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Binding of a Nonionic Detergent to Membranes: Flip-Flop Rate and Location on the Bilayer

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ABSTRACT: The kinetic aspects of amphiphile interaction with intact membranes (unilamellar and multilamellar liposomes, sarcoplasmic reticulum vesicles) were studied, with the nonionic detergent octa(ethylene glycol) dodecyl monoether ($C_{12}E_8$) as a prototype. $C_{12}E_8$ was bound to these membranes noncooperatively and with a maximum of 0.6–0.8 mol per mole of phospholipid, before the onset of solubilization. Binding was not affected by ultrasonication to expose internal binding sites on the inner leaflet. All detergent could be removed from the membranes by treatment with hydrophobic beads. Furthermore, bound detergent, also from the inside of multilayered liposomes, comprising 10–20 bilayers, was quickly released by dilution of the membranes, followed by gel filtration. The time course of these processes was investigated with a rapid-filtration apparatus, using glass fiber filters to deposit membrane material. Both detergent binding and removal could be described by a monoexponential process with a half-time of approximately 350 ms for all types of membranes. Binding of detergent enhanced the intrinsic fluorescence of sarcoplasmic reticulum vesicles. This occurred in less than 100 ms, probably as the result of direct interaction of $C_{12}E_8$ with Ca^{2+}-ATPase at a few binding sites. The data show that flip-flop of $C_{12}E_8$ across lipid membranes is a rapid process that cannot account for incomplete detergent removal in reconstitution experiments [Ueno, M., Tanford, C., & Reynolds, J. A. (1984) *Biochemistry 23*, 3070–3076]. It is also suggested that other nonionized amphiphiles, including those with an anesthetic action, rapidly gain access to membrane proteins on the inside of the cell, even when used at low, clinical doses.

Nonionic detergents are widely used to purify and characterize membrane proteins [for a recent review see Møller et al. (1986)]. Like Triton X-100, the homogeneous detergent octa(ethylene glycol) dodecyl monoether ($C_{12}E_8$)[1] is an efficient solubilizer of membrane proteins. It was originally reported to be useful in the preparation of active Ca^{2+}-ATPase, first in an oligomeric state (le Maire et al., 1976) and subsequently in a monomeric state (le Maire et al., 1978; Dean & Tanford, 1978; Andersen et al., 1982). The detergent has also been used at subsolubilizing concentrations to perturb membrane structure and function, thereby providing a new approach to the characterization and understanding of the role of partial reactions during enzyme turnover. This line was followed, for example, for Ca^{2+}-ATPase (Lüdi et al., 1982; Andersen et al., 1983; McIntosh & Davidson, 1984; Champeil et al., 1986) and Na^+,K^+-ATPase (Huang et al., 1985).

Another promising application of nonionic detergents is their use in reconstitution studies after solubilization of membrane protein and lipid. Originally, octyl glucoside was used for this purpose (Helenius et al., 1981; Mimms et al., 1981; Jackson & Litman, 1982), due to its high cmc, which facilitates removal by dialysis or similar procedures. However, in many cases this carbohydrate detergent is too abrasive to maintain membrane proteins in an active state. Instead, successful attempts have been made to use $C_{12}E_8$ as a membrane protein and lipid solubilizing agent for reconstitution studies (Cornelius & Skou, 1984; Andoh & Yamamoto, 1985). In a comprehensive study of factors affecting vesicle size and other properties of reconstituted liposomes, Ueno et al. (1984) noted that it was difficult to remove a significant portion of $C_{12}E_8$ from the reconstituted vesicles. The authors suggested that

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[1] Abbreviations: $C_{12}E_8$, octa(ethylene glycol) dodecyl monoether; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EPC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SR, sarcoplasmic reticulum; cmc, critical micelle concentration; Ca^{2+}-ATPase, calcium ion activated adenosinetriphosphatase; HPLC, high-performance liquid chromatography; ESR, electron spin resonance; DTT, dithiothreitol.

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this residual $C_{12}E_8$ might represent detergent trapped inside the reconstituted vesicles. This view implies a slow movement of detergent across the bilayer, similar to that of phospholipids. In the present paper we have addressed ourselves to the kinetic features of the removal and interaction of $C_{12}E_8$ with liposomes and a protein-containing membrane, the sarcoplasmic reticulum. We have approached the question by procedures that would ensure access of $C_{12}E_8$ to the inner side of the closed vesicles, and we have followed how the detergent was released from the membranes under these conditions. To our surprise, we found that $C_{12}E_8$ is quickly distributed between the leaflets even if there is 10–20 bilayers to be crossed. Our investigation questions the concept that the lipid bilayer in general constitutes a significant barrier for passage of nonionized amphiphiles.

Experimental Procedures

Materials. $C_{12}E_8$ was obtained from Nikko Chemicals, Tokyo, Japan, and [1-^{14}C]$C_{12}E_8$ from CEA, Saclay, France. The purity of the labeled compound was checked by HPLC. Egg yolk phosphatidylcholine (EPC) was prepared as described elsewhere (Singleton et al., 1965), and phosphatidyl[*N-methyl*-^{3}H]choline, [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC), and [6,6′-^{3}H(N)]sucrose were obtained from Amersham (Les Ulis, France); SM2 Bio-Beads were from Bio-Rad; PD-10 columns prepacked with Sephadex G-25M were obtained from Pharmacia, Uppsala, Sweden. GF/F filters were from Whatman. All other chemicals were of standard reagent grade.

Preparation of Membranes. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as previously described (Champeil et al., 1985). Different types of liposomes (unilamellar or multilamellar liposomes, with or without inclusion of detergent) were also prepared:

Type I. Large unilamellar vesicles (1000–2000-Å diameter) were prepared, essentially as described by Rigaud et al. (1983), but with modifications to permit inclusion of detergent: EPC (12.5 mg) containing [^{3}H]DPPC was solubilized in 1.5 mL of ether, followed by addition of 0.5 mL buffer (10 mM Tes, pH 7.5, 100 mM KCl, and 0.1 mM Ca^{2+}), containing 2 mg/mL $C_{12}E_8$ and [^{14}C]$C_{12}E_8$. The resulting suspension was sonicated for 2–3 min on ice and then slowly evaporated under N_2 until a gel phase appeared, followed by a more fluid suspension. One milliliter of detergent-free buffer was added and residual ether evaporated. The vesicles were then filtered on 0.4- and 0.2-μm nucleopore filters to obtain the large unilamellar vesicles.

Type II. Large unilamellar vesicles (1000–2000-Å diameter) were prepared exactly as described above, except that $C_{12}E_8$ was either completely omitted or added at the end of the procedure, at the same grams of detergent to grams of lipid ratio as for type I liposomes (0.08 g/g of lipid).

Type III. Multilamellar vesicles with inclusion of detergent were prepared as follows: EPC (12.5 mg) was solubilized in a mixture of 650 μL of chloroform, 250 μL of methanol, and 20 μL of a solution containing 50 mg of $C_{12}E_8$/mL of water. This preparation was evaporated to dryness, and 1.5 mL of buffer without detergent was added. Vigorous vortexing produced the multilamellar vesicles.

Type IV. Standard multilamellar vesicles were prepared as described above, except that $C_{12}E_8$ was omitted from the preparation or added at the beginning of the experiment at 0.08 g of detergent/g of lipid.

Liposomes were checked for size, homogeneity, and absence of structural effects of the detergent when it was used at 0.08 g/g of lipid by freeze–fracture electron microscopy in the presence of 30% glycerol, as previously described (le Maire et al., 1981).

Stoichiometry and Kinetics of Detergent Binding. Glass fiber filters with an average pore radius of 0.7 μm (Whatman GF/F filters) were placed in buffer a few minutes before use in either a usual Millipore filtration device or the Biologic rapid-filtration system (Dupont, 1984). The membranes (containing 50 μg of phospholipid) were deposited on the filters under light vacuum. To measure binding stoichiometry, 4–5 mL of buffer, containing [^{14}C]$C_{12}E_8$, was perfused in about 30 s through these filters, which were then counted in a scintillation fluor (Picofluor). For rapid kinetic studies of detergent removal, [^{14}C]$C_{12}E_8$-equilibrated vesicles were perfused with small volumes of detergent-free buffer at a rapid rate (4 mL/s). The assay included appropriate controls to check for nonspecific binding and wetting of the filters as described in legends to figures.

Small Columns or Hydrophobic Beads Used for Detergent Removal. (A) Columns. Liposomes of types I–III, prepared as described above at 0.4 mg/mL [^{14}C]$C_{12}E_8$ and 5 mg/mL ^{3}H-labeled lipid, were diluted 500-fold (10 μL in 5 mL of buffer). An aliquot of 1 mL was counted, and 2.5 mL was loaded onto a PD-10 column, preequilibrated with buffer. In the standard procedure the first 2.5-mL fraction was discarded. The membranes were collected in the second fraction (2.5–6 mL), corresponding to the void volume, and an aliquot was counted to measure the retention of $C_{12}E_8$ in the eluted liposomes. In more detailed studies on column behavior, fractions of 1 mL were collected.

(B) Bio-Beads. Bio-Beads were thoroughly rinsed with methanol and buffer before use, and the finest beads were removed. Liposomes of types I–III were diluted 5 times with buffer so that 1 mL of the final suspension contained 1 mg of lipid, 0.08 mg of total $C_{12}E_8$, and about 200 mg of Bio-Beads. The mixture was continuously and gently stirred, except when 25-μL aliquots were pipetted off after various periods of incubation. The density of the methanol-treated Bio-Beads is such that in the absence of stirring they rapidly sediment due to gravity, thus enabling a supernatant solution devoid of Bio-Beads to be pipetted off.

Other Methods. The partitioning of $C_{12}E_8$ between an aqueous phase and various organic solvent phases was examined by vigorous shaking for $^1/_2$–2 h of equal volumes (5 mL) of buffer, containing [^{14}C]$C_{12}E_8$ and heptane, octanol, diethyl ether, chloroform, or benzene, followed by low-speed centrifugation (1000*g*) to separate the phases. Phospholipids were determined by the micromethod of Bartlett (1959). Protein concentrations were measured either according to Lowry et al. (1951), corrected as previously described (Andersen et al., 1982), or by light absorption at 280 nm (Thorley-Lawson & Green, 1977). Gel filtration columns (2.5 × 15 cm; Sephacryl S-300, Pharmacia, Uppsala, Sweden) were used to measure detergent binding to ATPase vesicles prepared from sarcoplasmic reticulum as previously described (Andersen et al., 1983).

Results

Binding Stoichiometry. Figure 1 shows that a gel filtration column can be used to measure the amount of detergent bound to membranous material, irrespective of concomitant partial solubilization. In these experiments ATPase vesicles were loaded onto preequilibrated Sephacryl S-300 columns as described in the legend to Figure 1. The same binding level (0.25 ± 0.02 g of $C_{12}E_8$/g of protein) was obtained for vesicles eluting in the void volume at 50 μg/mL $C_{12}E_8$ (Figure 1A), corresponding to the cmc of the detergent, as at 70 μg/mL

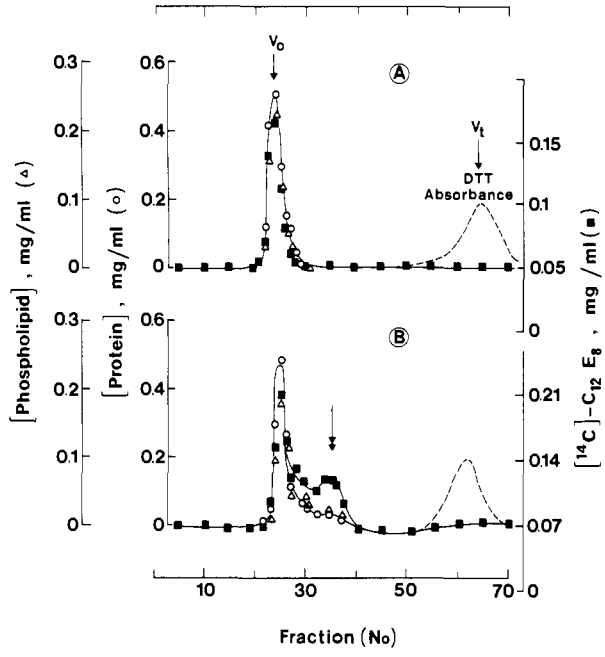

FIGURE 1: Gel chromatography of ATPase vesicles on a Sephacryl S-300 column at or slightly above the cmc. A small amount of $C_{12}E_8$ (0.65 mg in panel A and 0.75 mg in panel B) was slowly added to the vesicles containing 3 mg of protein, and 3 mg of DTT was then added before application to the column. (○) Protein; (△) phospholipid; (■) [^{14}C]$C_{12}E_8$. Panel A: Detergent concentration was 0.05 mg/mL, corresponding to the cmc of $C_{12}E_8$. Panel B: Detergent concentration was 0.07 mg/mL. Double arrow indicates solubilized protein, solubilized phospholipid, and the associated increase in the concentration of $C_{12}E_8$ in the micelles. Binding of $C_{12}E_8$ to the void volume material was 0.25 ± 0.02 g/g of protein in four experiments, for both panel A and panel B, which thus represents the maximal amount that can be incorporated into the ATPase membranes before solubilization. Note that the amount of detergent initially added to the sample compensated for binding, so that the free concentration of detergent was maintained constant throughout the column (otherwise a trough would have occurred, extending from the total volume to the elution volume of ATPase and lipid). In addition to $C_{12}E_8$, the column eluant had the following composition: 10 mM Tes (pH 7.5), 100 mM KCl, 0.1 mM Ca^{2+}, and 1 mM N_3^-. V_0 void volume; V_t, total volume. The temperature was 20 °C.

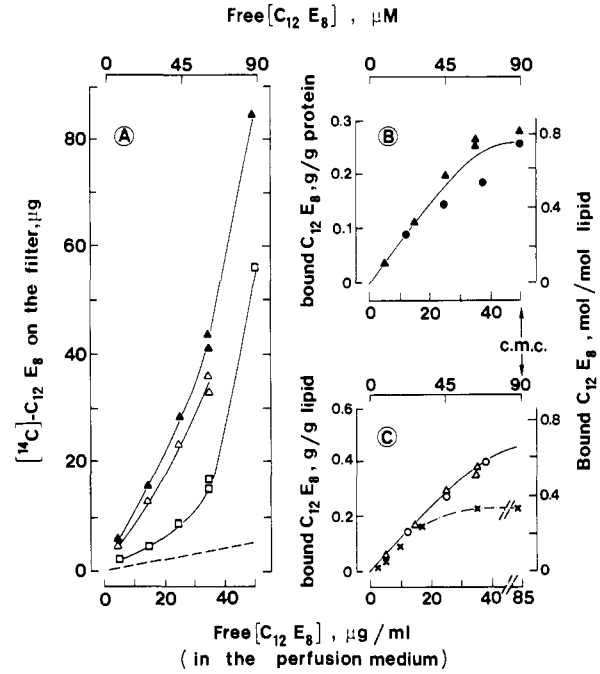

FIGURE 2: Detergent binding to SR vesicles and liposomes. The medium contained 100 mM KCl, 0.1 mM $CaCl_2$, and 10 mM Tes, pH 7.5 (20 °C). First step: One milliliter of buffer (□), 1 mL of buffer containing SR vesicles at 100 μg of protein/mL (▲), or 1 mL of buffer containing large unilamellar liposomes (type II) at 50 μg of lipid/mL (△) was filtered through a GF/F filter (pore diameter, 0.7 μm). Second step: Four to five milliliters of buffer containing various concentrations of [^{14}C]$C_{12}E_8$ was perfused in about 30 s through these filters, which were then counted in a scintillation fluid. Panel A: Radioactivity is expressed in terms of total weight of $C_{12}E_8$ retained on each filter (buffer alone, □; SR vesicles, ▲; liposomes, △). Dashed line indicates the expected radioactivity if the detergent was not truly bound but only trapped in the wetting volume of the filter (about 100 μL as measured with [^{3}H]sucrose). Panels B and C: Detergent binding to the membrane after background subtraction is expressed on a gram per gram basis (left scale) or mole per mole basis (right scale) for the SR vesicles (panel B) and the liposomes (panel C). Arrows indicate the cmc of $C_{12}E_8$ in the absence of lipid, which corresponds approximately to the onset of solubilization (see Figure 1). Data were obtained at 20 °C. Circles in panels B and C represent the values measured by column chromatography [Figure 1 and Andersen et al. (1983)]. Crosses in panel C correspond to the data of Ueno et al. (1984) (see Discussion).

(Figure 1B), where partial solubilization occurred. Thus the experiment defines the maximal amount of the detergent that can be bound by the membranes before the onset of solubilization. Note that $C_{12}E_8$ binding to the solubilized material entering the gel is considerably larger and would have affected the binding result, if vesicles eluting in the void volume had been contaminated by solubilized material. Size fractionation by gel filtration under these special conditions was therefore adequate for measurement of binding to the nonsolubilized vesicles.

Figure 2 establishes filtration through glass fiber filters (Whatman GF/F) as an alternative and new method of measuring detergent binding to membranes. In the first step, SR vesicles (100 μg of protein) or liposomes (50 μg of lipid) were deposited on the filters with close to 100% retention of vesicular material. Then buffer, containing $C_{12}E_8$ at various subsolubilizing concentrations, was perfused through the filter for approximately 30 s. Retention of $C_{12}E_8$ by the filter as a function of detergent concentration is shown in Figure 2A (closed triangles = SR vesicles and open triangles = liposomes). When the same experiment was repeated in the absence of membranes (squares in Figure 2A), some radioactivity was also retained on the filter, especially at concentrations close to the cmc. This represents (i) the amount of detergent trapped in the filter together with the buffer (approximately 100 μL with GF/F filters; see dashed line in Figure 2A) and (ii) nonspecific binding of detergent to the filter itself. Detergent binding to vesicular material is calculated by simple subtraction of this background level (right side of Figure 2) and is expressed on a gram per gram or a mole per mole basis for SR vesicles (panel B) and liposomes (panel C). It can be seen from the figure that the results are identical with those obtained by column chromatography for the binding isotherm before solubilization. Binding is characterized as a noncooperative process (in contrast to binding of detergent to the glass fiber that shows positive cooperativity near the cmc). This suggests that detergent–detergent interactions are not a prominent feature after uptake of $C_{12}E_8$ into the membrane, which argues against formation of inverted micelles (Hunt, 1980) or hydrophilic channels (Schlieper & Robertis, 1977) by incorporation of detergent into the membrane. The maximal binding capacity corresponds to 0.6–0.8 mol of detergent/mol of phospholipid. As can be seen from the figure, it is higher than reported by Ueno et al. (1984) for binding of $C_{12}E_8$ by reconstituted egg lecithin liposomes, but it is within the range of 0.5–1.4 mol/mol reported for binding of other amphiphiles to vesicles (Helenius & Simons, 1975; Stubbs & Litman, 1978).

In other experiments we found that the extent of nonspecific adsorption precluded the use of a number of other filter types, based on cellulose and plastic materials. Only glass fiber filters had sufficiently low adsorption, and the results obtained by this method seemed reliable and allowed kinetic experiments to be performed (see below). Compared to column chromatography, the glass fiber technique has one serious drawback: it cannot be used for detergent concentrations above the cmc, since solubilized membrane proteins and mixed detergent–lipid micelles may also be adsorbed on the filter and bind extra detergent.

Effect of Ultrasonication on Amount of Bound Detergent. Experiments to elucidate if $C_{12}E_8$ is able to cross the hydrocarbon phase of the intact liposomal membrane included measurements of detergent binding before and after ultrasonication. In these experiments, SR vesicles or type II liposomes pretreated with $C_{12}E_8$ to give a free concentration of around 35 μg/mL were filtered through the GF/F filters before or after sonication for 3 min on ice. During the formation of smaller vesicles or liposomes by ultrasonication, their interior aqueous phase is necessarily in contact with the suspension medium containing detergent. Ultrasonication did not lead to any increase in $C_{12}E_8$ binding (data not shown), indicating that $C_{12}E_8$ is rapidly equilibrated across the membrane. Indeed, in the case of unilamellar liposomes some decrease in detergent binding was observed (less than 20%), suggesting that the vesicles with smaller diameters, formed by ultrasonication, bind slightly less detergent. A possible explanation could be somewhat less binding to the inner leaflet due to increased surface curvature, which would leave less room available for insertion of detergent molecules on the inside of the bilayer (see Discussion). In this connection it should be noted that the diameters of the unilamellar vesicle preparations used were large (1000–2000 Å) so that, before ultrasonication, surface curvature presumably would be an even less important factor for differences in detergent binding between the two layers.

Release of $C_{12}E_8$ from Liposomes. In regard to the kinetic aspects of detergent binding and release, the location and flip-flop rates are important factors to be considered. In a release experiment, a very slow flip-flop rate, of the order of several hours, as has been observed for phosphatidylcholine at 37 °C (Rousselet et al., 1976; Van Meer & Op den Kamp, 1982; Carmel et al., 1985), would imply that most of the detergent on the inner leaflet of the bilayer would still be bound to the membrane many hours after removal of detergent from the outer leaflet. Figure 3 shows the time course of $C_{12}E_8$ removal from various types of liposomes in the presence of Bio-Beads: first, unilamellar liposomes to which $C_{12}E_8$ had been added either "externally" (type II) or both "internally" and "externally" (type I); second, multilamellar liposomes to which $C_{12}E_8$ had been added both "internally" and "externally" (type III). The experiments were performed at a low detergent to lipid ratio (0.08 g/g). This ensures that the liposomes remain essentially impermeable to small molecules [as tested with $^{45}Ca^{2+}$ in Andersen et al. (1983)]. It also means that we are examining detergent release under conditions corresponding to removal of residual detergent in a reconstitution experiment. Figure 3 shows that the major part of the detergent is removed from the medium within 30 min by processes that give rise to an essentially monoexponential decay curve. In the case of multilayered liposomes, $C_{12}E_8$ release is only slightly impeded. There is no difference between type I and type II liposomes, suggesting that internally located $C_{12}E_8$ readily leaves the liposomes. This observation is not in agreement with

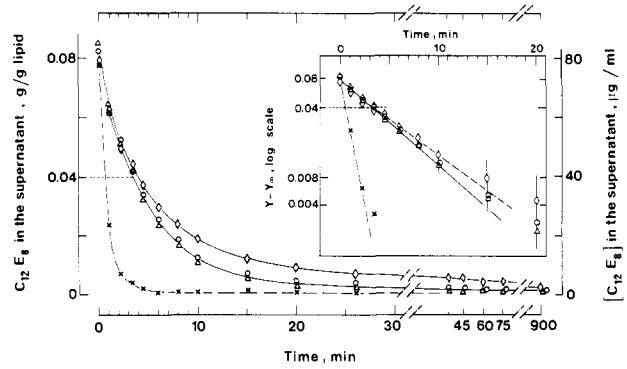

FIGURE 3: Time course of detergent removal by hydrophobic beads. Unilamellar liposomes (type I) and multilamellar liposomes (type III) were prepared in the presence of detergent; unilamellar liposomes were also prepared in the absence of detergent and were ultimately submitted to detergent perturbation after vesicle formation (type II liposomes). In all cases, the medium contained 100 mM KCl, 0.1 mM $CaCl_2$, 10 mM Tes (pH 7.5), 1 mg/mL [^{3}H]phospholipid, and 0.08 mg/mL [^{14}C]$C_{12}E_8$. Bio-Beads were added at 200 mg/mL, and aliquots from the supernatant were counted after various periods. (○) Type I liposomes formed in the presence of [^{14}C]$C_{12}E_8$; (△) type II liposomes externally perturbed with [^{14}C]$C_{12}E_8$. (◇) Type III multiwalled liposomes formed in the presence of [^{14}C]$C_{12}E_8$; (×) same experiment performed in the absence of phospholipid but at the same initial detergent concentration of 0.08 mg/mL. Inset: Semilog representation of the results. Data were obtained at room temperature.

the results published by Ueno et al. (1984), in which only half of the $C_{12}E_8$ present in reconstituted lipid vesicles was found to be released after many hours.

It is also seen from Figure 3 that adsorption of $C_{12}E_8$ by the Bio-Beads occurs more quickly from a solution not containing phospholipid (crosses). This fast rate in the absence of phospholipid is only indicative of the process by which the Bio-Beads interact with detergent: the delayed uptake rate of $C_{12}E_8$ in the presence of liposomes largely can be accounted for by assuming that only nonbound detergent interacts with the hydrophobic resin.[2] Thus the rate of detergent removal by Bio-Beads cannot be taken as a measure of the release rate of $C_{12}E_8$ from the liposomes.

Detergent removal was also examined after 500-fold dilution of the vesicles, followed by separation of the vesicles from the medium on a Sephadex column (Figure 4). The gel filtration procedure resulted in a further decrease in the free concentration of detergent, due to additional dilution (by a factor of 1.4–2) and adsorption of $C_{12}E_8$ by the gel material (in the absence of liposomes, only about 16% of the detergent eluted after collection of the first 15 mL of eluant, corresponding to twice the total volume of the column). Virtually all $C_{12}E_8$ was released from the liposomes within a few minutes, both from unilamellar (I, II) and multilamellar (III) liposomes, under conditions where the initial detergent/lipid ratio was 0.08 g/g (the initial detergent total concentration was 0.4 mg/mL). This was also the case for multilamellar liposomes (III*) which had been prepared with a very low detergent concentration (total detergent concentration was 0.0013 mg/mL), showing that binding of $C_{12}E_8$ to any high-affinity sites in the liposome

[2] With 1 mg/mL lipid and 0.08 mg/mL total $C_{12}E_8$ roughly 90% detergent is bound to the membrane as deduced from Figure 2, and accordingly free $C_{12}E_8$ is about 10% of total $C_{12}E_8$. Therefore, in the presence of lipid, the concentration of free detergent, i.e., the detergent pool that interacts with Bio-Beads, is 10 times lower than in the absence of lipid. Even if the rate of exchange between the two detergent pools (membrane bound and free) is very fast, the kinetics of detergent removal will be 10 times slower. This corresponds quite well to the slower rate of removal of $C_{12}E_8$ in the presence of lipid (Figure 3, open symbols), which is about 7 times slower than in the absence of lipid (crosses).

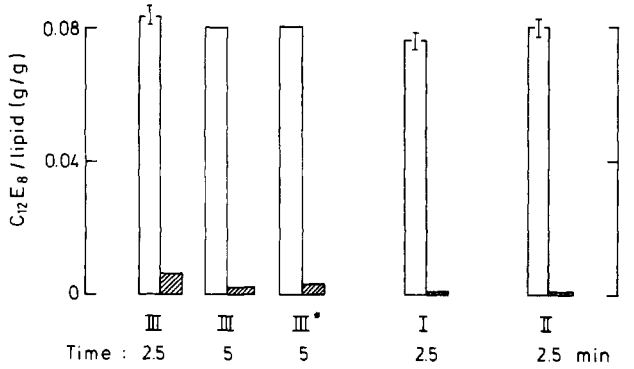

FIGURE 4: Histogram representing the effect of dilution followed by Sephadex G-25 chromatography on the amount of detergent bound to various types of liposomes. Liposomes I, II, or III were prepared as described in the legend to Figure 3, except that the samples contained 5 mg/mL [^{3}H]phospholipid and 0.4 mg/mL [^{14}C]$C_{12}E_8$ (except III*, where the detergent concentration was lowered to 1.3 μg/mL). About 5 min after detergent addition to type II liposomes, all samples were diluted 500-fold in buffer without detergent. Aliquots (2.5 mL) were loaded onto equilibrated PD-10 columns (1.5 × 5 cm) and eluted in the above detergent-free buffer. Fractions were collected in 2.5 or 5 min and [^{3}H]phospholipid and ^{14}C-labeled detergent radioactivity was counted on 2-mL aliquots. The void volume fractions contained all the ^{3}H label and only a small fraction of the $C_{12}E_8$ initially bound. The detergent/lipid ratio in the void volume (hatched area) was compared to the same ratio before chromatography (open area). The scale does not apply to histogram III*, for which the initial $C_{12}E_8$/lipid ratio was 0.00026 g/g.

material does not give rise to a delay in detergent removal. It was ascertained in control experiments (not shown) that close to the expected amount of $C_{12}E_8$ (0.074 mg of $C_{12}E_8$/mg of lipid) remained bound to the multilayered vesicles if chromatography was performed with a column preequilibrated with a concentration of $C_{12}E_8$ corresponding to that present in the undiluted sample (0.006 mg/mL free $C_{12}E_8$, calculated from the binding data of Figure 2). It may be presumed that both outer and inner bilayers of type I unilamellar vesicles and type III multilayered vesicles (typically containing 10–20 bilayer shells as evidenced by a freeze–fracture analysis of our sample) were equilibrated with $C_{12}E_8$. Thus the release experiment unequivocally shows passage of $C_{12}E_8$ through successive bilayers within a limited time period.

Partition Experiments. In other experiments the lipophilicity of $C_{12}E_8$ was examined by partition experiments. Complete (>99%) extractability from an aqueous phase was observed with ether, chloroform, benzene, and octanol. $C_{12}E_8$ was quickly extracted to equilibrium (within $^1/_2$ h) into a heptane phase with a partition coefficient of 13.7 ± 1.9 (SD) over a range of final detergent concentrations of 4–70 μM, suggesting solubilization of detergent in the organic phase in a nonassociated form. At higher $C_{12}E_8$ concentrations stable emulsions of $C_{12}E_8$ and heptane were formed during prolonged shaking. The uptake of $C_{12}E_8$ by the heptane phase suggests that the entire detergent molecule is capable of crossing the hydrocarbon interior of the lipid bilayer.

Kinetics of Detergent Interaction with Membrane. The kinetics of detergent binding and removal from the membranes were measured with a recently introduced rapid-filtration device (Dupont, 1984), combined with the GF/F filter technique described in Figure 2. An example of a release experiment is shown in Figure 5A,B. In this experiment SR vesicles (100 μg of protein) were loaded onto a filter in the presence of a concentration of [^{14}C]$C_{12}E_8$ of 41 μg/mL, corresponding to about 35 μg/mL free $C_{12}E_8$ (calculated from Figure 2B). [^{3}H]Sucrose was included in this loading buffer as a control of the behavior of a nonbound substance. Then

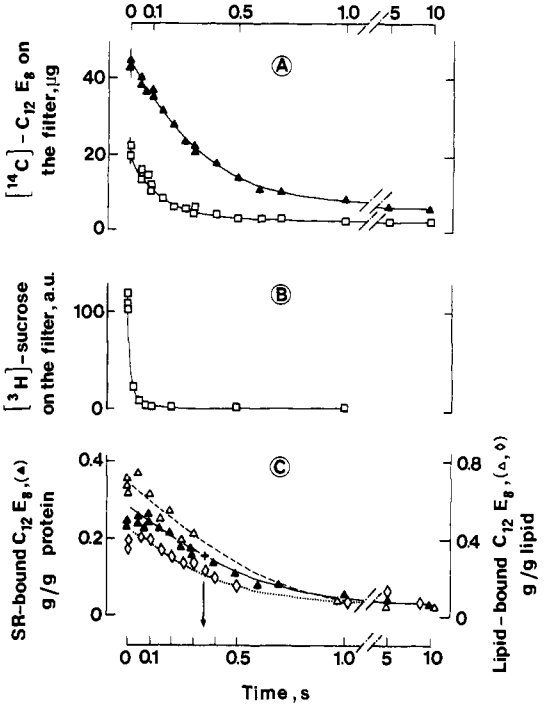

FIGURE 5: Kinetics of detergent release from various types of membranes. First step: Loading of the GF/F filters with the membrane plus bound [^{14}C]$C_{12}E_8$. This was done by perfusing the filter with 3.3 mL of buffer (10 mM Tes, pH 7.5, 100 mM KCl, and 0.1 mM $CaCl_2$) containing 30 μg of protein/mL of SR vesicles (▲) or 15 μg of phospholipid/mL of unilamellar vesicles (△) or multilamellar vesicles (◇) and 41 μg/mL. [^{14}C]$C_{12}E_8$, which corresponds to a free detergent concentration of about 35 μg/mL. The latter concentration was used for the control experiment in the absence of lipid. Second step: Rapid washing of the filters with buffer but no detergent. The rapid-filtration device (Biologic, Grenoble) was set up to deliver buffer for specific periods at a flow rate of 3 or 4 mL/s up to a total volume of 5 mL. Panel A: Data are expressed as total $C_{12}E_8$ bound per filter, in the presence of SR vesicles (▲) or in their absence (□). Panel B: Same experiment, but the buffer contained 1 mM [^{3}H]sucrose in addition to SR vesicles and detergent. Panel C: Data are expressed in terms of grams of detergent bound per gram of protein or lipid, after background subtraction (see panel A). The arrow corresponds to the approximate half-time of detergent removal. The average of two experiments performed at 20 °C is shown. The slightly lower initial amount of $C_{12}E_8$ bound to the multilayered vesicles (◇) may be due to decreased binding of $C_{12}E_8$ as the result of slight changes in the properties of the lipid bilayer in the multilayered liposomes (see Discussion).

buffer without detergent and [^{3}H]sucrose was perfused through the filter for specific periods, and the amount of detergent and sucrose remaining on the filters was measured (Figure 5A, closed triangles). The blank, run in the absence of membranes, is also shown (Figure 5A, open squares). As shown by Figure 5B, sucrose was removed in less than 50 ms. Therefore, the persistence of detergent on the filter for a longer period can be attributed to binding and not, for example, to an insufficient rinsing volume. In Figure 5C detergent bound to different types of vesicles is expressed on a gram per gram basis after background subtraction, both for SR vesicles (as in panel A) and for type II unilamellar liposomes or type IV multilamellar liposomes previously equilibrated with detergent. Detergent removal from all types of membrane is virtually complete and monoexponential with a half-time of about 350 ms in all three cases.

The kinetics of detergent binding to SR vesicles are shown in Figure 6. In this case, SR vesicles without detergent were first loaded on the filter and buffer containing 35 μg/mL detergent was then flushed through the filter for various periods (see panel A for original data and panel B after back-

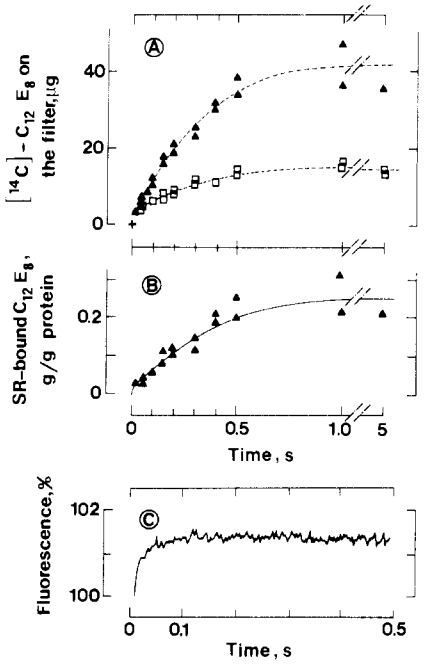

FIGURE 6: Kinetics of detergent binding to SR vesicles. Data were obtained as described in Figure 5 except that the detergent was absent during the first step but present during the second step at a concentration of 35 μg/mL. The buffer was delivered at 3 to 4 mL/s so that the initial points (up to 150 ms) must be considered as minimal binding. Panel A: Data are expressed as total $C_{12}E_8$ bound per filter in the presence of SR vesicles (▲) or in their absence (□). Panel B: Data are expressed in terms of grams of detergent bound per gram of protein, after background subtraction (see panel A). Panel C: Stopped-flow recordings of the changes in fluorescence observed after adding 0.06 mg/mL $C_{12}E_8$ to 0.06 mg/mL purified ATPase vesicles, both in 10 mM Tes, pH 7.5, 100 mM KCl, and 0.1 mM $CaCl_2$ (20 °C). The final $C_{12}E_8$ concentration was therefore 0.03 mg/mL (free concentration about 0.025 mg/mL). Excitation wavelength, 290 nm; emission wavelength, 330 nm.

ground subtraction). As expected in a situation of dynamic equilibrium, the kinetics of binding were identical with the kinetics of removal, occurring with a half-time of about 350 ms.

We previously noticed that binding of $C_{12}E_8$ to SR vesicles slightly enhanced the intrinsic fluorescence of Ca^{2+}-ATPase, the major protein in these membranes (Andersen et al., 1983). Panel C in Figure 6 shows the result of a similar binding experiment analyzed by fluorescence in a stopped-flow apparatus. In this case, the small rise in Ca^{2+}-ATPase intrinsic fluorescence, attributable to $C_{12}E_8$ binding, was completed within the first 100 ms. Thus the interaction of $C_{12}E_8$ with the protein is kinetically distinguished from the interaction with lipid. The reason why this rapid phase is not observed in the [^{14}C]$C_{12}E_8$ experiments presumably is due to predominant interaction of $C_{12}E_8$ with the lipid component of the SR membrane (see Discussion).

Discussion

At the onset of this work it was important to define conditions that would allow us to perform fast and reliable measurements of detergent binding to vesicles or liposomes. The gel filtration method previously used [Figure 1 and Andersen et al. (1983)] is an excellent, but slow, procedure (elution takes 6 h). Ultracentrifugation of membrane equilibrated with detergent is slightly faster for membranes that contain protein, but at least in our hands it is not very reliable, due to difficulties in complete sedimentation and adsorption of $C_{12}E_8$ to the ultracentrifugation tubes. Ultracentrifugation is even less suited for the centrifugation of pure unilamellar liposomes. As is apparent from this work, adsorption of $C_{12}E_8$ by various materials constitutes an obstacle to accurate study of detergent behavior. The finding that GF/F glass fiber filters retain sarcoplasmic reticulum vesicles or liposomes without high nonspecific binding of detergent (Figure 2A) therefore opens up new possibilities. Related techniques of hygroscopic desorption have been used to measure amphipath binding (Conrad & Singer, 1981; Bondy & Remien, 1981). To test the present filtration method, we reproduced the previously established curve for $C_{12}E_8$ binding to both sarcoplasmic reticulum vesicles and liposomes (compare triangles and circles in Figure 2B,C). An additional advantage of our filtration method is that it is directly applicable to the rapid-filtration device developed by Dupont (1984) and allows kinetic studies of detergent binding and removal in the 50 ms–10 s range.

With this procedure and other techniques, we addressed ourselves to the question whether the closed lipid bilayer constitutes a significant barrier to the attainment of equilibrium binding of $C_{12}E_8$ on both sides of the membrane. A slow flip-flop of $C_{12}E_8$ across the membrane would have been expected to result in biphasic binding and release of $C_{12}E_8$ from membranes previously equilibrated with detergent. Rapid kinetic experiments showed that binding and release of $C_{12}E_8$ from unilamellar liposomes or SR vesicles were complete and occurred by a monoexponential time course with a half-time of 350 ms. We also found that maneuvers to expose internal, latent binding sites (ultrasonication), or addition of $C_{12}E_8$ before liposome formation, caused no increase in the binding capacity or the release rate of detergent from the liposome. It may be concluded that $C_{12}E_8$ is capable of passing the phospholipid bilayer very quickly. But the alternate hypothesis, viz., that the detergent is predominantly bound on the outside of the unilamellar vesicles, due to the asymmetry of the lipid bilayer, must also be considered. However, even if as the result of curvature there is a strain on the hydrocarbon chains and a higher density of lipid head groups on the inside leaflet (Huang & Mason, 1978), this cannot reasonably account for our results. This follows from the large diameter of our unilamellar vesicles which makes it very unlikely that detergent binding could be reduced almost to zero by bilayer asymmetry. Furthermore, the release experiments on multilayered liposomes (typically consisting of 10–20 bilayer shells) indicate that $C_{12}E_8$ must be able to cross successive bilayers quickly. On the other hand, bilayer strain might be a significant factor in the slight difference in behavior between unilamellar and multilayered liposomes (see Figure 5C).

If we consider that the monoexponential $C_{12}E_8$ release in fact reflects a two-stage process for the $C_{12}E_8$ molecules that are located on the inner leaflet, i.e., initial flip-flop followed by the release from the outer leaflet to the medium, the half-time for flip-flop per se must by shorter than the half-time estimated for the whole process (350 ms). This conclusion follows also from the observed kinetics of detergent binding. Surprisingly, there is very little information about the flip-flop rate of various amphiphiles (Helenius & Simons, 1975) although for a nonionized amphiphile a fast flip-flop is not unexpected. For example, from ESR and NMR measurements it can be concluded that a protonated fatty acid, unlike an ionized fatty acid, has a flip-flop half-time whose higher limit falls in the 1–10-ms range and that uncharged *N*-acylamino acids (*N*-palmitoyl-L-glycine, *N*-palmitoyl-L-alanine, or protonated *N*-palmitoyl-L-glutamic acid) also display flip-flop, although at a slower rate depending on the amino acid (Sanson, unpublished results; Sanson et al., 1987). Parinaric

acid (a C_{18} fatty acid with four double bonds) is also considered to diffuse to all the leaflets of multilamellar liposomes (Sklar et al., 1977) without kinetic discrimination due to a slow flip-flop. A rapid flip-flop accords with the pronounced tendency for the whole molecule of $C_{12}E_8$ to be taken by an organic liquid phase.

Note, however, that neither the rapid flip-flop nor the bulk transfer into an organic phase contradicts the idea that the detergent molecule is mainly anchored at the membrane–water interface: First, the bulk partition coefficient of detergent between heptane and water (14) is very much lower than what can be calculated between membrane and water (10^4);[3] this indicates that detergent location is not restricted to the purely hydrophobic inner portion of the bilayer. Second, the ^1H NMR signals arising from the detergent polar head group were found adequately sensitive to quenching by the addition of Mn^{2+} ions (data not shown).

The present conclusion regarding transmembrane passage of $C_{12}E_8$ contrasts with the report of Ueno et al. (1984), in which an exceedingly slow flip-flop rate, comparable to that of phospholipids, was suggested, essentially because of the inability to remove more than 50% of the $C_{12}E_8$ which remained attached to the reconstituted vesicles. But clearly this is not the case in our experiments, and it is rather difficult to explain this discrepancy. Ueno et al. (1984) recognize that one of their experiments is in fact contradictory to slow flip-flop, since they did not observe a twofold higher binding ratio after reconstitution where both halves of the bilayer should have been accessible to detergent. The question arises whether the phenomenon is in some way dependent on the use of hydrophobic beads to remove $C_{12}E_8$ in the reconstitution procedure. Perhaps one could propose that during reconstitution very small portions of the polymer, which tightly bind the detergent, become trapped within the vesicles during their formation (the beads themselves are much too large), resulting in sequestration of detergent inside the newly formed liposomes. At any rate, the tenacity with which $C_{12}E_8$ remains attached to the reconstituted vesicles appears, on the basis of the present study, to be attributable to some other factor than a slow flip-flop.

From the point of view of use of $C_{12}E_8$ for reconstitution procedures, our data show that it is possible to remove with Bio-Beads virtually all $C_{12}E_8$. In any case, some residual detergent does not seem to be a critical factor for demonstration of transport properties (Cornelius & Skou, 1984).

In addition to the radiochemical measurement of the rate of detergent incorporation into SR vesicles (Figure 6), we were able to follow the rate of detergent interaction with SR vesicles by monitoring in a stopped-flow fluorometer the change in Ca^{2+}-ATPase intrinsic fluorescence (Figure 6C). Remarkably, the fluorescence rise was completed within 100 ms, which is clearly shorter than the half-time of bulk binding of the detergent to the membrane. Our interpretation of this early event is that we probably monitor the fast binding of detergent molecules to the Ca^{2+}-ATPase itself at very few more or less specific binding sites. This would account for the absence of a rapid phase in the radiochemical experiments, in agreement with a previous detailed analysis of the interaction of $C_{12}E_8$ with sarcoplasmic reticulum membranes (Andersen et al., 1983). It has also been suggested that functional consequences of detergent perturbation mainly are the consequence of binding of $C_{12}E_8$ in close proximity to the protein, either in crevices or at the phospholipid–protein interface (Andersen et al., 1983; Champeil et al., 1986). Similar conclusions have been drawn concerning the binding of hydrophobic drugs to Ca^{2+}-ATPase vesicles (Rooney & Lee, 1983; Lee et al., 1983).

Finally, it is of interest to recall that detergents are considered to belong to the category of anesthetics (Seeman, 1972). For example, halothane has similar effects on Ca^{2+}-ATPase function (Malinconico & McCarl, 1982) as those reported by low amounts of $C_{12}E_8$ and Triton X-100 (Andersen et al., 1983; McIntosh & Davidson, 1984); conversely, Triton X-100 presents the characteristics of local anesthetics in other systems (Brisson et al., 1975). Local anesthetic drugs form a heterogeneous group of compounds among which many are amphiphilic and surface active (Helenius & Simons, 1975). Although it has often been considered that anesthetics produce their stabilizing effects on nerve membranes by interaction with the lipid phase [e.g., Bean et al. (1981), Haydon and Urban (1983a–c), and Vassort et al. (1986)], correlative studies on structural membrane and anesthetic effects suggest a direct interaction of these compounds with membrane proteins or at the lipid–protein interface (Lee, 1976; Richards et al., 1978; Franks & Lieb, 1979, 1985). From our study it can probably be inferred that noncharged anesthetics are immediately distributed (i) on both halves of the plasma membranes and (ii) on all the membranes within the cells (mitochondria, lysosomes, etc.). Thus a large number of membranous enzymes may rapidly become perturbed if susceptible to interaction with anesthetics.

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[3] At 0.04 mM free detergent, 0.3 g of detergent is bound to 1 g of lipids (Figure 2); therefore, the concentration of bound detergent (0.3 g/1.3 g total) is around 0.25 g/mL, i.e., around 0.5 M; the partition coefficient is thus about 10^4.

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