius & Simons (1975), Lichtenberg et al. (1983), and Lichtenberg (1985)]. In stage I, the detergents distributed between the phospholipid bilayers and the solution until such point as sufficient detergent has been added to saturate all the bilayers. At that point, i.e., stage II, additional detergents induce the formation of mixed micelles. Further detergent addition promotes gradual lipid solubilization, resulting in the coexistence of mixed lipid–detergent micelles and lipid bilayers saturated with detergent. Stage III is defined as that point where all the phospholipid bilayer phase has disappeared and only mixed detergent–phospholipid micelles are present.

One important general conclusion of our study is that when turbidity measurement is systematically performed as a function of the phospholipid concentration, it is a simple and useful technique not only for qualitative evaluation of the lamellar to micellar transitions but also for quantitative evaluation of the amounts of detergent bound either to the bilayer or to the micelles. According to Lichtenberg (1985) [see also Stubbs and Litman (1978)] it might be possible to calculate the critical effective detergent to phospholipid ratios at which the lamellar to micellar transition occurs and finishes from the linear dependence of the critical detergent concentration at which phase transformation occurs (D_T) over the lipid concentration [L]. The striking feature of our results is that such linear relationships were obtained from turbidity measurements for a wide range of phospholipid concentrations (from 0.2 to 20 mM) and for both the onset and completion of phospholipid solubilization by the three detergents studied. These linear dependencies can be described by the equation

$$D_T = D_w + R_{eff}[L]$$

where R_{eff} is the detergent to phospholipid molar ratio in the detergent-saturated bilayer ($R_{eff} = R^{Sat}$ at the onset of solubilization, i.e., when the turbidity started to decrease sharply) or in the mixed micelles ($R_{eff} = R^{Sol}$ at the total solubilization, i.e., for optically transparent solutions). Further, D_w represents the concentration of the monomeric detergent in equilibrium with either the saturated bilayer or the mixed micelles. The values for R^{Sol} deduced from the slopes of curves 1 in Figures 3B, 4, and 5 are 0.64, 1.3, and 0.30 mol of detergent/mol of lipid for TX 100, OG, and cholate, respectively. The corresponding values for R^{Sol} deduced from the slopes of curves 2 in the same figures are 2.5, 3.6, and 0.9. Our ^{31}P NMR measurements (^{31}P NMR is known to be a very accurate technique for determining the percent of solubilization for membranes) are in complete agreement with the turbidity measurements (Figure 7). Furthermore, the critical effective ratios, R^{Sat} and R^{Sol}, reported in this paper are also in agreement with the previously reported values measured by centrifugation, chromatography, and ^{31}P NMR studies (Jackson et al., 1982; Goni et al., 1986; Schubert et al., 1986; Dennis, 1974; Almog et al., 1986; Stubbs & Litman, 1978). The turbidity method has several advantages over sedimentation and ^{31}P NMR methods for quantitative determination of the solubilization process: It is time and material saving, it requires only a simple spectrophotometer, it permits the variation of phospholipid concentrations within a wide range, and it does not require the physical separation of the micelle and bilayers phases, thus eliminating the possible intrinsic artifacts of centrifugation techniques.

Another important result of the present studies is that extrapolation to zero lipid concentrations of the straight lines characterizing the dependence of D_T on the lipid concentration allows an accurate determination of the aqueous monomer detergent concentration in equilibrium with the saturated bilayer or the saturated micelles. Note that these concentrations, which were 0.18, 17, and 3 mM for TX 100, OG, and sodium cholate, respectively, are comparable to the critical micelle concentrations of these detergents (Møller et al., 1986; Helenius et al., 1979). Consequently, our experimental results support the generally admitted assumption (Lichtenberg, 1985) that the concentration of free detergent has to rise to the critical micelle concentration for solubilization to occur. Nevertheless, our results confirm previous observations (Andersen et al., 1983) that in the presence of phospholipid the

aqueous monomer concentrations are slightly but significantly lower than the critical micelle concentrations of these detergents determined in aqueous solutions. The correction is significant and must be taken into consideration when low phospholipid concentrations are studied or for detergents with high critical micelle concentration, such as OG.

A consequence of the perturbations induced by detergents is the large increase in liposome permeability observed at subsolubilizing concentrations. We believe that the real importance of the detergent-induced changes in the basic permeability of the liposomes illustrated in Figure 8 and Table I concerns the implications for detergent-mediated reconstitutions of membrane proteins. The results indicate that very small amounts of detergents may have drastic effects upon the basic permeability and therefore the transport efficiency of reconstituted proteoliposomes. Removal of traces of detergent is obviously an absolute necessity for valid transport studies in reconstituted proteoliposomes.

In addition, our detailed study of the effects of subsolubilizing detergent concentrations on the basic permeability of liposomes indicates that detergent interaction with the membrane in this stage can be described by assuming an equilibrium distribution of detergent between the bilayers and the aqueous phase. Partition coefficients have been derived from the linear dependencies of the critical detergent concentrations at which different degrees of liposome permeabilization occurred on the phospholipid concentration: $K = 3.5$, 0.09, and 0.11 mM^{-1} for TX 100, OG, and cholate, respectively. However, calculations should only be regarded as a rough estimate of the effective partition coefficient of the detergent (the mixing of lipids and detergents is not necessarily ideal, especially when the detergent concentration in the bilayer is not significantly lower than the phospholipid concentration). Nevertheless, these approximate values are comparable with those reported in the literature and measured by centrifugation techniques (Stubbs & Litman, 1978; Jackson et al., 1982; Schurtenberger et al., 1985; Almog et al., 1986). Furthermore, at least for TX 100, the calculated values of the partition coefficient are constant over a wide range of R_{eff} values (up to the onset of solubilization), indicating that at subsolubilizing concentrations the detergent intercalates into the membrane according to an equilibrium distribution, i.e., that the concentration of detergent in the bilayer phase is directly proportional to the concentration in the aqueous phase.

Another useful aspect of our results is that they allow the effects of the three detergents to be compared on the same preparation. For example, TX 100, OG, and cholate ruptured EPC/EPA liposomes at ratios of 0.64, 1.25, and 0.30, respectively, in spite of the large differences in critical micellar concentrations, which were 0.18, 18, and 3 mM. In other words, the detergent monomeric concentration does not correlate with its solubilizing power; the amount of detergent that a phospholipid bilayer can accommodate depends on the detergent–phospholipid interaction. As suggested by Lichtenberg (1985), interaction of cholate may involve the introduction of polar hydroxyl groups into the hydrophobic core of the bilayer, which might explain why relatively low amounts of cholate are necessary to transform lamellar structures into micellar ones.

In summary, the data presented in this paper define quantitatively the different steps in the solubilization of large liposomes by Triton X-100, octyl glucoside, and sodium cholate. In the following paper, the present experimental conditions and results will be used to gain insight into the mechanisms underlying protein reconstitution into functional proteoliposomes by means of detergent-mediated procedures.

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Mechanisms of Membrane Protein Insertion into Liposomes during Reconstitution Procedures Involving the Use of Detergents. 2. Incorporation of the Light-Driven Proton Pump Bacteriorhodopsin†

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ABSTRACT: A method has been developed for identifying the step in a detergent-mediated reconstitution procedure at which an integral membrane protein can be associated with phospholipids to give functional proteoliposomes. Large liposomes prepared by reverse-phase evaporation were treated with various amounts of the detergents Triton X-100, octyl glucoside, or sodium cholate as described in the preceding paper [Paternostre, M.-T., Roux, M., & Rigaud, J. L. (1988) *Biochemistry* (preceding paper in this issue)]. At each step of the solubilization process, we added bacteriorhodopsin, the light-driven proton pump from *Halobacterium halobium*. The protein–phospholipid detergent mixtures were then subjected to SM_2 Bio-Beads treatments to remove the detergent, and the resulting vesicles were analyzed with respect to protein insertion and orientation in the membrane by freeze-fracture electron microscopy, sucrose density gradients, and proton pumping measurements. The nature of the detergent used for reconstitution proved to be important for determining the mechanism of protein insertion. With sodium cholate, proteoliposomes were formed only from ternary phospholipid–protein–detergent micelles. With octyl glucoside, besides proteoliposome formation from ternary mixed micelles, direct incorporation of bacteriorhodopsin into preformed liposomes destabilized by saturating levels of this detergent was observed and gave proteoliposomes with optimal proton pumping activity. With Triton X-100, protein insertion into destabilized liposomes was also observed but involved a transfer of the protein initially present in phospholipid–Triton X-100–protein micelles into Triton X-100 saturated liposomes. Our results further demonstrated that protein orientation in the resulting proteoliposomes was critically dependent upon the mechanism by which the protein was incorporated.

The data presented in the preceding paper (Paternostre et al., 1988) define the conditions for a stepwise solubilization of large liposomes by different detergents. The lipid–detergent interactions can be analyzed in terms of a three-stage model (Lichtenberg, 1985). Accordingly, as increasing amounts of detergents are added to a suspension of liposomes, the detergent is first incorporated into the bilayers, leading to alterations in membrane permeability without solubilization. At a critical detergent concentration, when the bilayers are saturated with detergent, gradual disintegration of the membrane starts and lipid–detergent mixed micelles begin to form. The end point of solubilization is reached when all the membrane material is converted into mixed micelles. It is generally assumed that this solubilization process represents the reverse of detergent-mediated reconstitution of pure liposomes or proteoliposomes (Helenius & Simons, 1975; Eytan, 1982). One possible sequence of events during detergent-mediated reconstitution is the following: Initially, lipid–detergent and lipid–protein–detergent micelles are formed and the detergent is then removed. At a certain point, the micelles are no longer soluble, and structures containing lipids and proteins are formed that, upon reorganization, are transformed into vesicles. The last phase of detergent removal corresponds to the removal of residual detergent from detergent-saturated proteoliposomes.

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