Phospholipid Vesicle Formation Using Nonionic Detergents with Low Monomer Solubility. Kinetic Factors Determine Vesicle Size and Permeability†

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ABSTRACT: The method developed previously for formation of unilamellar vesicles from mixed micelles of egg lecithin and octyl glucoside [Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry 20*, 833–840] has been extended to allow for (1) use of nonionic detergents with much lower critical micelle concentrations and (2) variation in the time course of detergent removal. The results demonstrate the importance of kinetic factors, especially in the determination of vesicle size: initially formed vesicles are small, but the size increases slowly thereafter if detergent is not removed too quickly. Vesicle size remains fixed when the molar detergent/lipid ratio falls below about 1/1, and detergent removal becomes increasingly difficult thereafter, presumably because flip-flop of detergent from the inner to the outer leaflet of the bilayer membrane is very slow. Residual detergent (to about 25 mol %) has surprisingly little effect on anion permeability but increases cation permeability to the point where the normal discrimination between anions and cations (in pure lipid vesicles) is lost. Detergent added to initially detergent-free vesicles readily partitions into vesicular membranes (presumably only into the outer leaflet) and has a qualitatively similar effect on permeability. Vesicles produced by this method, regardless of residual detergent level, were found to be predominantly unilamellar: no multilamellar liposomes or other lipid aggregates could be detected within the accuracy of the methods employed.

A previous paper from this laboratory (Mimms et al., 1981) has described a method for preparing unilamellar phospholipid vesicles by detergent removal from mixed micelles of lipid and the nonionic detergent octyl glucoside. We now report on an extension of this method to allow for the use of other nonionic detergents with a much lower critical micelle concentration (cmc),[1] i.e., a much lower monomer solubility, than octyl glucoside. Detergents of this kind cannot be easily removed from a micellar solution by dialysis (which depends on establishment of a high monomer concentration gradient across the dialysis membrane), so that a different method of detergent removal is required. Adsorption by hydrophobic resin beads has been used previously for removal of Triton X-100, which has a cmc of 3×10^{-4} M (Holloway, 1973; Cheetham, 1979). The same method is employed here. The detergent used is *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$), which has a cmc of 9×10^{-5} M, significantly lower than the cmc of Triton X-100.

One of the reasons for undertaking this study is its close relationship to the functional reconstitution of membrane proteins after they have been purified in detergent solution. With this application in mind, the method described in a previous paper (Mimms et al., 1981) was explicitly designed to permit incorporation of membrane proteins into vesicle membranes with virtually no change in the preparative protocol. Octyl glucoside is, however, a poor detergent for this purpose, because many membrane proteins require a detergent with a longer alkyl chain length (and therefore lower cmc) if they are to retain their native structure and function during the purification process (Reynolds, 1981). Thus, although this paper is not concerned with reconstitution per se, the methods developed here are expected to be important for future reconstitution studies of proteins, such as the sarcoplasmic reticulum Ca pump protein, which retain their activity in $C_{12}E_8$ and similar polyoxyethylene detergents (Dean & Tanford, 1978) but not in octyl glucoside.

A more general aspect of this work is that the use of adsorbent beads provides a convenient means for varying the time course of detergent removal, and thereby a means for investigating kinetic factors that are important for vesicle formation. It is reasonably well established that bilayers represent the thermodynamically favored state of association for most phospholipids, including phosphatidylcholines, which is the lipid variety used in this study. However, bilayers can give rise to different types of macrostructures, including multiwalled liposomes and small or large single-walled vesicles, depending on the experimental conditions. This aspect of lipid association appears to be under kinetic rather than thermodynamic control. Some of the results obtained in this investigation will provide initial information on kinetic factors in the formation of unilamellar vesicles and on how they affect vesicle size and permeability.

Experimental Procedures

Materials

n-Dodecyl octaethylene glycol monoether ($C_{12}E_8$) was obtained as a homogeneous product from Nikko Chemicals Co., Tokyo, Japan. [^{14}C]$C_{12}E_8$ and [^{14}C]octyl glucoside were purchased from Research Products International Corp., Mount Prospect, IL. XAD-2 hydrophobic beads and nonradioactive octyl glucoside were from Sigma Chemical Co. Sephacryl S-1000 was from Pharmacia Fine Products, Sweden. Egg yolk lecithin (EYL) was supplied by Lipid Products, Nutley, England, and contained less than 0.5 mol % contamination by lysolecithin or fatty acid when analyzed by HPLC. All

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[1] Abbreviations: cmc, critical micelle concentration; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; EYL, egg yolk phosphatidylcholine; HPLC, high-pressure liquid chromatography.

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other chemicals were standard reagent grade.

Methods

Preparation of Micellar Solution. Solutions of mixed lipid–detergent micelles were obtained as described previously (Mimms et al., 1981) by incubating a detergent solution at the desired concentration with a lipid film deposited on the walls of a Corex tube. Our previous study showed that micellar solutions with detergent/lipid ratios of 10/1 or greater produced virtually 100% single-walled vesicles, whereas lower ratios were insufficient for complete dispersal of the lipid and led to the appearance of multilamellar liposomes in the final product. Micellar solutions in the present study generally contained 15 mM EYL and 150 mM $C_{12}E_8$.

Use of Hydrophobic Beads for Detergent Removal. Econo columns from Bio-Rad of dimensions 0.7 × 10 cm containing polyethylene support films were filled with an appropriate weight of wet adsorbent beads. The beads were washed exhaustively with buffer solution, and excess liquid was removed by applying gentle pressure with a syringe attached to the Luer fitting on the column reservoir cap. The sample containing detergent–lipid mixed micelles was then applied to the top of the column and rapidly forced through the moist beads by using the attached syringe. Removal of most of the aqueous solution from the beads prior to addition of the sample prevents undesirable dilution. However, caution must be exercised to avoid drying the beads completely.

Since the small sample volume does not come in contact with all of the adsorbent when this procedure is used, multiple passes were used to remove the detergent to the desired level. Other reasons for using multiple passes will become apparent later in the paper. Note that the short time of exposure to the bead surface in multiple passes is insufficient to achieve equilibrium (Cheetham, 1979).

Alternative methods for exposing the sample to hydrophobic beads have proved generally undesirable. Batch mixing and subsequent removal of the solution depleted of detergent by either filtration or centrifugation are experimentally difficult. Filtration often leads to severe foaming and losses of lipid vesicles on the filter surface. Since the volume of liquid is small relative to the volume of adsorbent, centrifugation results in large volume losses of the vesicle solution.

Binding Capacity of Beads. Titration of XAD-2 beads with $C_{12}E_8$ yielded an adsorptive capacity of 3×10^{-4} mol of $C_{12}E_8$ per g of wet beads, similar to that measured for Triton X-100 (Cheetham, 1979). Our procedures did not involve use of an amount of beads exactly equivalent to the amount of detergent to be removed, so that precise evaluation of binding capacity and investigation of possible surface heterogeneity with respect to binding affinity were not necessary.

Vesicle Size. Gel filtration chromatography on Sephacryl S-1000 was used to determine vesicle size, as described by us previously (Reynolds et al., 1983). This method provides a more objective measure of average size than can be obtained by electron microscopy and is especially advantageous for the determination of sample heterogeneity because a single elution profile contains information about all of the lipid present in the sample. Electron micrographs were obtained for some vesicle preparations, and size estimates based on them were found to be consistent with the gel chromatography results.

Ion Flux Measurements. Ionic permeability was determined by electrometric measurement of Cl^- efflux into $NaNO_3$ solutions, as described previously (Mimms et al., 1981), and by similar electrometric measurement of Na^+ efflux into choline chloride solutions. When used with vesicles prepared by octyl glucoside dialysis, the latter method gave essentially the same results as the isotope exchange method used previously. Solutions used for Na^+ efflux measurements contained 0.5 M Cl^-, which is a more permeant ion than Na^+ under all conditions employed, so that counterdiffusion of choline cations would not be expected to be a limiting factor in the measurement. Electrodes for monitoring Cl^- concentration were obtained from Orion Research, and Na^+ electrodes were obtained from Beckman Instruments or Lazar Research Labs., Inc. Unimolecular rate constants (k) for ion efflux were obtained from logarithmic plots of $C_\infty - C$ vs. time, where C is the extravesicular concentration at time t and C_∞ is the final equilibrium concentration after the vesicles were lysed by addition of excess detergent. The permeability coefficient (P) was obtained from the relation

$$P/k = \frac{\text{internal volume/vesicle}}{\text{membrane area/vesicle}} \quad (1)$$

Internal Volume. Total trapped volume was determined from the difference between C_∞ and C_0, the external concentration at zero time. Zero time was defined as the midpoint of the time required to exchange the external medium (replacing Cl^- by NO_3^- or Na^+ by choline), which was usually about 7 min before the first potentiometric measurement. $C_\infty - C_0$ was obtained by extrapolating ion efflux plots (e.g., Figure 4) to this midpoint. The trapped volume per mole of phospholipid was used as a measure of the apparent vesicle diameter by calculations similar to those in Table I of Mimms et al. (1981).

Other Methods. Radioactivity was monitored by counting appropriate aliquots of the sample in ACS (Amersham) scintillation fluid in a Beckman LS 100C scintillation counter. Phospholipid was determined by the micro method of Bartlett (1959).

Results

Protocol for Detergent Removal. As noted under Methods, the molar concentration of detergent (in micellar form) must exceed the molar lipid concentration by at least 10-fold if the lipid is to be dispersed sufficiently to remove preexisting multilamellar aggregates. Usually 1 mL of micellar solution was used, containing 150 mM $C_{12}E_8$ and 15 mM EYL. For the experiments with octyl glucoside, the initial detergent content was 175 mM, to allow for the approximately 25 mM monomeric octyl glucoside that would be present because of this detergent's high cmc. The quantity of XAD-2 beads that would be barely sufficient to remove all the detergent from such a micellar solution would be about 0.5 g of wet beads, but in practice it proved desirable to use an excess, typically 2.8 g of wet beads. In some experiments, sequential treatments with less than 0.5 g were used. Regardless of the precise protocol, contact between beads and the micellar solution and subsequent separation of detergent-depleted supernatant from the beads were achieved by the short-column technique described under Methods.

Several protocols were tried, the simplest involving long incubation of the micellar solution with an excess of beads. This method efficiently removed detergent but was unsatisfactory because it resulted in large loss of lipid by adsorption to the beads. It was found that lipid loss can be minimized (though not entirely avoided) by rapid passage of the micellar solution through the beads. Several rapid passes through the same bead volume can remove as much detergent as a single long incubation, but loss of lipid is significantly decreased. Another advantage of this procedure is to permit incubation of the solution for any desired length of time between passes

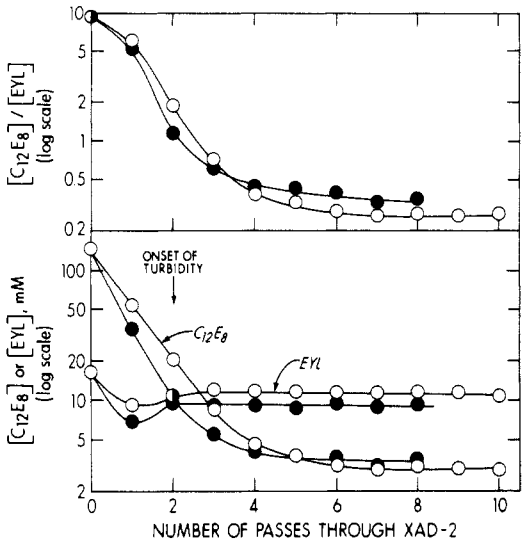

FIGURE 1: Time course of detergent removal and EYL concentration. Open circles represent the average of two experiments using 2.8 g of wet beads; filled circles represent one experiment using 5.6 g of wet beads.

through the beads, so that time-dependent changes at different levels of detergent depletion can be studied.

In some experiments, the quantity of beads employed was less than that required for complete removal of detergent, and fresh beads were added for successive passes through the bead-filled volume. This procedure appeared to have no advantage for the experiments reported here but may be useful for future experiments in which a more precise interplay of incubation time and residual detergent level is desired.

Time Course of Detergent Removal and Vesicle Formation. Figure 1 is representative of several experiments designed to follow the time course of detergent removal. An excess of wet beads (2.8 g) was used, and the experiment was repeated with 5.6 g to make certain that the availability of free bead surface was not a limiting factor. The micellar solution was passed through the beads as rapidly as possible (about 20 s), so that only part of the detergent was removed in a single pass. Solutions were allowed to stand 30–60 s between passes. The results demonstrate that detergent removal and vesiculation occur in three distinct stages.

(1) Detergent removal from the initially clear solution is relatively fast, but coadsorption of lipid (about 30% of the original lipid content) cannot be avoided. This stage ends at an average detergent/lipid ratio of 2/1, when the solution becomes turbid, indicative of self-association of the lipid. One cannot be certain whether this initial turbidity represents formation of closed vesicles.

(2) Detergent removal continues at nearly the same rate in the now turbid suspension, but there is no further loss of lipid. On the contrary, the overall lipid content of the suspension is seen to increase. This result is consistent with the supposition that closed vesicles, relatively slow to exchange lipid with the adjacent solution, must now exist. A reasonable explanation for the increase in the lipid content of the suspension is that the additional lipid is derived from the volume and/or surfaces within the pores of the adsorbent beads. Mixed micelles at the beginning of vesiculation are certainly to some extent heterogeneous, and it is presumably the micelles with the highest lipid content that form vesicles. Vesicles are too large to enter the resin pores and, in effect, constitute a separate phase. Only the nonvesicular (and lipid-depleted) part of the bulk suspension is capable of rapid exchange with the pore contents, and net lipid movement from pores to bulk suspension

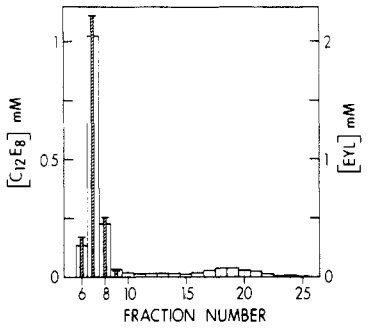

FIGURE 2: Gel chromatography of vesicle suspensions on Sephadex G-200. The principal peak (fraction 7) represents vesicles eluting at the void volume: hatched areas represent EYL concentrations and open areas $C_{12}E_8$ concentrations. The minor peak (fractions 18 and 19) represents monomeric $C_{12}E_8$, the concentration being slightly below the cmc. The initial $C_{12}E_8$/EYL ratio of the sample placed on the column was 0.57; the ratio in the vesicle peak (void volume) is 0.46; rechromatography of the peak tube (data not shown) reduced the ratio in the vesicle peak to 0.35.

is then a consequence of the drive toward equilibrium in the exchange process.

(3) When the detergent/lipid ratio is reduced to about 1/1, there is a sharp decrease in the rate of detergent removal. In some experiments (data not shown), fresh beads were added during this third stage, without significant effect on the residual detergent content. No such anomalous retention of detergent outside the bead pores is observed in the absence of lipid: repeated passage through the beads in this case readily reduces the detergent content to close to 10^{-5} M, i.e., well below the cmc and a factor of 100 below that seen in Figure 1.

The detergent/lipid ratio at the onset of this slow stage is roughly 50% of the ratio when vesicles are first formed, so that one possible explanation for the slow rate is that the detergent that is hard to remove is bound to the inner leaflet of the vesicular membrane and that slow permeation through the bilayer becomes rate determining. This explanation will be seen to be consistent with much of the data to be presented below, but, as the Discussion will show, it leaves one result unexplained.

Additional evidence for the tenacity of detergent retention in stage 3 was provided by gel chromatography on Sephadex G-200 and by ultracentrifugal separation. For chromatrography, a 0.1-mL solution with an initial detergent/lipid ratio of 0.57 was used at a flow rate that required 1 h for elution of the total volume. Vesicles elute in the void volume on Sephadex G-200, and, as Figure 2 shows, most of the $C_{12}E_8$ eluted with the lipid at this position. (The $C_{12}E_8$/lipid ratio was essentially constant across the peak at 0.46.) The peak tube from this experiment was rechromatographed, and only a small further loss of detergent was observed, in spite of the fact that the free $C_{12}E_8$ concentration in contact with the vesicles was sharply reduced: the $C_{12}E_8$/lipid ratio in the void volume peak fell from 0.46 to 0.35. There is a 10-fold dilution between the first chromatography (data of Figure 2) and the rechromatography (data not shown), and the $C_{12}E_8$ concentration spread across the column behind the lipid peak was reduced to about 2 μM.

Sedimentation experiments were carried out at 45 000 rpm, by using 0.65 mL of a vesicle suspension with an initial detergent/lipid ratio of 0.45. Six experiments were carried out, at successively greater dilutions of the suspension, which in turn led to successively lower $C_{12}E_8$ concentrations in the supernatant recovered after centrifugation. It required 16 h to pellet the vesicles, and a larger loss of detergent was observed than in the 1-h chromatography experiment. The re-

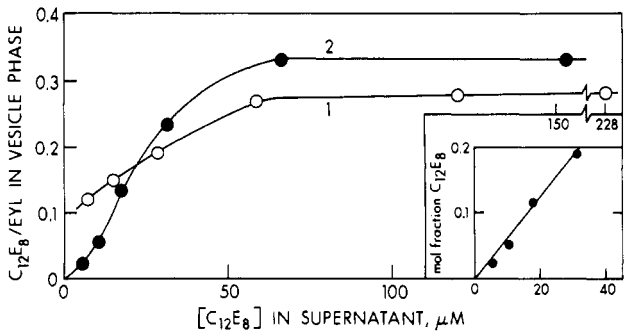

FIGURE 3: $C_{12}E_8$ content of the pellet after centrifugation as a function of the free $C_{12}E_8$ concentration in the supernatant. Curve 1: Loss of detergent from vesicles with an initial $C_{12}C_8$/EYL ratio of 0.45. Data were generated by dilution with buffer to varying degrees before centrifugation. Curve 2: Incorporation of $C_{12}E_8$ into initially detergent-free vesicles prepared by use of octyl glucoside. Data were generated by addition of varying amounts of $C_{12}E_8$ prior to centrifugation. Inset: The first four points of curve 2 replotted with the mole fraction of $C_{12}E_8$ as the ordinate.

sults are given in Figure 3, and they show that the final $C_{12}E_8$/EYL ratio attained in the pellets depends on the monomeric $C_{12}E_8$ concentration in the solution (as observed in the supernatant): the ratio in the pellet was found to be 0.28 at all free $C_{12}E_8$ concentrations above the cmc, where the monomer concentration is essentially independent of the total supernatant concentration, but lower ratios were observed below the cmc, where all of the free $C_{12}E_8$ exists as monomer. It should be noted that the $C_{12}E_8$/EYL ratio does not approach zero detergent concentration, which serves to demonstrate that the results in the Figure are not equilibrium data.

It is important to point out that the residual $C_{12}E_8$ associated with the vesicles in all of these experiments is mostly membrane bound. The intravesicular volume is small, and free $C_{12}E_8$ trapped in this volume when vesicles are first formed (when the concentration of $C_{12}E_8$ is 5–10 mM) could contribute no more than 0.01–0.02 to the overall $C_{12}E_8$/EYL ratio.

Vesicle Size. Vesicle size was determined by gel exclusion chromatography using S-1000 Sephacryl. Particles with diameters up to 3000 Å are retarded by this resin, and average diameters can be measured as previously described (Reynolds et al., 1983). With columns that are fully presaturated with lipid, no adsorptive loss of lipid occurs when vesicles are chromatographed. A significant result obtained with the preparations in this study (Figure 4) was that no lipid was eluted in the void volume, which indicates the absence of large multilamellar vesicles or aggregates. Essentially 100% of the lipid initially placed on the column eluted as a single peak, presumably containing only single-walled vesicles (see below for additional confirmation).

Vesicles formed by rapid detergent removal, as in Figure 1, were found to be relatively small, with an average diameter of 500–600 Å. When detergent removal was interrupted at various detergent/lipid ratios, it was found that the 500–600-Å size represented the size of vesicles when first formed, at detergent/lipid ratios between 2/1 and 1/1; i.e., no change in size occurs when the subsequent reduction in detergent content is carried out rapidly. However, if vesicles were allowed to stand for 1 or more days at a detergent/lipid ratio near 1.5, the preparations became much more heterogeneous (curve 2 in Figure 4B), with a gradual increase in average diameter. These results presumably reflect slow fusion of the vesicles, due to the still high content of detergent in the membranes. Average diameters up to 880 Å were measured in the limited number of experiments summarized in Table I. Incubation at a detergent/lipid ratio of 3/1, i.e., just before

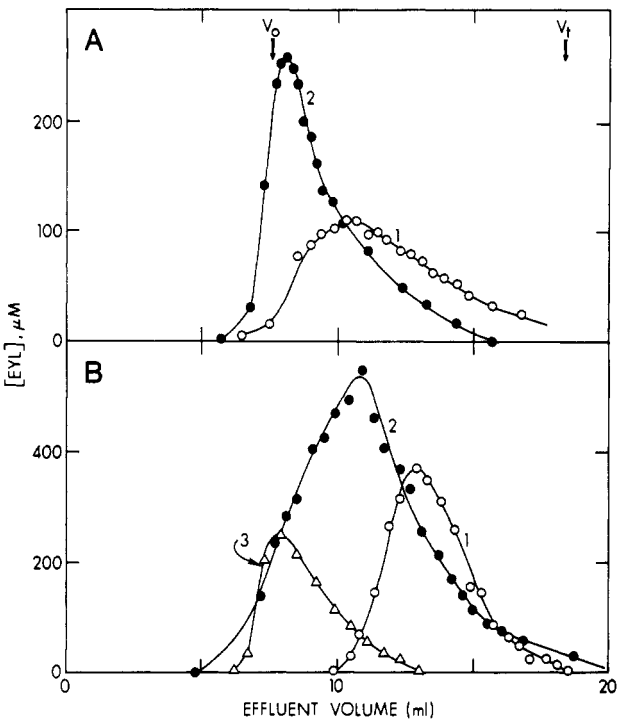

FIGURE 4: Representative results from gel chromatography on Sephacryl S-1000. (A) Vesicles from octyl glucoside–EYL mixed micelles. Curve 1 represents rapid detergent removal by use of XAD-2 beads; curve 2 represents slow removal by dialysis. Peak positions correspond to diameters of 1000 and 2100 Å, respectively. (B) Vesicles from $C_{12}E_8$–EYL mixed micelles. Curve 1 represents rapid detergent removal without incubation at any stage, curve 2 represents a suspension incubated at stage 2 for 1 day, and curve 3 represents a similar suspension prepared at an ionic strength of less than 0.001. Peak positions correspond to diameters of 560, 880, and 2200 Å, respectively.

Table I: Effect of Interruption of Detergent Removal on Vesicle Size

detergent removal protocol			
$C_{12}E_8$/EYL molar ratio after initial rapid removal	period of incubation (days)	$C_{12}E_8$/EYL molar ratio after subsequent rapid removal	average diameter (Å)
0.3	0		560
0.3	1		560
0.5	0		540
1.0	4	0.4	540
1.4	0[a]		610
1.4	1		880
1.7	1		830
1.8	1	0.5	780
2.0	2	0.5	790
2.2	0		680
3.0	1	0.9	670

[a] Elution time on the S-1000 column was 1–2 h. At the $C_{12}C_8$/EYL level of this sample, a small increase in the average size would be expected to occur during the elution process.

the onset of turbidity, had only a small effect on the vesicles produced by subsequent rapid detergent removal. More important, once the detergent/lipid ratio falls to 1.0 and less (beginning of stage 3 of detergent removal), incubation produces no further change in size: vesicles passed rapidly through stage 2 remain small for at least several days. This result is consistent with the possibility that the slow rate of detergent removal in stage 3 of this process arises from a slow rate of transfer from the inner leaflet of the bilayer: absence of detergent from the external surface of the vesicle would presumably make the rate of fusion as low as it is for vesicles containing no detergent at all.

Table II: Vesicle Size from Measurement of Trapped Volume

average diameter (Å)	
from trapped volume	similar prepn,[a] S-1000 column
500	560
600	560
700	780
1400[b]	1000[b]
2000[c]	2100[c]

[a] Sample prepared by similar detergent removal protocol. [b] Vesicles prepared by use of octyl glucoside with rapid detergent removal by XAD-2 beads. The S-1000 value is from curve 1 of Figure 4; a similar preparation had a peak position corresponding to 1300 Å. [c] Vesicles prepared by octyl glucoside and slow dialysis.

Intravesicular Trapped Volume. Internal volume was measured as described under Methods. The measurement is useful as a discriminant between unilamellar and multilamellar vesicles. If all of the analytically measured phospholipid is present in the form of unilamellar vesicles, then the internal volume per mole of phospholipid becomes a unique function of vesicle diameter, as given in Table I of Mimms et al. (1981), and one can use the volume measurement as a measure of vesicle size. Multilamellar vesicles have a much smaller internal volume per mole of phospholipid than single-walled vesicles and, if present in a significant amount, would by this method yield an apparent diameter substantially below the true value. Table II gives representative data and shows that the diameters calculated from internal volume measurements are in good agreement with diameters obtained by S-1000 gel chromatography. The result demonstrates that multilamellar vesicles (and also lipid in any form unable to trap solvent) cannot comprise a significant fraction of the vesicle preparations.

Effect of Low Ionic Strength. A few measurements were made to investigate the effect of ionic strength, using micellar solutions at a total salt concentration of less than 1 mM. A similar time course for vesicle formation was observed, but, surprisingly, the vesicles formed were found to be much larger than those obtained at higher ionic strength: i.e., vesicles that were not incubated during stage 2 of detergent removal had an average diameter of 1400 Å; vesicles incubated for 4 days at a detergent/lipid ratio of 1.5/1 had an average diameter of 2200 Å (curve 3 of Figure 4B).

Experiments with Octyl Glucoside. The preceding results suggest that spontaneous vesiculation occurs when the detergent/lipid ratio is still quite large and that vesicles formed initially are relatively small. To see whether this is a general phenomenon, we reinvestigated vesicle formation from mixed micelles of phospholipid with octyl glucoside, by using XAD-2 beads for rapid detergent removal (as in Figure 1) instead of the slow dialysis method used before (Mimms et al., 1981). The results confirmed the generality of the phenomenon: vesicles in preparations still containing a 1/1 detergent/lipid ratio were found to have an average diameter of only about 1000 Å, and the trailing of the elution peak (Figure 4) suggests that many vesicles were smaller than that. The same micellar solution was then subjected to the slow dialysis method, where the time of exposure to all intermediate detergent levels is inevitably long. As Figure 4 shows, an average vesicle diameter above 2000 Å was obtained, as before. (In the previous study, vesicle size was estimated by electron microscopy.)

Ionic Permeability. Figure 5 shows typical plots for the first-order efflux of Cl^- and Na^+ from vesicles formed by use of $C_{12}E_8$. Table III gives permeabilities calculated from such data, together with comparable results for vesicles prepared by the octyl glucoside dialysis method. The measured

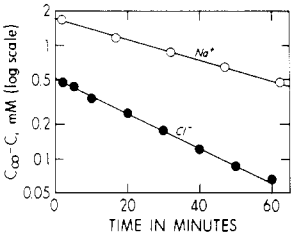

FIGURE 5: Representative logarithmic plots for measurement of Na^+ and Cl^- efflux from vesicles.

Table III: Ion Flux Measurements

ion	$C_{12}E_8$/EYL molar ratio	average vesicle diameter (Å)	efflux rate constant, k (s^{-1})	permeability coeff, P (cm/s)
	Vesicles Prepared by Use of Octyl Glucoside			
Cl^-	0	2100	2.3×10^{-5}	0.8×10^{-10}
	0[a]	2300	8.3×10^{-5}	3.0×10^{-10}
Na^+	0	2100	4.4×10^{-7}	1.4×10^{-12}
	0[b]	2300	2.6×10^{-7}	1.0×10^{-12}
	0[a]	2300	4.4×10^{-7}	1.6×10^{-12}
	Vesicles Prepared by Use of $C_{12}E_8$			
Cl^-	0.26	600	5.0×10^{-4}	3.7×10^{-10}
	0.43	550	9.6×10^{-4}	6.4×10^{-10}
	0.49	710	5.9×10^{-4}	5.5×10^{-10}
	0.57	710	1.0×10^{-3}	9.2×10^{-10}
Na^+	0.23	620	3.5×10^{-4}	2.7×10^{-10}

[a] Mimms et al. (1981), showing the effect of incorporation of 1 molecule of glycophorin per 6000 molecules of EYL. [b] Mimms et al. (1981), measured by isotope exchange. The present work (preceding entry) used an electrometric method.

permeabilities are extraordinarily low, considering the high detergent content of the membranes of vesicles formed by use of $C_{12}E_8$ (20–40% of the lipid content). Equally remarkable is that the discrimination between small cations and small anions that is characteristic of detergent-free vesicular membranes (Bangham et al., 1965; Papahadjopoulos & Watkins, 1967; Hauser et al., 1973; Mimms et al., 1981) has become greatly reduced; i.e., the presence of detergent has clearly affected Na^+ permeability more than Cl^- permeability, to the point where the two are nearly equal. (Table III shows two results for octyl glucoside vesicles with membrane-incorporated protein. The incorporated protein raises P_{Cl^-} to about the same level as is observed for $C_{12}C_8$-containing vesicles at a $C_{12}E_8$/EYL ratio of 0.26, but in this case, there is no disparate effect on P_{Na^+}.)

Titration of Detergent-Free Vesicles with Detergent Added from the Outside. It was of interest to see whether $C_{12}E_8$ added to initially detergent-free vesicles has a similar effect on permeability as $C_{12}E_8$ remaining in the vesicular membrane when vesicles are prepared as described above. Vesicles prepared by dialysis from octyl glucoside–EYL micelles were used, with a diameter of about 2000 Å and containing essentially no residual detergent. Incorporation of $C_{12}E_8$ was measured by using the ultracentrifugal separation method described earlier, and the results are shown in Figure 3. As was expected, there is no evidence for a kinetic barrier to incorporation (added detergent presumably enters the outer leaflet of the bilayer), and the data below the cmc of $C_{12}E_8$ in fact closely correspond to what would be expected for an ideal equilibrium distribution curve. Ideal mixing within the bilayer (Tanford, 1980) would make the mole fraction of detergent in the bilayer (X_D) a linear function of the monomer concentration (C_{mon}):

$$X_D = C_{mon}/\text{cmc} \qquad (2)$$

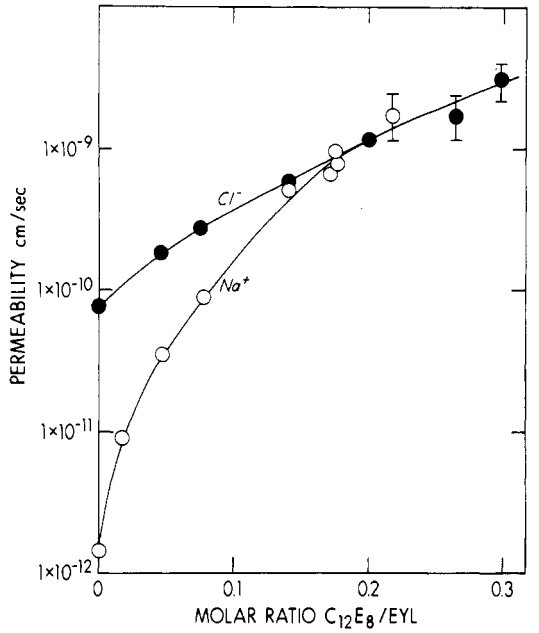

FIGURE 6: Effect of $C_{12}E_8$ incorporation into initially detergent-free vesicle membranes on permeability coefficients for Na^+ and Cl^-.

with C_{mon} equal to the total free detergent concentration as long as one is below the cmc. A linear relation within experimental error is observed (Figure 3, inset), but the slope is only half the theoretical slope of 1/cmc that is predicted by the equation. This result is consistent with the idea that only half the bilayer volume (outer leaflet) is accessible to the detergent in the time of the experiment.

Figure 6 shows the Cl^- and Na^+ permeabilities measured for vesicles with detergent incorporated in this way, and the results are qualitatively consistent with the data in Table III: detergent incorporation has a greater effect on P_{Na^+} than on P_{Cl^-}, and the discrimination between them essentially disappears when the vesicular $C_{12}E_8$/EYL ratio reaches 0.2. There is, however, a quantitative difference between the two sets of results: permeabilities at the upper end of Figure 6 are about 2×10^{-9} cm/s, whereas in Table III, at a similar $C_{12}E_8$/EYL ratio, they are about 3×10^{-10} cm/s.

Discussion

This paper has demonstrated that the method of vesicle formation described in a previous paper (Mimms et al., 1981) can be extended to nonionic detergents with a low cmc if hydrophobic beads are used for removal of the detergent. There is an unavoidable disadvantage in this method of detergent removal because lipid and detergent can both be adsorbed by the beads, whereas dialysis membranes (used in the previous paper for removal of octyl glucoside) discriminate in favor of the smaller detergent molecules. Loss of lipid in the procedure can therefore not be avoided, but our results show that lipid loss can be minimized if one does not try to remove as much detergent as possible in a single incubation with excess beads but instead passes the micellar solution several times rapidly through the same bead volume.

The protocol that was employed (in part to avoid lipid loss) is well suited for investigating the time course of vesiculation. The results show that vesicle size is not an equilibrium property of the system but is kinetically determined. Initially formed vesicles are small, but fusion and enlargement occur spontaneously if detergent removal after initial vesiculation is sufficiently slow. This result is probably quite general. The data of this paper show that it applies to octyl glucoside as well as to $C_{12}E_8$, and preliminary data using sodium dodecyl sulfate suggest it applies to ionic detergents as well. Zwizinski & Wickner (1977), in a study of reconstitution of a viral coat protein, demonstrated the same phenomenon (somewhat less directly than here) in a procedure where a bile salt anion was used as detergent.

When $C_{12}E_8$ is used as detergent, both the initial and final vesicle sizes were found to be significantly larger if the preparative procedure was carried out at very low ionic strength. Since neither the detergent nor the lipid head group of EYL carries a net charge, this result may at first seem surprising. The probable explanation is that the negatively charged phosphate moieties of the phosphatidylcholine head groups are much closer to the hydrocarbon core of the bilayer than the $N(CH_3)_3^+$ groups of the choline moiety. In small vesicles, the surface area per head group is very small at the inner bilayer surface, bringing the phosphate groups close together. At low ionic strength, electrostatic repulsion between the negative charges would tend to prevent such close approach between them, but a high salt concentration exerts a screening effect and would permit closer approach and therefore a smaller vesicle diameter.

A striking feature of the results with $C_{12}E_8$ is the inability to remove all of the detergent from the vesicle membranes in a reasonably short time. This phenomenon is consistent with the established fact that the rate of flip-flop of lipid and other amphiphilic molecules across a bilayer is slow, which would make removal of detergent from the inner leaflet of the vesicular bilayer a very slow process. The data of Figure 3 for incorporation of detergent into initially detergent-free vesicles support this explanation: the slope of the linear plot in the inset suggests that detergent added from the outside may partition exclusively into the outer leaflet.

A comparison between octyl glucoside and $C_{12}E_8$ also supports this explanation. There is no impediment to complete removal of detergent when vesicles are prepared from mixed micelles of octyl glucoside and EYL (Mimms et al., 1981), which means that octyl glucoside, unlike $C_{12}E_8$, must be able to exchange relatively rapidly between the two leaflets of the bilayer. Partition measurements analogous to those of Figure 3 have been made (unpublished results). Partitioning is in this case reversible, i.e., independent of whether one starts with detergent-free vesicles or with vesicles containing a detergent excess, which proves that the data represent thermodynamic equilibrium. The plateau level of incorporation is more than double the maximal detergent/EYL ratio in Figure 3, and the low-concentration points, when plotted as in the inset of Figure 3, have a slope that slightly *exceeds* the slope calculated from eq 2 for ideal partitioning, in contrast to the 50% of theoretical slope that is observed with $C_{12}E_8$.

The one observation that is inconsistent with an explanation based on flip-flop rates is that the plateau levels of the two curves in Figure 3 (above the cmc) are about equal. This is consistent with the suggestion that curves 1 and 2 reflect detergent bound to only one leaflet of the bilayer. However, if there is spontaneous partitioning of $C_{12}E_8$ into the outer leaflet when the free detergent concentration is 100 μM (as in curve 2), why is there not also spontaneous incorporation at the same free detergent concentration into the outer leaflet of vesicles that contain hard to remove detergent on the inner leaflet, which would make the overall $C_{12}E_8$/EYL ratio for curve 1 higher than that for curve 2? Does detergent bound to the inner leaflet somehow affect the outer leaflet so as to prevent reasonably rapid incorporation of additional detergent?

In view of this anomalous result, it is desirable to explore the possibility that the tenacity of $C_{12}E_8$ retention in the

vesicular membrane is not related to a slow flip-flop rate at all but is instead a result of nonideal mixing in the bilayer. In this case, both plateau levels in Figure 3 could represent a unique equilibrium state, with both halves of the bilayer having the same composition, and one could then rationalize the existence of the state by supposing that a mixture with an EYL/$C_{12}E_8$ ratio of about 3/1 corresponds to a molecular complex of some kind that has unusual thermodynamic stability. Such a possibility is in fact untenable. The permeability data of Figure 6 and Table III show that the vesicular membranes formed by detergent removal (corresponding to curve 1 of Figure 3) and by detergent addition (curve 2 of Figure 3) are not the same and therefore do not represent a single equilibrium state: ion permeabilities at the same detergent/lipid ratio differ by nearly an order of magnitude, depending on which pathway was used to arrive at the final composition. Furthermore, formation of a stable complex would be expected to lead to deviations from ideality (when $C_{12}E_8$ is added from the outside) opposite to what is observed in the inset to Figure 3: partitioning into the bilayer at low $C_{12}E_8$ concentrations should be more favorable than predicted by eq 2, rather than less.

Regardless of the ultimate explanation for this one anomalous observation, there is an unambiguous implication of our results: disruption of a vesicular lipid bilayer by addition of detergent is clearly not the symmetrical opposite of the vesiculation process. If the slow flip-flop hypothesis is correct, then detergent added to an existing bilayer would first accumulate in the outer leaflet, and transfer to the inner leaflet would be much slower. Direct evidence for biphasic detergent incorporation of this kind has been reported for protein-containing cellular membranes (Billington et al., 1977).

The relatively low ionic permeabilities of vesicular membranes containing as much as 0.4 mol of $C_{12}E_8$ per mol of lipid represent a surprising result. Observed permeabilities were in the range of 10^{-10}–10^{-9} cm/s, larger than those for detergent-free membranes but much to small to merit the term "leaky". [The sarcoplasmic reticulum membrane is an example of a leaky in vivo membrane. Its permeability coefficients are 5×10^{-8} cm/s for Na^+ and K^+ and 3×10^{-6} cm/s for Cl^- (Kasai & Kometani, 1979).] Perhaps the most interesting aspect of our permeability data is the near-equalization of the permeability coefficients for Na^+ and Cl^- when the detergent content is high. An explanation consistent with this observation is that the presence of detergent creates an alternate nonspecific permeation pathway across the membrane, perhaps reflecting the existence of transient membrane defects. The observed value of P would then be the sum of two components, P_o, the intrinsic permeability, and P_d, the "defect" permeability. If P_d is on the order of 10^{-10}–10^{-9} cm/s, it would make P much larger than P_o for Na^+, but not for Cl^-. The same kind of phenomenon is observed when planar bilayers mounted on a solid support are compared with vesicular membranes (Brunner et al., 1980), the defect here being most likely the torus around the planar membrane at the locus of attachment to its support.

We should like in conclusion to reemphasize the need for systematic studies of the kind reported here and in our previous paper (Mimms et al., 1981) for the purpose of creating a sound scientific basis for "reconstitution" of protein-containing vesicles. Empirical procedures often yield less than satisfactory results, as is illustrated for example by recent work on reconstitution of erythrocyte adenylate cyclase, using the detergent Lubrol PX (a commercial product similar to $C_{12}E_8$) and using SM-2 hydrophobic beads for detergent removal (Keenan et al., 1982). In this study, 50% of the vesicles obtained were multilamellar, and the remainder were unilamellar vesicles with a diameter of only 300 ± 100 Å. Many aspects of the preparative procedure were quite different from those employed in the work described here. In particular, the detergent/lipid ratio was too low to produce proper lipid dispersal, which undoubtedly accounts for the high content of multilamellar vesicles. The preparative method described in this paper satisfies all the criteria that make the procedure well suited for membrane protein incorporation. A preliminary experiment (not described) indicates that the beads do not adsorb typical membrane proteins when they are first incorporated into detergent micelles and when phospholipid is introduced into the same micelles before detergent is removed.

Registry No. $C_{12}E_8$, 3055-98-9; octyl glucoside, 29836-26-8; chloride, 16887-00-6; sodium, 7440-23-5.

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