Phospholipid Vesicle Solubilization and Reconstitution by Detergents. Symmetrical Analysis of the Two Processes Using Octaethylene Glycol Mono-*n*-dodecyl Ether

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ABSTRACT: The processes of liposome solubilization and reconstitution were studied by using *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$). The solubilization of large unilamellar liposomes prepared by reverse-phase evaporation was systematically investigated by turbidity, ^{31}P nuclear magnetic resonance, and centrifugation experiments. The solubilization process is well described by the three-stage model previously proposed for other detergents, and our results further demonstrate the validity of some of the postulates related to this model. In stage I, the detergent distributes between the bilayers and the aqueous solution with a partition coefficient of 1.6 mM^{-1}. In stage II, the detergent-saturated liposomes convert into mixed micelles, the conversion being complete by stage III where all the phospholipids are present as mixed micelles. The agreement between the three methods was excellent, and the results allowed quantitative determination of the effective detergent to phospholipid ratios at which the lamellar to micellar transformation begins and is complete, which amounted to 0.66 and 2.2 (mol/mol), respectively. Furthermore, compositional analysis determined from centrifugation experiments directly demonstrate that the properties of detergent-saturated liposomes and mixed micelles remain constant throughout most of stage II: the $C_{12}E_8$ to phospholipid ratios in the pelleted vesicles and in micelles are constant during stage II and similar to the ratios at which stage II was initiated and complete, respectively. On the other hand, bilayer formation upon detergent removal from mixed $C_{12}E_8$–phospholipid micelles by SM_2 Bio-Beads is demonstrated to be the symmetrical opposite of bilayer solubilization. The threshold values at which the three opposite stages occurred as well as the composition of the species present in each stage of the vesiculation process perfectly match those determined during the solubilization process. However, the sizes of reconstituted liposomes as analyzed by freeze–fracture electron microscopy were found to depend drastically upon the rate of detergent removal, demonstrating the importance of kinetic factors in the determination of the vesicle size during reconstitution experiments.

A method has been developed in this laboratory (Paternostre et al., 1988; Rigaud et al., 1988) for identifying the steps in a detergent-mediated reconstitution procedure at which an integral membrane protein can be associated with phospholipids to give functional proteoliposomes. The protocol employed is based on the idea that such reconstitution procedures represent the reverse of the 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 that has to be reconstituted is then added, and its incorporation can be suitabilly studied in each step of the lamellar-to-micellar transition. We analyzed three detergents, namely, Triton X-100, octyl glucoside, and sodium cholate. We now report results for an extension of this method to the use of another nonionic detergent, *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$).[1]

This nonionic detergent has gained widespread use in solubilization and reconstitution studies for the last 10 years. Originally reported to be useful in the preparation of active Ca^{2+}-ATPase (Le Maire et al., 1976; Dean & Tanford, 1978), it was found to be a suitable detergent for the solubilization of many other functionally active proteins (Cornelius & Skou, 1984; Krämer, 1984; Krämer et al., 1986). It has also been used at subsolubilizing concentrations to perturb membrane structure and function, providing a new approach to the characterization of the turnovers of the Ca^{2+}-ATPase (Champeil et al., 1986) and of the Na^+·K^+-ATPase (Huang et al., 1985). Another promising application of $C_{12}E_8$ is its use in reconstitution studies, and some reports appeared in the literature dealing with formation of active proteoliposomes after detergent removal from solubilized mixtures of $C_{12}E_8$, membrane proteins, and lipids (Andoh & Yamamoto, 1985; Cornelius & Skou, 1984; Krämer & Heberger, 1986; Dierks & Krämer, 1988).

However, surprisingly, despite all these studies that employed $C_{12}E_8$ very little information is available that quantitatively characterizes the solubilization of phospholipids by this detergent, and contradictory results exist concerning the interaction of $C_{12}E_8$ with bilayers upon its removal (Ueno et al., 1984; Lemaire et al., 1987).

In this paper, we first report our findings for the solubilization by $C_{12}E_8$ of large unilamellar liposomes prepared by reverse-phase evaporation (Rigaud et al., 1983). The methods used include turbidity measurements, magnetic resonance

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[1] Abbreviations: EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; NMR, nuclear magnetic resonance; R_{eff}, effective molar ratio of detergent to phospholipid; R_{sat}, R_{eff} at the onset of the lamellar-to-micellar transition; R_{sol}, R_{eff} at complete solubilization; cmc, critical micelle concentration; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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spectroscopy, and centrifugation experiments. The results of these studies were related to the "three-stages" model describing the interaction of detergents with membranes and analyzed in light of recent papers on phospholipid solubilization by detergents (Lichtenberg, 1985; Paternostre et al., 1988). Furthermore, the experimental data presented in this paper allow us to define accurately the different steps of the solubilization process and to quantify the mixed bilayer–mixed micelle interconversion, thus definitely supporting many of the asumptions proposed in the general model of liposome solubilization (Lichtenberg et al., 1983; Dennis, 1974).

In the second place we have analyzed the process of liposome reconstitution upon detergent removal from mixed $C_{12}E_8$–phospholipid micelles using SM_2 Bio-Beads as the detergent-removing agent (Holloway, 1973; Levy et al., 1990). The vesiculation process appears qualitatively as the symmetrical opposite of the disruption process of liposome by $C_{12}E_8$: three steps in the reconstitution process are observed at detergent to phospholipid ratios similar to those observed during solubilization. However, the results confirm the importance of kinetic factors, related to the rate of detergent removal, in the determination of the final vesicle size (Ueno et al., 1984; Lasic, 1988). From the point of view of the use of $C_{12}E_8$ for reconstitution studies our data have important implications since they demonstrate that unilamellar liposomes, homogeneous in size and devoid of significant residual detergent, can be produced.

Materials and Methods

Chemicals. Purified egg yolk phosphatidylcholine (EPC) and derived phosphatidic acid (EPA) were isolated according to the methods of Singleton et al., (1965) and Allgyers and Wells (1979), respectively.

The chemicals used in this study and their sources were as follows: $C_{12}E_8$ (Nikko Chemical, Tokyo, Japan), [1-^{14}C]$C_{12}E_8$ (CEA, Saclay, France), and [^{3}H]dipalmitoylphosphatidylcholine (Amersham, France). SM_2 Bio-Beads were obtained from Bio-Rad. All other chemicals were of analytical grade.

Preparation of Liposomes. Large unilamellar liposomes were prepared by reverse-phase evaporation as described previously (Rigaud et al., 1983; Paternostre et al., 1988). Buffers used were 10 mM Pipes–KOH, pH 7.0, supplemented with 120 mM K_2SO_4. Liposomes (about 20 mM, i.e., 16 mg of lipid/mL) were sized through 0.4- and 0.2-μm polycarbonate membranes before use.

Turbidity Measurements. The turbidity of the phospholipid vesicle suspension as a function of detergent concentration was measured between 400 and 700 nm with a Philips (PU 8740) spectrophotometer. The vesicles were suspended at concentrations ranging from 1.25 to 10 mM phospholipid. The detergent concentration was raised in increments, and absorbance was continuously recorded until a steady-state levels was reached. For phospholipid vesicle concentrations below 1.25 mM light-scattering changes were also monitored with a Perkin-Elmer (MPF 44A) spectrofluorometer set at 450 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 as described previously (Paternostre et al., 1988). 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 in 10 mM Pipes and 120 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).

Centrifugation Experiments. Aliquots (200 μL) of the phospholipid–detergent suspensions were spun down (at 4 °C) at 95 000 rpm for 1 h in a TLA 100 rotor using a Beckman TL 100 ultracentrifuge (400 000g). The clear supernatants were removed and assayed for radioactive phospholipids and/or detergents. The pellets were resuspended in 100 μL of buffer and analyzed for radioactivity.

Detergent Removal. Bio-Beads were thoroughly rinsed with methanol and buffer before use (Holloway, 1973), and the finest beads were discarded. In order to remove $C_{12}E_8$, desired amounts of moist copolymer beads were added directly to detergent-containing solutions and gently stirred at room temperature except when aliquots were pipetted off after the desired periods of incubation. The density of the washed Bio-Beads is such that in the absence of stirring they rapidly sediment, thus enabling a supernatant solution devoid of Bio-Beads to be pipetted off (similar results were obtained whenever the beads were centrifuged at 10000g). The different aliquots were then assayed for radioactive detergents and/or phospholipids.

Results

(I) Solubilization of Liposomes by $C_{12}E_8$. (A) Turbidity Measurements. As a first approach to the problem of liposome solubilization, we analyzed the detergent-induced changes in turbidity of preformed liposome suspensions. The results are shown in Figure 1A for phospholipid concentrations ranging from 1.25 to 10 mM phospholipid. As increasing amounts of $C_{12}E_8$ are added to preformed liposomes, the turbidity showed an initial increase, passed through a maximum, and then decreased to a minimal value.

Previous studies of the interactions of detergents with model membranes (Lichtenberg, 1985; Jackson et al., 1982; Goni et al., 1986; Paternostre et al., 1988) indicated that each curve can be interpreted in the light of a "three-stage" model. Stage I involves partitioning of detergent monomers between the aqueous medium and the lipid bilayer. During this stage (up to the detergent concentrations denoted by black arrows) turbidity increased 1.2-fold as the $C_{12}E_8$ concentration rose. At the end of this stage, liposomes are saturated with $C_{12}E_8$. During stage II, detergent addition promotes gradual liposome solubilization and mixed phospholipid–detergent micelles of much lower molecular weight begin to form with subsequent decrease in turbidity. Stage III is characterized by complete solubilization of lipids into mixed micelles, and the suspensions become optically transparent. At this point (white arrows in Figure 1A) excess detergent has no effect on the turbidity of the suspensions.

For comparison, the changes in turbidity of multilamellar suspensions and sonicated liposomes caused by the addition of $C_{12}E_8$ are shown in the inset of Figure 1A. The $C_{12}E_8$ concentrations at which turbidities start to decrease are identical for all samples, as are the $C_{12}E_8$ concentrations needed for complete solubilization. However, the behavior of turbidity in stage I is clearly dependent on the mode of preparation and thus on the size of the liposomes. Indeed, during stage I, the turbidity of large liposomes increased 1.2-fold as the $C_{12}E_8$ concentrations rose; that of sonicated liposomes, about 1.8-fold; while that of multilamellar did not vary significantly. We have checked by freeze–fracture electron microscopy that the changes in turbidity occurring after the addition of subsolubilizing $C_{12}E_8$ concentrations to

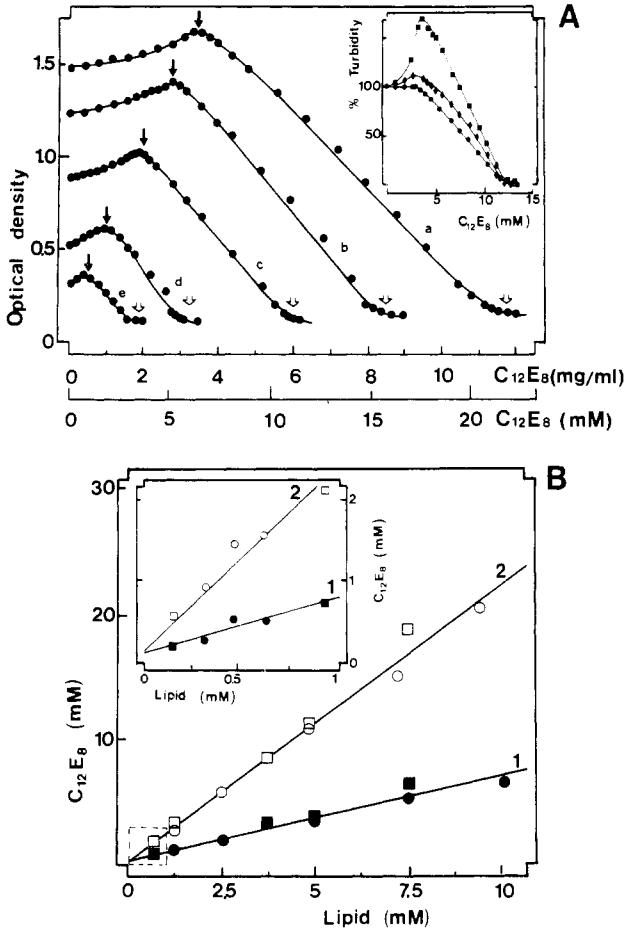

FIGURE 1: Process of liposome solubilization by $C_{12}E_8$ as analyzed by turbidity measurements. (A) Turbidity changes induced by stepwise addition of $C_{12}E_8$ to liposome suspension containing 10 (a), 7.5 (b), 5 (c), 2.5 (d), or 1.25 mM lipid (e). Liposomes prepared by reverse-phase evaporation were resuspended at the desired concentration in a spectrophotometer cell. Then $C_{12}E_8$ from a concentrated stock solution was added stepwise to the liposome suspension under constant stirring at 20 °C. The turbidity was measured at 500 nm after detergent equilibration. Black and white arrows denote the threshold detergent concentrations for the onset and total liposome solubilization, respectively, at each phospholipid concentration. (Inset) Percent changes in turbidity of multilamellar (●), sonicated (■), and reverse-phase evaporation liposomes (♦) upon $C_{12}E_8$ addition. Total phospholipid in all samples: 4 mg/mL, i.e., 5 mM (100%, turbidity in the absence of detergent; the initial turbidities were 0.16, 0.840, and 2.5 for sonicated, reverse-phase, and multilamellar liposomes, respectively). (B) $C_{12}E_8$ to phospholipid relationships describing the solubilization process. The $C_{12}E_8$ concentrations corresponding to the onset (filled symbols, curve 1) and complete solubilization (open symbols, curve 2) are plotted as a function of the 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. Different symbols refer to different experiments. (Inset) Phospholipid concentrations range up to 1 mM (light scattering measurements).

liposomes prepared by reverse phase were not due to massive aggregation and/or fusion unlike what was reported for sonicated liposomes (data not shown).[2]

[2] As already reported for Triton X-100, octyl glucoside, and cholate (Paternostre et al., 1988), the changes that occurred in light scattering after addition of subsolubilizing detergent concentration may conceivably be caused by slight changes in vesicle size or liposome aggregation not detected by electron microscopy (data not shown) or by changes in the refractive index of water and/or the membranes after detergent incorporation. This is in contrast with the massive vesicle aggregation and/or fusion observed with all kind of detergents on small, highly curvated sonicated liposomes (Alonso et al., 1981).

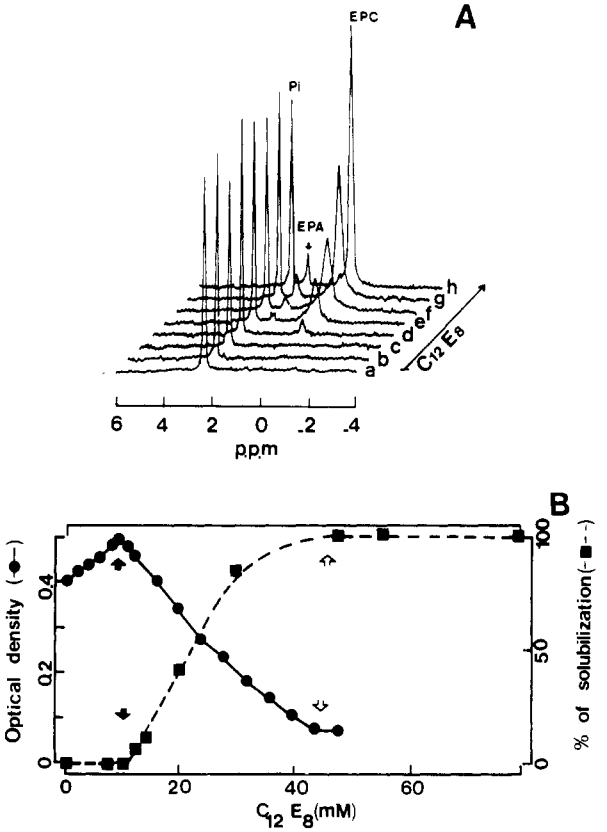

FIGURE 2: Process of liposome solubilization by $C_{12}E_8$ as analyzed by ^{31}P NMR measurements. (A) ^{31}P NMR spectra of liposomes in the presence of different amounts of $C_{12}E_8$. Liposomes prepared by reverse-phase evaporation (12.5 mg of lipid/mL, i.e., 15.62 mM) were treated by (a) 0, (b), 9.25, (c) 10.4, (d) 11.1, (e) 18.5, (f) 27.75, (g) 40.5, and (h) 74 mM $C_{12}E_8$. Inorganic phosphate (P_i) was included as a reference, and the ppm were given by the resonance of H_3PO_4. (B) Comparison of turbidity and ^{31}P NMR studies of the solubilization process of liposomes by $C_{12}E_8$. 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 concentration in all samples was 15.6 mM. Black and white arrows indicate the threshold detergent concentrations for onset and total solubilization.

It is evident from Figure 1A that the detergent concentrations corresponding to the onset of solubilization and total solubilization are dependent on the phospholipid concentration. When these critical concentrations (denoted by black and white arrows, respectively, in Figure 1A) are plotted versus the total lipid concentration, striking linear relationships are obtained (Figure 1B) whose slopes give the detergent to phospholipid ratio either in the detergent-saturated bilayers (R_{sat} at the onset of solubilization) or in the mixed micelles (R_{sol} at the total solubilization). The values for R_{sat} and R_{sol} deduced from the slopes of curves 1 and 2 in Figure 1B are 0.66 and 2.2 mol of $C_{12}E_8$/mol of phospholipid, respectively. Further extrapolations of curves 1 and 2 to zero phospholipid concentration give values of about 0.2 mM, which may be interpreted to be the aqueous monomer detergent concentration (D_w) in equilibrium with either the saturated liposomes or the mixed micelles.

(B) NMR Measurements. The lamellar-to-micellar transition caused by $C_{12}E_8$ addition to preformed liposomes was also documented by using ^{31}P NMR, a method allowing a clear distinction to be made between phospholipid in micelles and in vesicles (Roux & Champeil, 1984; Jackson et al., 1982; Goni et al., 1986; Paternostre et al., 1988).

Some ^{31}P NMR spectra for samples with a fixed phospholipid concentration (12.5 mg/mL, i.e., 15.6 mM) and various $C_{12}E_8$ concentrations are shown in Figure 2A. In the absence of detergent, only the narrow resonance signal of inorganic phosphate is visible (P_i has been included together with the liposomes to provide an internal reference). Under the conditions of these scans (3000 Hz sweep width) the broad ^{31}P resonance of the phospholipid head group is not visible. Up to 10.5 mM $C_{12}E_8$ (traces a–c in Figure 2A) no significant changes appeared in the spectra. Above this concentration, two narrow and symmetrical peaks (30 Hz width) appeared, indicative of isotropic phospholipid motion. These two new peaks with chemical shifts from external H_3PO_4 of −0.28 and +1.74 ppm corresponded respectively to phosphatidylcholine and phosphatidic acid molecules in micelles. The percentage of solubilized phospholipids was estimated by comparing the integral of micellar phosphatidylcholine ^{31}P signal (area of the peak) to the integral of the P_i signal and was plotted (Figure 2A) as a function of added $C_{12}E_8$. The solubilization started at about 11 mM $C_{12}E_8$ ($R_{sat} \simeq 0.7$) and was virtually complete at about 40 mM $C_{12}E_8$ ($R_{sol} = 2.5$) in agreement with the values estimated form the turbidity measurements reported in the same figure.

Additionally, 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 is complete are about 25 Hz and can be compared to the values of 22.5, 16, and 6 Hz previously reported for Triton X-100, octyl glucoside, and cholate, respectively (Paternostre et al., 1988), reflecting the large difference in the size of the micelles of these four 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 15 Hz (data not shown); this enhanced resolution might be related to the general reduction in the size of the phospholipid–detergent micelles (Roux & Champeil, 1984).

(C) Centrifugation Experiments. Finally, the solubilization process was also characterized by ultracentrifugation experiments. Liposome suspension (3.5 mg of [^{3}H]lipid/mL) containing increasing concentrations of [^{14}C]$C_{12}E_8$ were centrifuged at 400 000*g* for 1 h and the amounts of lipid and detergent determined both in the supernatants and in the pellets. Under these centrifugation conditions bilayer vesicles pellet while mixed micelles remain in the supernatant.

As shown in Figure 3A (see also Figure 4), when liposomes were treated by detergent concentrations lower than 2.7 mM $C_{12}E_8$, only a small percentage of the lipids was found in the supernatants (about 5–10% of liposomes never sediment in our experimental conditions). Above this detergent concentration the amount of lipids found in the supernatants increased linearly, finally plateauing for samples treated by more than 9.25 mM $C_{12}E_8$. Correspondingly (Figure 3B), the amount of lipids in the pellets decreased rapidly between these two detergent concentrations, and no pellets were recovered at 100% lipid solubilization. At this point it is important to note that the detergent to phospholipid ratios corresponding to the threshold values for the onset ($R_{sat} = 0.62$) and total solubilization ($R_{sol} = 2.1$) are similar to those obtained by turbidity and ^{31}P NMR experiments.

Interestingly, the $C_{12}E_8$ to phospholipid molar ratios in the pelleted vesicles have a constant value of about 0.7 throughout most of stage II, i.e., throughout the lamellar-to-micellar transition, and this value is identical with the point at which

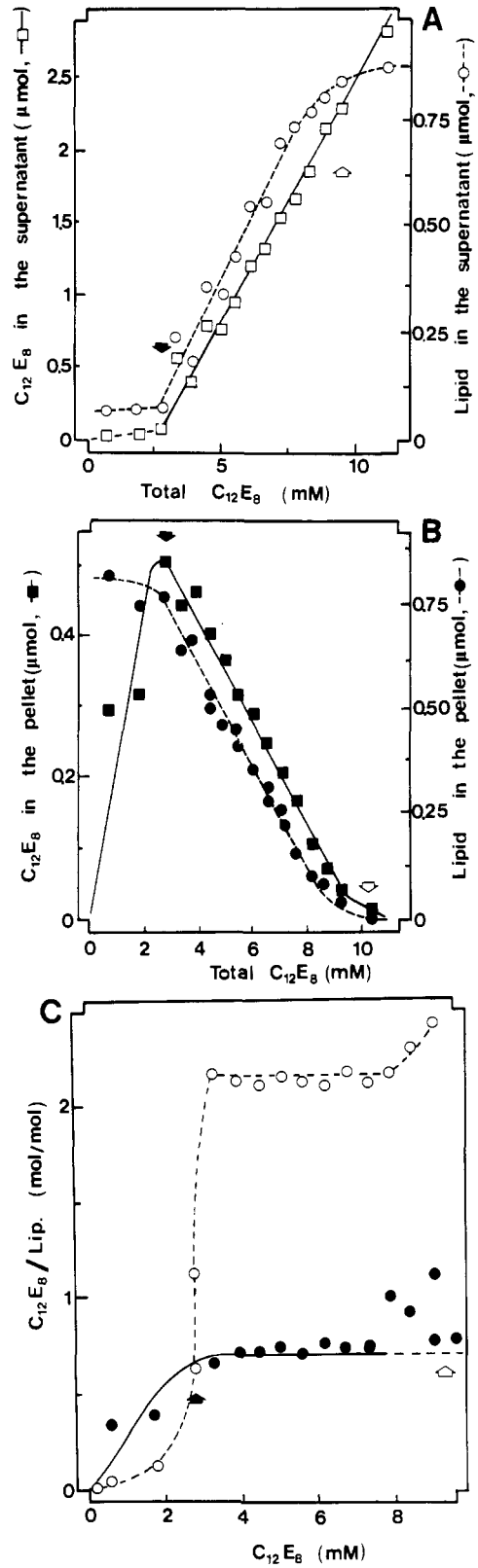

FIGURE 3: Process of liposome solubilization by $C_{12}E_8$ as analyzed by centrifugation experiments. Liposomes prepared by reverse-phase evaporation in the presence of [^{3}H]phosphatidylcholine were resuspended at 4.375 mM phospholipid and treated by different amounts of [^{14}C]$C_{12}E_8$; 200-μL aliquots of each sample were then centrifuged at 400000*g* for 1 h and analyzed for their radioactivity in the supernatants and in the pellets as described under Material and Methods. (A) Phospholipid (○) and $C_{12}E_8$ (□) in the supernatants. (B) Phospholipid (●) and $C_{12}E_8$ (■) in the pellets. (C) Detergent to phospholipid ratios (mol/mol) in the pellets (●) and in the supernatants (○). These ratios were deduced from results in (A) and (B). Black and white arrows denote the threshold values for onset and total solubilization.

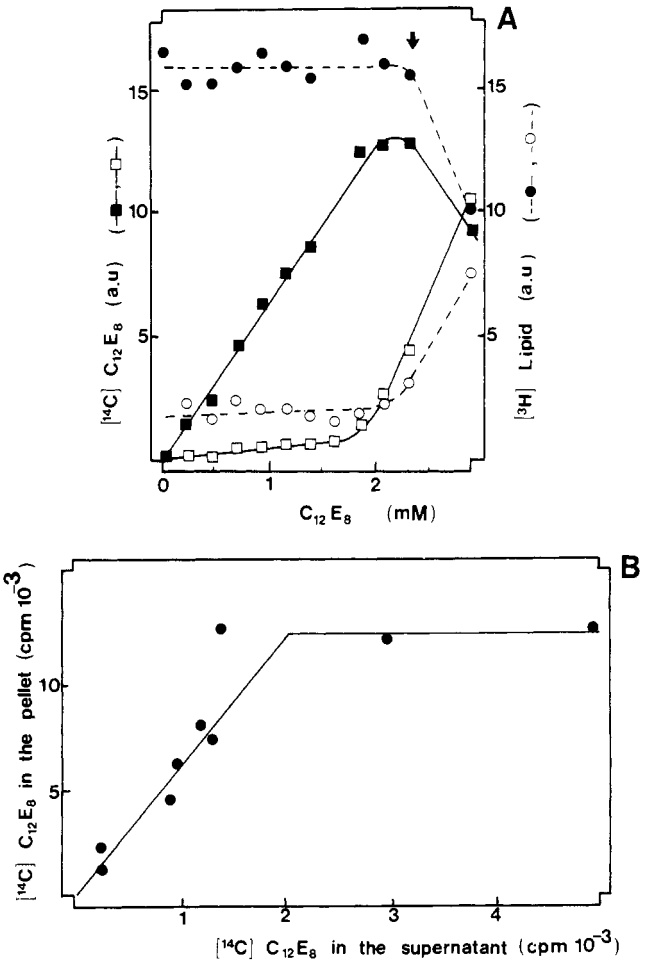

FIGURE 4: Distribution of $C_{12}E_8$ between pellet and supernatant for subsolubilizing levels of detergent. (A) Radioactivity analysis of the pellets and the supernatants. Liposomes prepared by reverse-phase evaporation were resuspended at 3.75 mM and treated by the indicated amounts of $C_{12}E_8$. Aliquots (150 μL) of each sample were then centrifuged at 400000g for 1 h and analyzed for their radioactivity in both the pellets and the supernatants. Arrow indicates the onset of liposome solubilization. Circles: Phospholipids in the pellets (●) and in the supernatants (○). Squares: Detergent in the pellets (■) and in the supernatants (□). (B) Distribution of $C_{12}E_8$ between pellet and supernant at different total $C_{12}E_8$ concentrations. Values deduced from (A). (10^3 cpm corresponds to about 10 μg of $C_{12}E_8$, i.e., 0.37 μmol.)

stage II is initiated (Figure 3C). Similarly, the $C_{12}E_8$ to phospholipid ratios in the supernatants, i.e., in mixed micelles, remain constant throughout stage II and are found to be 2.2, i.e., similar to the point at which stage II is complete. Thus during the bilayer-to-micelle conversion, the properties of the micelles and of the detergent-saturated liposomes remain constant; only the relative percentage of each species varies during the solubilization process.

We have also analyzed more systematically the subsolubilizing range of detergent concentration, i.e., stage I, where it is generally admitted (Jackson et al., 1982; Lichtenberg, 1985; Goni et al., 1986) that detergent partitions between the vesicles and the aqueous medium. Figure 4A reports the data from ultracentrifigation experiments for the range of subsolubilizing $C_{12}E_8$ concentrations. The most important feature of these data is that, up to the onset of liposome solubilization (R_{sat} = 0.66), the amount of lipids that is pelleted is constant and similar to that obtained when centrifuging untreated liposomes while the concentration of $C_{12}E_8$ in the pellets is found to increase rapidly and linearly. Concomitantly, the amount of $C_{12}E_8$ in the supernatants increases but much more slowly. A plot of the $C_{12}E_8$ level associated with the liposomes ($D_{Bilayer}$) against the aqueous detergent concentration (D_w) yields a straight line which passes through the origin of the axes (Figure 4B). The slope of this line ($D_{Bilayer}/D_w$) is about 6. Assuming an equilibrium distribution of $C_{12}E_8$ between the bilayer and aqueous medium, a distribution coefficient K has been defined (Schurtenberger et al., 1985):

$$K = D_{Bilayer}/D_w[L]$$

Thus a coefficient of about 2 (mg/mL)$^{-1}$, i.e., 1.6 mM^{-1}, can be calculated from the slope of the straight line in Figure 4B.[3]

(II) Reconstitution of Liposomes. (A) Time Course of Detergent Removal and Vesicle Formation. In order to analyze the different steps in the process of liposome reconstitution, the method previously developed for formation of unilamellar vesicles from mixed phospholipid–Triton X-100 micellar solutions (Rigaud et al., 1988; Levy et al., 1990) has been applied to $C_{12}E_8$. This method relies on the procedure originally described by Holloway (1973) and is based on the adsorption of $C_{12}E_8$ onto hydrophobic SM_2 Bio-Beads.

The standard procedure was the following. Solutions of mixed lipid–detergent micelles were obtained by adding 12 mM [^{14}C]$C_{12}E_8$ to a suspension of liposomes containing 5 mM [^{3}H]lipid; under these conditions no sedimentable material could be detected, and the good dispersal of lipids was checked by freeze–fracture electron microscopy (data not shown). Then Bio-Beads were added at a low bead to detergent ratio (≃5 w/w) both to allow slow detergent removal during the micellar-to-lamellar transition and to avoid phospholipid losses by adsorption to the beads during the micellar stage. After 90-min incubation, additional Bio-Beads were added to remove residual detergent. During detergent removal, aliquots were pipetted off and analyzed for their lipid and detergent contents before and after centrifugation experiments. Results are given in Figure 5.

Part A of this figure shows the time course of detergent removal together with the continuous changes in absorbance of the reconstitution solution: after 1-h incubation at the low bead to detergent ratio about 8.5 mM $C_{12}E_8$ is adsorbed, which corresponds to the maximal adsortive capacity of the hydrophobic beads.[4] Then adding fresh beads (arrow in Figure 5A) allows rapid elimination of residual detergent. Interestingly, the turbidity of the initially clear detergent–phospholipid mixture increases upon detergent removal, finally plateauing after 1-h incubation with Bio-Beads, additional fresh beads having no further significant effect. If we assume that the S-shaped increase in turbidity represents the transition from the micellar to vesicular state, then it would appear that this transition is complete after 1-h incubation. It is noteworthy, that the detergent to phospholipid ratio attained at this stage is about 0.7 (mol/mol), characteristic of the level of $C_{12}E_8$

[3] From the definition of the effective detergent to phospholipid ratio, $R_{eff} = D_{Bilayer}/[Lip]$, it follows that $K = R_{eff}/D_w$. From the values obtained for R_{sat} and D_w in Figure 1, a partition coefficient K of 3.5 mM^{-1} can be calculated, which can be compared to that obtained by direct measurements of the partition of $C_{12}E_8$ between bilayer and water.

[4] Titration of SM_2 Bio-Beads with ^{14}C-labeled $C_{12}E_8$ yielded an absorptive capacity of 3 × 10^{-4} mol of $C_{12}E_8$/g of wet beads. On the other hand, titration of SM_2 beads with [^{14}C]dipalmitoyllecithin yielded an absorptive capacity of 1.25 × 10^{-6} mol of phospholipid/g of wet beads for pure liposomes and 2.5 × 10^{-6} mol of phospholipid/g of wet beads for phospholipid–$C_{12}E_8$ micelles. This indicated that in our experimental conditions, starting from 5 × 10^{-6} mol of phospholipid/mL, lipid loss in the presence of 90 mg of Bio-Beads is negligible. Interestingly, in the experiments described in Figure 5, less than 5–7 residual $C_{12}E_8$ molecules per 100 lipid molecules were detected in the reconstituted liposomes.

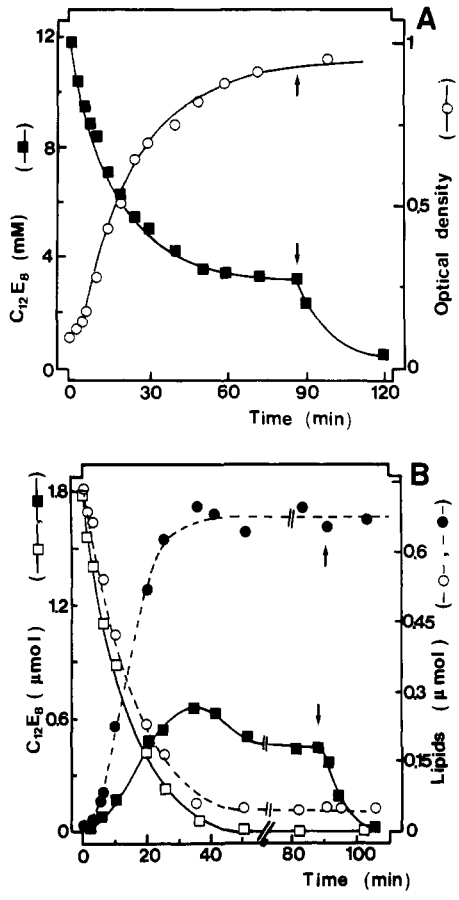

FIGURE 5: Process of liposome formation during $C_{12}E_8$ removal. (A) Time course of $C_{12}E_8$ removal by SM_2 Bio-Beads. Micellar solutions were prepared by adding [^{14}C]$C_{12}E_8$ (12 mM) to a suspension of liposomes prepared by reverse-phase evaporation (5 mM). Then Bio-Beads were added at 30 mg/mL, and aliquots from the supernatant were collected as a function of time and analyzed for turbidity (○) and residual $C_{12}E_8$ (■). The arrow indicates when additional fresh Bio-Beads (60 mg/mL) were added to the phospholipid–detergent mixture. (B) Analysis of the reconstitution process by centrifugation experiments. Same samples as in (A). Aliquots (150 μL) were pipetted off as a function of time and centrifugated for 1 h at 400000*g*. The pellets (closed symbols: ●, ■) and the supernatants (open symbols: ○, □) were analyzed for their phospholipid (circles) and $C_{12}E_8$ (squares) radioactivity. The arrow corresponds to the addition of fresh beads.

saturation of preformed liposomes. Upon addition of fresh beads, detergent removal continues in the now turbid suspension and would correspond to the elimination of residual detergent from $C_{12}E_8$-saturated liposomes. All these assumptions are seen to be consistent in the light of centrifugation data presented in Figure 5B. Starting from initial $C_{12}E_8$–lipid micellar solution, no pellet is obtained. As detergent is removed, the amount of phospholipid in the pellets increased rapidly, finally plateauing after 40-min contact with Bio-Beads, where almost all phospholipids initially present pellet. Correspondingly, the amount of phospholipid in the supernatants decreases rapidly, and after 40-min contact with Bio-Beads, only a small percentage of lipid was found in the supernatants. Furthermore, at this stage, most residual $C_{12}E_8$ is found in the pellets and very low $C_{12}E_8$ radioactivity is detected in the supernatants, indicating that no mixed micelles exist and that vesiculation is complete, in agreement with the turbidity measurements performed under the same experimental conditions. The slight increase in turbidity observed after 40-min incubation may however be real and may be due to a postvesiculation size growth of $C_{12}E_8$-saturated liposomes although this has been reported as a very slow process (Ueno et al., 1984). After 40-min incubation with Bio-Beads, the amount of detergent in the pellet was found to decrease, which appears more evident upon addition of fresh beads. This corresponds to the detergent removal from the detergent-saturated liposomes already formed.

Besides providing information about the time course of the vesiculation process, the data reported in Figure 5B allow us to determine $C_{12}E_8$ to phospholipid ratios in both the pellets and the supernatants, i.e., in both liposomes and micelles; throughout all the range of vesiculation the detergent to phospholipid ratios in the pellets and in the supernatants remain roughly constant and are 0.7 and 2.2 (mol/mol), respectively. These values can be compared to the values obtained above for the levels of $C_{12}E_8$ associated with liposomes and micelles during the lamellar-to-micellar transition and demonstrate that solubilization and reconstitution are two symmetrical processes.

(*B*) *Influence of the Rate of Detergent Removal upon Liposome Size.* The size and unilamellarity of liposomes reconstituted by the method described above have been analyzed by freeze–fracture electron microscopy as a function of the rate of detergent removal. Systematic studies indicated that the rate of detergent removal was critically dependent upon the amount of Bio-Beads present in solution. For example, starting from the same experimental conditions described in Figure 5, the removal of the detergent was essentially complete after 1-h incubation with 80 mg of Bio-Beads/mL while the detergent could be eliminated in 30 min in the presence of 160 mg of Bio-Beads/mL and in only 5 min in the presence of 600 mg of Bio-Beads/mL (data not shown).

Independently of the initial Bio-Bead concentrations used in this study (20–600 mg of beads/mL), reconstituted liposomes consisted of unilamellar vesicle populations. However, the sizes of the vesicles formed upon $C_{12}E_8$ removal drastically depended upon the rate of detergent elimination (Figure 6): at increased detergent removal rate, liposomes became smaller. At the slowest rate of detergent removal analyzed, the vesicles obtained consist of fairly homogeneous unilamellar vesicle population with a mean diameter of about 80 nm (panel A) while at higher detergent removal rates small unilamellar vesicles are formed with mean diameters around 25 nm (panels B and C in Figure 6).

Discussion and Conclusions

A large number of studies have been recently published that analyze the processes of detergent-mediated solubilization and reconstitution of liposomes, increasing the understanding of such phenomena [for reviews see Lichtenberg (1985), Lasic (1988), and Silvius and Allen (1988)]. However, these studies on phospholipid–surfactant interactions deal mainly with the three detergents most frequently used in solubilization and reconstitution studies, namely, Triton X-100, octyl glucoside, and sodium cholate. This paper is an extension of such studies to a detailed characterization of the phase behavior of mixed $C_{12}E_8$–phospholipid systems. This nonionic detergent has gained widespread interest during the last decade (see the introduction) with the advantage of being a homogeneous and not a polydisperse chemical species such a Triton X-100, and without the disadvantage of easily denaturing membrane proteins as reported for octyl glucoside (Reynolds, 1981; Dencher & Heyn, 1983; Rigaud et al., unpublished work).

In the first place in this work, we have studied the solubilization of large unilamellar liposomes prepared by reverse-phase evaporation. Such liposomes have been used in order to avoid problems associated with multilamellar dispersions where time of detergent equilibration has been reported to be

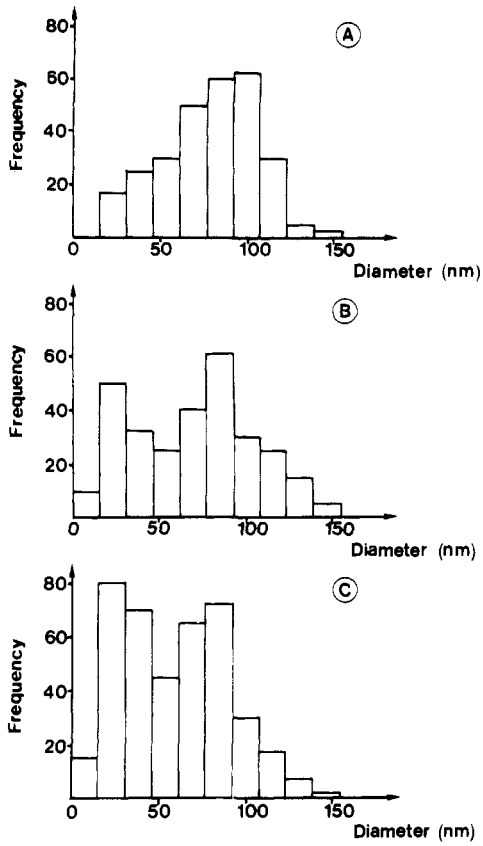

FIGURE 6: Size distribution histograms of reconstituted liposomes. Liposomes prepared by reverse-phase evaporation were solubilized as described in the legend of Figure 5 and treated by different amounts of beads. After total detergent ellimination, the samples were analyzed by freeze–fracture electron microscopy. Size distribution histograms of liposomes obtained after detergent removal by different amounts of SM_2 Bio-Beads. Panel A: Sequential additions of 20 mg of beads/mL every hour for 4 h followed by addition of 540 mg of beads/mL. Panel B: Addition of 160 mg of beads/mL for 2 h followed by addition of 440 mg of beads/mL. Panel C: Addition of 600 mg of beads/mL for 2 h. Bars are 16.54 nm.

very long (Goni et al., 1986; Lichtenberg et al., 1979) or with highly curved sonicated vesicles where important changes in size and morphology have been described (Alonso et al., 1981, 1982). The experimental data reported 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 occurs in three distinct stages [for reviews see Helenius and Simons (1975), Lichtenberg et al. (1983), and Lichtenberg (1985)]. In stage I, the detergent distributes between the bilayer and the aqueous solution. In stage II, the mixed bilayers convert into mixed micelles until all of the bilayer has disappeared. In stage III, the detergent to phospholipid ratio increases in the mixed micelles with a concomitant decrease in the size of these micelles.

The solubilization process was documented by different techniques including turbidity, NMR, and centrifugation, and the perfect agreement between these techniques allowed us to accurately determine two important parameters, namely, R_{sat} and R_{sol}. Indeed, from the linear dependencies of the critical detergent concentrations at which the lamellar-to-micellar transformations begin and finish, over the lipid concentration (Figure 1B), the process of solubilization was described by the equation:

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

where [L] and $[D_T]$ are the total lipid and detergent concentrations, D_w is the monomeric detergent concentration, and R_{eff} is the effective detergent to phospholipid ratio at which the phase transformations occur (R_{sat} at the onset of solubilization; R_{sol} at the total solubilization). The corresponding values for R_{sat} and R_{sol} were found to be 0.7 and 2.2 mol of $C_{12}E_8$/mol of lipid, respectively (i.e., 0.45 and 1.5 mg of $C_{12}E_8$/mg of lipid). These values can be compared to those obtained for other detergents under the same experimental conditions (Paternostre et al., 1988). For instance, detergent saturations of liposomes occur at ratios of 0.64, 1.3, and 0.3 mol of detergent/mol of phospholipid for Triton X-100, octyl glucoside, and sodium cholate, respectively, while micellizations occur at respective ratios of 2.5, 3.6, and 0.9 mol of detergent/mol of lipid. This would suggest that the solubilizing power of $C_{12}E_8$ is comparable to that of the nonionic detergent Triton X-100.

On the other hand, D_w, which represents the concentration of the monomeric detergent in equilibrium with either the saturated bilayers or the mixed micelles, was found to be about 0.2 mM $C_{12}E_8$ comparable to the critical micelle concentration of this detergent (Møller et al., 1986). Consequently, our experimental results support the generally admitted assumption (Lichtenberg, 1985) that the concentration of free detergent has to rise to the cmc for solubilization to occur.

Besides providing information about the general scheme of phospholipid solubilization and the solubilizing power of $C_{12}E_8$, the data reported in this paper allow us to characterize in more detail stages I and II of the solubilization process. During stage I, it is generally postulated that the detergent intercalates into the liposomes 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. Data reported in Figure 4 indicate that this is indeed the case for $C_{12}E_8$ and PC–PA liposomes, so that an apparent partition coefficient $\simeq 1.6\ mM^{-1}$ can be calculated. This partition coefficient is high when compared to the values reported for octyl glucoside and cholate ($K \simeq 0.05$–$0.1\ mM^{-1}$; Bayerl et al., 1989; Jackson et al., 1982; Schubert et al., 1986; Paternostre et al., 1988; Almog et al., 1986) but of the same order as that reported for Triton X-100. More interestingly, our data show that the $C_{12}E_8$ partition coefficient is constant for all $R_{Tot} < R_{sat}$, i.e., for all subsolubilizing detergent concentrations. This important result can be analyzed in the light of two recent papers dealing with the interaction of other detergents with vesicles during stage I, which indicate that the partition coefficient can be dependent on the detergent concentration except in a very low concentration region (Ueno, 1989; Bayerl et al., 1989). These last results suggest that the "postulate" of an equilibrium partition of detergents in a large range of subsolubilizing concentration should be taken with great care since it seems to depend upon the nature of the phospholipids (Bayerl et al., 1989), the kind of vesicles used (Ueno, 1989; Jackson et al., 1982), the nature of the detergent, and probably the method used. They also stress the need of a direct measurement of the detergent concentration in both phases over a wide concentration range in order to analyze accurately the partition behavior of a detergent. Thus, in this context, our systematic study allows us to conclude that the distribution of $C_{12}E_8$ is a perfect illustration of an ideal mixing of lipids and detergents even at high $C_{12}E_8$ concentrations. The present conclusion is agreement with the report of Le Maire et al. (1987) in which $C_{12}E_8$ binding to liposomes and sarcoplasmic reticulum vesicles was shown as a noncooperative process. Furthermore, it can be noted that, with use of the same liposome preparation as in this paper, indirect mea-

surements of the partition coefficient of Triton X-100 gave values constant over a range of R_{eff} values, up to the onset of solubilization (Paternostre et al., 1988).

During stage II, transformation of detergent-saturated bilayers into mixed micelles occurs with upper and lower limits corresponding to the experimentally observed R_{sat} and R_{sol}. Throughout all the range of detergent addition which causes this transformation both lamellar and micellar structures coexist as suggested by the agreement between turbidity measurements (indicative of the disappearance of lamellar structures) and NMR measurements (indicative of the appearance of micellar structures). This interpretation is clearly corroborated by the sedimentation experiments depicted in Figure 3, which furthermore allow us to get more insight the composition of the different molecular species present throughout the lamellar to micellar transition. Indeed, we have determined that the $C_{12}E_8$ to phospholipid ratios in the pelleted vesicles and in the mixed micelles are constant throughout most of stage II and correspond to the critical ratios at which stage II begins and finishes, respectively. Thus solubilization starts at R_{sat} (0.66 mol/mol), leading to the transformation of detergent-saturated liposomes into phospholipid-saturated micelles. During all the bilayer-to-micelle transition, both of these amphiphilic structures coexist and only the relative proportion of these structures varies with increasing detergent concentration. Finally, at R_{sol} (2.2 mol/mol) only mixed micelles are present in the solutions. Again, these results confirm the validity of the model proposed by Dennis (1974) and Lichtenberg et al. (1983). To our knowledge, the only systematic report of another commonly used detergent where stage II has been compositionnally characterized concerns phospholipid solubilization by octyl glucoside, with similar conclusions (Jackson et al., 1982).

The second important part of this work is related to the study of the process of liposome reconstitution upon detergent removal from $C_{12}E_8$–phospholipid mixed micelles. The protocol that was employed, namely, adsorption of $C_{12}E_8$ onto Bio-Beads by direct contact with the polystyrene beads, is well suited for investigating the time course of vesiculation and for following the composition and relative proportions of the different aggregates present in lipid–detergent samples. The most interesting finding is that liposome formation during detergent removal takes place in three distinct stages which are the symmetrical opposites of those observed during the solubilization process of preformed liposomes. Furthermore, the striking similarities of the critical $C_{12}E_8$ to phospholipid ratios at which phase transformations occur as well as of the composition of the detergent–phospholipid structures present in solution during the reconstitution and the solubilization processes demonstrate that the two processes are quantitatively symmetrical. Thus, during $C_{12}E_8$-mediated liposome reconstitution, the micellar-to-lamellar transition would begin at a $C_{12}E_8$ to phospholipid ratio of 2.2 (mol/mol) and end at a ratio of 0.7 (mol/mol). During all this transition, micellar and lamellar structures of a fixed composition coexist, only the relative proportion of each structures varying upon detergent removal. The last stage of the reconstitution process would correspond to detergent removal from the now formed detergent-saturated liposomes. At this point it is important to stress that the present results regarding the quantitative description of the intermediate stages during liposome formation do not preclude any information concerning the size, the structure, and the shape of the different intermediates formed, which can be very different in solubilization and reconstitution experiments. A large number of studies have been published that contributes to a knowledge of the possible intermediate structures in the vesicle formation, and different models have been proposed [for a recent review see Lasic (1988)]. Besides the influence of the physicochemical properties of the detergents (size, geometry, amphiphilicity, critical micelle concentration, fusogenic abilities) in determining the structure of the micelles and the size distribution of the reconstituted liposomes, it has been shown that for a given detergent kinetic factors can be of key importance in determining the final size of reconstituted samples. In this connection, our freeze–fracture electron microscopy studies demonstrate that the rate of detergent removal critically affects the final size distribution of the reconstituted liposomes. Small unilamellar vesicles (25 nm) are formed by rapid $C_{12}E_8$ removal, and larger liposomes (80 nm) are formed by slow removal. These observations can be interpreted in the light of the models proposed for vesicle formation by detergent depletion techniques (Lasic, 1988; Wrigglesworth et al., 1987): three steps may occur in the overall process, namely, micellar equilibration (micellar growth by fusion or phospholipid exchange), vesiculation (bilayer closure), and postvesiculization size growth (due to the residual detergent in the formed vesicle). In general, slower detergent depletion produces larger vesicles because micelle fusion (Lasic, 1988), lipid exchange (Almog et al., 1986), and postvesiculization (Ueno et al., 1984) are not instantaneous processes.

Besides providing new information concerning the way in which $C_{12}E_8$ interacts with phospholipid during liposome solubilization and reconstitution, we believe that another benefit of our study is the finding that the reconstitution method described in this paper provides a convenient and reproducible way for preparing unilamellar and fairly homogeneous liposomes with no significant amount of residual detergent (see Figure 5 and footnote 3). A strategy similar to that described here was previously published by Ueno et al. (1984), who studied vesicle formation upon $C_{12}E_8$ removal using XAD_2 hydrophobic columns. Our results contrast with this report in which an excedingly slow flip-flop rate of $C_{12}E_8$ was suggested essentially due to the inability to remove more than 50% of the detergent which remained bound to the reconstituted vesicles. Clearly, this is not the case in our experiments since our data show it is possible to remove with SM_2 Bio-Beads virtually all $C_{12}E_8$. In agreement, Le Maire et al. (1987) recently demonstrated that $C_{12}E_8$ flip-flop is a very rapid process ($\simeq$350 ms) that cannot account for incomplete detergent removal in reconstitution experiments.

In summary, the data presented in this paper serve to characterize accurately the various stages of solubilization and reconstitution of liposomes by the nonionic detergent $C_{12}E_8$. The present study completed the scarce information on the behavior of $C_{12}E_8$–phospholipid systems. In addition, the results not only corroborated much of the previous information on the behavior of other detergents but also confirmed many of the asumptions proposed in the general models of liposome solubilization and reconstitution using detergents. The present experimental conditions and results have been used in our laboratory to gain insight into the mechanisms underlying protein reconstitution into functional proteoliposomes and will be presented elsewhere.

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