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A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal

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Equilibrium and kinetic aspects of Triton X-100 adsorption onto hydrophobic Bio-Beads SM_2 were investigated in detail using the batch procedure originally described by Holloway, P.W. (1973) Anal. Biochem. 53, 304–308. The results demonstrated the importance of the initial detergent concentration, the amount of beads, the commercial source of the detergent, the temperature and the presence of phospholipids in determining the rates of Triton X-100 adsorption onto Bio-Beads. One of the main findings was that Bio-Beads allowed the almost complete removal of Triton X-100, whatever the initial experimental conditions. It was shown that monomeric as well as micellar detergent could be adsorbed and that a key factor in determining the rate of detergent removal was the availability of the free bead surface. Rates of detergent removal were found to be linearly related to the amount of beads even for bead concentrations above those sufficient to remove all the detergent initially present. Adsorptive capacity of phospholipids onto Bio-Beads SM_2 was also analyzed and found to be much smaller (2 mg lipid per g of wet beads) than that of Triton X-100 (185 mg TX 100 per g of wet beads). A more general aspect of this work was that the use of Bio-Beads SM_2 provided a convenient way for varying and controlling the time course of Triton X-100 removal. The method was further extended to the formation of liposomes from phospholipid-Triton X-100 micelles and the size of the liposomes was found to be critically dependent upon the rate of detergent removal. A general procedure was described to prepare homogeneous populations of vesicles. Freeze-fracture electron microscopy and permeability studies indicated that the liposomes thus obtained were unilamellar, relatively large and impermeable. Noteworthy, this new procedure was shown to be well suited for the reconstitution of different membrane transport proteins such as bacteriorhodopsin, Ca^{2+}-ATPase and H^+-ATPase.

Introduction

Triton X-100 is a non-ionic detergent which is often employed for the initial solubilization of membrane proteins [1,2]. Furthermore this detergent has been used for the reconstitution of a large number of functional membrane proteins [3–8]. However, the usefulness of this detergent in reconstitution experiments has often been claimed to be hampered by the difficulty of removing it entirely from the reconstituted membrane preparation. This problem is mainly due to its extremely low critical micelle concentration which makes its removal very difficult by dialysis [9]. Although a simple method, involving adsorption of TX 100 onto hydrophobic resin beads such as Bio-Beads SM_2 [10] or Amberlite XAD-2 [11] has been previously described to improve removal of this detergent, several authors have found that the method has inherent drawbacks. These include: (i) the residual detergent-to-phospholipid ratios obtained which are still higher than those obtained in reconstitution procedures employing other detergents [12,13]; (ii) large loss of lipids which also adsorb onto the beads [12,14] and (iii) formation of multilamellar structures apparently related to the rapid rate of detergent removal [7,8]. Due to these limitations, TX 100 is now rarely used for reconstitution despite its high solubilizing efficiency for membrane proteins.

However, in the course of our studies of the different mechanisms by which an integral membrane protein can associate with phospholipids in detergent-mediated reconstitutions [15,16] we have shown that TX 100 is a detergent of choice leading to the formation of proteo-

Abbreviations: TX 100, Triton X-100; EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; cmc, critical micelle concentration; Pipes, 1,4-piperazinediethanesulfonic acid.

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liposomes with fairly high transport activity; thus optimal reconstitutions of bacteriorhodopsin were obtained by a mechanism involving a transfer of the protein from mixed micelles to TX 100-saturated liposomes allowing a good unidirectional orientation of bacteriorhodopsin into the reconstituted proteoliposomes. Another advantage of the reconstitution procedure described previously was believed to rely on the 'batch procedure' using Bio-Beads SM_2 as detergent-removing agent. It provided a reproducible and easy way of achieving unilamellar, relatively large and impermeable liposomes: thus, light-induced pH gradients as large as 2 pH units could be generated across the membranes of the bacteriorhodopsin proteoliposomes.

Thus, in order to design a successful reconstitution procedure that would serve as a general guide for TX 100-mediated liposome and proteoliposome reconstitutions, we examined in detail the removal of Triton X-100 by Bio-Beads SM_2. The systematic study reported here shows that all the difficulties reported above can be minimized or overcome and get insight the mechanisms underlying TX 100 adsorption onto Bio-Beads. On the basis of these results, general rules for TX 100-mediated reconstitutions are proposed.

Materials and Methods

Materials

Purified egg-yolk phosphatidylcholine (EPC) and derived phosphatidic acid (EPA) were isolated according to Singleton et al. [17] and Allgyer and Wells [18], respectively.

L-α-Dipalmitoylphosphatidyl[*N-methyl*-^{14}C]choline (50 mCi/mmol) was purchased from Amersham. Triton X-100 was obtained from Sigma, unless otherwise stated, and [*phenyl*-^{3}H]Triton X-100 (1.3 mCi/mg) came from New England Nuclear. Pyranine came from Molecular Probes. Bio-Beads SM_2 were obtained from Bio-Rad.

Preparation of vesicles

Large unilamellar liposomes (100–200 nm) were prepared by reverse phase evaporation as described previously [19] using a mixture of EPC and EPA (molar ratio 9 : 1). Buffers used were 20 mM Pipes-KOH (pH 7.2) supplemented with 110 mM K_2SO_4. Liposomes (about 20 mM phospholipid) were sequentially sized through 0.4 μm and 0.2 μm polycarbonate membranes before use.

Use of hydrophobic beads for TX 100 removal

Bio-Beads were thoroughly rinsed with methanol and buffer before use (Holloway [10]) and the finest beads were discarded. In order to remove TX 100, desired amounts of moist copolymer beads were added directly to 1 ml of a detergent containing solution and gently stirred at room temperature except when 25 μl 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 devoided of Bio-Beads to be pipetted off (similar results were obtained whenever the beads were centrifuged at $10\,000 \times g$). The different aliquots were then assayed for radioactive detergents and/or phospholipids.

Standard procedure for liposomes reconstitution

Liposomes prepared by reverse-phase evaporation were diluted to a final concentration of 4 mg lipid/ml (i.e., 5 mM) in a K_2SO_4-Pipes buffer (pH 7.2) supplemented with the desired amount of TX 100 (8 mg TX 100/ml, i.e., 12.5 mM for complete solubilization) and containing 200 μM pyramine when stated. To remove detergent, Bio-Beads were added directly to the lipid-detergent mixtures at a concentration of 80 mg of wet beads/ml. The mixtures were gently stirred at room temperature. After 3 h of incubation, a second portion of 80 mg of wet beads were added for an additional incubation period of 2 h. The turbid suspensions containing reconstituted liposomes were then pipetted off and collected for analysis.

Fluorescence measurements of proton fluxes

Changes in internal pH after an acidic pulse were measured as changes in the fluorescence intensity of the pH-sensitive fluorescent probe pyranine trapped within the vesicle, as detailed elsewhere [20]. Removal of non encapsulated pyranine was performed after the Bio-Bead treatment by passage through PD 10-Sephadex G-25 columns (Pharmacia).

Freeze-fracture electron microscopy

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

Results

Characterization of TX 100 removal by Bio-Beads SM_2

Incomplete detergent removal by copolymer beads might be due to many factors: (1) saturation of the bead capacity, (2) too slow detergent adsorption, (3) adsorption of micellar and not monomer species, (4) failure to remove detergent impurities and/or (5) too strong interaction of detergent with phospholipids in reconstitution experiments. In order to get insight the mechanisms underlying TX 100 adsorption onto Bio-Beads we have analyzed in more detail both kinetic and equilibrium

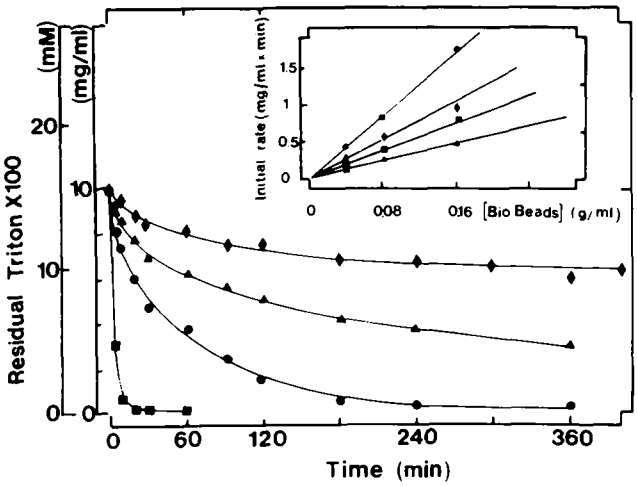

Fig. 1. Triton X-100 binding to Bio-Beads SM_2. Aliquots of 1 ml buffered solutions (110 mM K_2SO_4, 20 mM Pipes (pH 7.2)) each containing 10 mg Triton X-100 and 1 μg [^{3}H] TX 100 were treated with Bio-Beads SM_2 in different proportions. The beads were continuously maintained in suspension with a magnetic stirrer and aliquots from the supernatant were counted after various periods. Each curve corresponds to a specific amount of Bio-Beads: 20 (◆), 40 (▲), 80 (●) and 320 (■) mg of wet beads. (Inset) Rates of Triton X-100 adsorption as a function of Bio-Bead concentrations for different initial detergent concentrations (8 (●), 5 (◆), 2 (■) and 1 (▲) mg TX 100/ml). Initial rates of TX 100 adsorption were deduced from the amount of detergent adsorbed after 1 min incubation in the presence of the indicated amount of beads.

aspects of the detergent binding to Bio-Beads, first of all in the absence of phospholipids.

(a) Binding capacity of Bio-Beads SM_2. The adsorptive capacity of Bio-Beads was first examined. Fig. 1 depicts the time course of detergent removal from aqueous solutions containing 10 mg/ml TX 100 and various amounts of Bio-Beads. There is clearly an optimal detergent-to-bead ratio beyond which detergent removal cannot be improved. The plateau values reached in the presence of 20 and 40 mg beads/ml allowed determination of the adsorptive capacity of the polystyrene beads which corresponded to a value of 185 mg TX 100/g of wet beads (i.e., $3 \cdot 10^{-4}$ mol of TX 100 per g of wet beads) which agreed with the values previously reported [11,21].

Interestingly, the data reported in Fig. 1 indicate that the rate of detergent removal is drastically dependent upon the amount of beads present in the solution. Starting from a micellar detergent solution containing 10 mg TX 100/ml it took 3–4 hours to remove virtually all the detergent initially present using 80 mg beads per ml. However, in the presence of 320 mg beads/ml, detergent can be eliminated in about 20 min. Since in these later experimental conditions Bio-Beads are used in large excess as compared to their adsorptive capacity, it could be simply assumed that a limiting factor in the rate of TX 100 removal by Bio-Beads SM_2 is the availability of the free bead surface: in other words the accessibility of TX 100 to the pores of the beads is hampered by the prefixation of other detergent molecules and raising the bead concentration may therefore accelerate TX 100 removal. From the inset in Fig. 1, it is obvious that the initial rate of detergent removal is linearly related to the bead concentration whatever the initial TX 100 concentration. Lastly it can be stressed from Fig.1 that when the amount of beads exceeds their adsorptive capacity virtually all TX 100 initially present can be removed: thus starting from 10 mg TX 100/ml less than 0.025 mg/ml (i.e., 0.04 mM) of residual TX 100 was detected which was well below the cmc of this detergent (0.1–0.2 mg/ml [1,2]).

(b) Monomer and/or micelle adsorption. To compare the rates of detergent removal under monomeric and micellar form, experiments as a systematic function of TX 100 concentration were performed: to this end, a fixed amount of Bio-Beads SM_2 (80 mg/ml) was added to different detergent solutions containing from 0.05 to 2 mg TX 100 per ml. The amount of detergent removed was analyzed and representative results are shown in Fig. 2. The evolution of the initial rates of detergent removal with initial TX 100 concentrations is shown in the inset of this figure. The results reveal that up to an initial detergent concentration of about 0.2 mg/ml, the rate of removal is linearly related to the detergent concentration. Above this critical value the rate of adsorption still increases linearly but much more slowly.

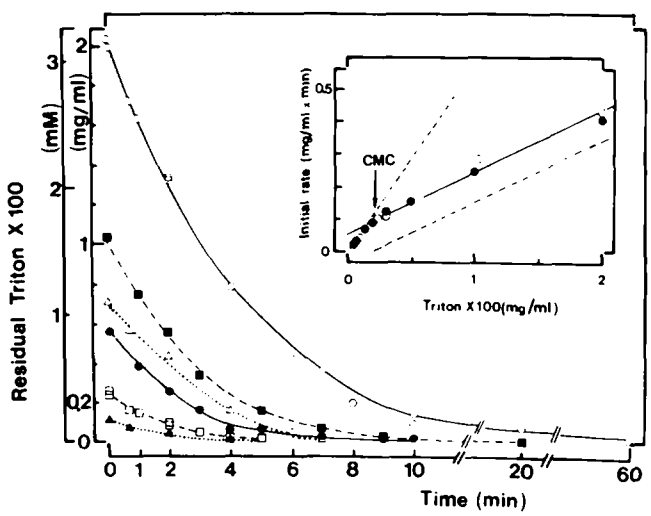

Fig. 2. Kinetics of Triton X-100 release from monomeric and micellar detergent solutions by Bio-Beads SM_2. 80 mg Bio-Beads were added to 1 ml samples containing the indicated amount of TX 100 supplemented with 1 μg [^{3}H] TX 100 in a buffer 20 mM Pipes, 110 mM K_2SO_4 (pH 7.2). Aliquots were removed at various time intervals for determination of TX 100 concentration. (Inset) Initial rates of TX 100 adsorption as a function of initial detergent concentrations. Initial rates were derived from data in Fig. 2 and other experiments by determining the amount of detergent adsorbed after 1 min incubation in the presence of 80 mg Bio-Beads ((●) and (○) represent two sets of experiments). The arrow indicates the TX 100's critical micelle concentration deduced from such a plot (for explanation see text).

We believe the break in the curve is caused by the different adsorption rates of Bio-Beads SM_2 towards TX 100 monomers and micelles *. Thus the critical concentration at which this break occurs may reasonably be related to the critical micelle concentration of TX 100: the cmc determined by the present method was about 0.2 mg TX 100/ml (i.e., 0.312 mM) and was comparable with the cmc determined by other methods [2,22,23]. The linear dependences of the adsorption rates on total detergent concentration above and below the cmc indicate that both monomeric and micellar TX 100 are adsorbed. This indicates that the process involving dissociation of micelles into monomers followed by adsorption of these monomers is not significant under these conditions. Indeed if only monomers were adsorbed onto the beads, the rates of detergent absorption should have been constant above the break point observed in the inset of Fig. 2 since the monomer concentration did not vary with total detergent concentration above the cmc [1]. However from the dotted lines in Fig. 2, slopes of 0.57 min^{-1} and 0.2 min^{-1} can be calculated reflecting the difference between the rates of monomeric and micellar TX 100 adsorption.

(c) Effect of temperature upon adsorption. Since reconstitution experiments are often performed at low temperatures to avoid protein denaturation, it is of interest to examine the binding efficiency of Bio-Beads SM_2 as a function of temperature. As reported in Fig. 3, the rate of TX 100 adsorption increased remarkably with temperature (5°C–37°C). From the Arrhenius plot of the initial rates of detergent removal reported in the inset of Fig. 3, a high temperature dependence was found, corresponding to an activation energy of about 8 kcal · mol^{-1} (this means that the rate of TX 100 adsorption doubled every 12 C°). This property has never been previously emphasized and points out the need to optimize the temperature of the experiment when dealing with TX 100-mediated reconstitutions using Bio-Beads as the detergent removing agent. In particular, it can be noted that reconstitutions of membrane proteins which require detergent removal at low temperature must be performed with high Bio-Bead concentrations in order to remove all the detergent in a reasonable short time (under the experimental conditions described in Fig. 3 it takes more than 5 h to remove all the detergent at 5°C).

(d) Influence of the detergent source. Triton X-100 is a non-ionic detergent with a heterogeneous nature, e.g.,

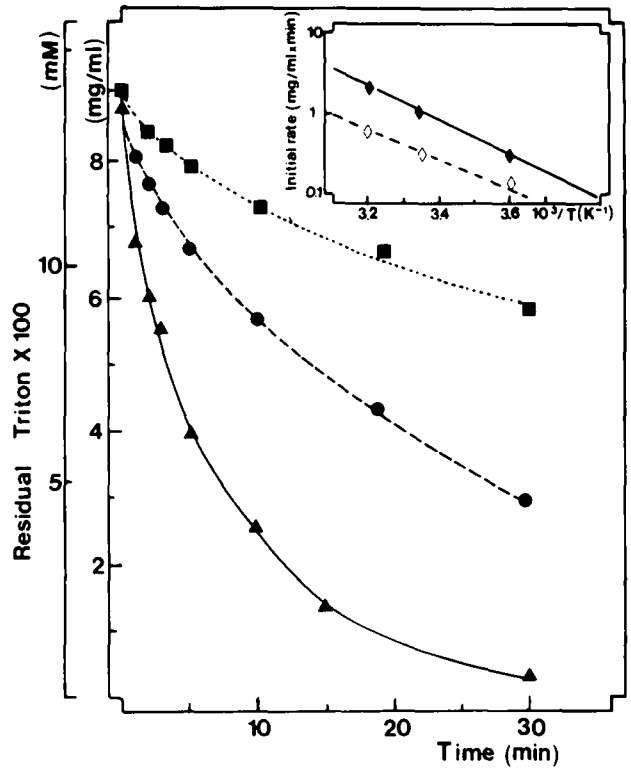

Fig. 3. Time-course of detergent removal as a function of temperature. Aliquots of 1 ml buffered solutions containing 9 mg TX 100 and 1 μg [^{3}H] TX 100 were incubated at different temperatures [(■) 5°C), (●) 25°C and (▲) 37°C] in the presence of 80 mg Bio-Beads and detergent removal analyzed as a function of time. (Inset) Arrhenius plot of initial rates of detergent adsorption. (◆) and (◇) corresponded to initial TX 100 concentrations of 9 and 1 mg/ml, respectively.

containing many different polymers as well as impurities. According to Ashani and Catravas [24], there are marked differences among the various TX 100's as regard to their oxidizing and carbonyl impurities depending on the supplier. Furthermore, it has been shown [13] that the ease with which the various molecular components of TX 100 are removed is a function of the molecular weight of the species. We therefore analyzed adsorption onto Bio-Beads of TX 100's from three different suppliers and found that adsorption clearly depended upon the nature of the detergent (Fig. 4). For example our data indicate that the rates of detergent adsorption may vary by a factor of 2–3, depending upon the TX 100's suppliers (compare TX 100's from Sigma and from Boehringer *). At the present time we have no definite explanations for the observed dif-

* Similar results were obtained when initial rates of TX 100 adsorption were measured after 30 s, 1 or 2 min contact with Bio-Beads. These observations indicated that in the experiments depicted in Fig. 2, TX 100 adsorption during the first minute was not hampered by the pre-fixation of other detergent molecules even at the highest detergent concentration analyzed.

* Preliminary experiments indicated that similar adsorption time courses were observed when starting from constant unlabelled Triton X-100's (range studied: 0.25 to 8 mg Triton X-100/ml) but various ^{3}H-labelled Triton X-100 concentrations (10-fold variations were used). These results (not shown) indicated that adsorption of labelled Triton X-100 reflected adsorption of unlabelled detergents.

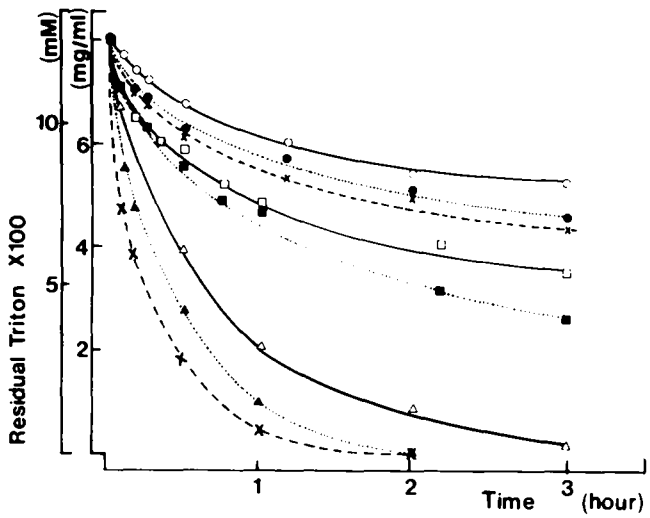

Fig. 4. Time-course of detergent removal as a function of TX 100's suppliers. 1 ml samples containing 8 mg TX 100 and 1 μg [^{3}H] TX 100 from different suppliers were treated with different amounts of Bio-Beads. Triton X-100 from Sigma (open symbols): detergent removal by 30 (○), 50 (□) and 80 (△) mg beads. Triton X-100 from Pierce (closed symbols): detergent removal by 30 (●), 50 (■) and 80 (▲) mg beads Triton X-100 from Boehringer (crosses); detergent removal by 30 (×) and 80 (×) mg beads.

ferences since they may depend upon the detergent composition, its structure (cmc, micellar size) and/or its purity.

Detergent removal in the presence of phospholipids

All the experiments reported above were performed in the absence of phospholipids and the results clearly indicated that it was possible to remove with Bio-Beads virtually all Triton X-100 whatever its concentration, its monomeric or micellar state, its source and/or the temperature of the experiment. We therefore addressed ourselves to the question whether the presence of phospholipids can noticebly change the processes by which Bio-Beads interact with Triton X-100 and approached the process of TX 100 adsorption during liposomes or proteoliposome reconstitution experiments.

Fig. 5 is representative of several experiments designed to follow the time course of detergent removal from various TX 100-phospholipid mixtures in the presence of Bio-Beads SM_2 (closed symbols). For this purpose, ^{3}H-TX 100 was added externally to preformed liposomes at three different detergent-to-phospholipid ratios. After equilibration, the different solutions were mixed with a fixed amount of beads (80 mg wet beads/ml) and TX 100 adsorption was monitored as a function of time (Data obtained in the absence of phospholipids at similar detergent concentrations are also presented: open symbols in Fig. 5). At this point, it is important to recall that the three detergent concentrations selected in Fig. 5 correspond to three characteristic stages of the solubilization process of preformed liposomes by TX 100 [15,25]. For a given concentration of liposomes (4 mg lipid/ml, i.e., 5 mM in Fig. 5), three stages in the solubilization process are apparent depending upon TX 100 concentration: (i) partitioning of non-micellar detergent between the aqueous medium and the lipid bilayer until such point as sufficient detergent has been added to saturate all the bilayer (this corresponds to a detergent-to-phospholipid weight ratio of 0.5, i.e., 0.62 mol/mol; (ii) gradual lipid solubilization, resulting in the coexistence of lipid-detergent mixed micelles and detergent-saturated liposomes (at a detergent-to-phospholipid weight ratio of 1.25, i.e. in the presence of 5 mg TX 100/ml about 50% of the preformed liposomes have been solubilized) and (iii) complete solubilization of the lipids which are now present as mixed micelles (this onset of total solubilization occurs at a detergent-to-phospholipid weight ratio of 2 (2.5 mol/mol), i.e. in the presence of 8 mg TX 100/ml).

It is seen from Fig. 5 that starting from an initial detergent-to-phospholipid weight ratio of 0.5 the major part of the detergent was removed from the medium by a process which gave rise to an essentially monoexponential decay curve (data not shown) with a half time of about 10 min. Interestingly, less than 0.015 mg/ml of residual detergent (0.35 TX 100 per 100 lipid molecules) was detected after 3 h of incubation. At this initial detergent-to-phospholipid ratio which corresponded to the onset of liposome solubilization [15] all the detergent present in solution was monomeric. These results

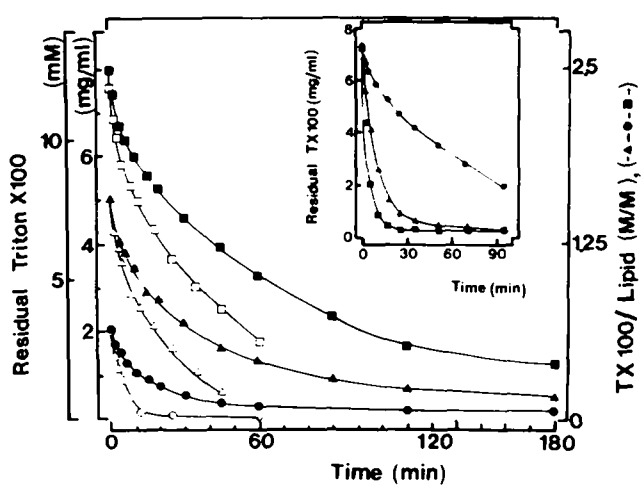

Fig. 5. Time-course of Triton X-100 removal by Bio-Beads SM_2 in the presence of phospholipids. Liposomes prepared by reverse-phase evaporation were resuspended at a concentration of 4 mg lipid/ml and treated by different amounts of TX 100 (8 (■), 4 (▲) and 2 (●) mg TX 100/ml). Then 160 mg Bio-Beads were added to 2 ml of each lipid-detergent mixture and aliquots from the supernatant analyzed for their radioactivity. Open symbols: detergent removal in the absence of phospholipid measured under the same experimental conditions. (Inset) Influence of Bio-Bead SM_2 concentrations on TX 100 removal. Liposomes (4 mg lipid/ml) were solubilized in the presence of 8 mg TX 100/ml and then mixed with 80 (●), 240 (▲) or 460 (■) mg beads/ml.

indicate that even in the presence of phospholipids, non-micellar detergent can be adsorbed efficiently onto Bio-Beads and consequently that in detergent-mediated reconstitution experiments removal of residual TX 100 can be almost complete.

In the case of higher detergent concentrations (5 and 8 mg TX 100/ml, i.e., detergent-to-phospholipid molar ratios of 1.5 and 2.5, respectively) the process of TX 100 removal is no longer mono-exponential and its half-time increases drastically with the initial detergent concentration. For example starting from isotropic detergent phospholipid micellar solutions containing 8 mg TX 100/ml, 1.2 mg TX 100/ml is still present outside the beads after 3 h of incubation. Since the adsorptive capacity of the beads is not exhausted under these experimental conditions (see above) perhaps one could propose that the detergent that is hard to remove is bound to the inner leaflet of the vesicular membrane formed during detergent removal from mixed micelles. In this connection, Ueno et al. [14] suggested a slow movement of non ionic detergent (*n*-dodecyl octaethylene glycol) across the bilayer of reconstituted vesicles. However, this possibility is not consistent with the data reported in the inset of Fig. 5 which demonstrates that the rate of detergent removal is critically dependent upon the amount of Bio-Beads. Thus it took 10 h to remove all the detergent initially present by 80 mg Bio-Beads/ml. Comparatively, in the presence of 240 mg beads/ml removal of the detergent can be achieved in about 2 h and in 20 min in the presence of 360 mg beads/ml. Noteworthy, starting from detergent-phospholipid micelles less than 0.25 mg/ml of residual detergent was detected corresponding to about 7 TX 100 molecules per 100 lipid molecules in the reconstituted liposomes. Furthermore at the highest bead concentration analyzed, the time course of detergent adsorption was found mono-exponential down to detergent-phospholipid ratio of about 0.1 (data from inset in Fig. 5, not shown). These results contrast clearly with the report of Ueno et al. [14] which describes a break in the adsorption rate of their non-ionic detergent at a detergent to phospholipid ratio of 1 and with about 0.3 mol detergent per mol lipid which remained attached to the reconstituted vesicles and cannot be removed. Evenmore, Lemaire et al. [30] reported later a flip-flop rate of less than 100 ms for this detergent. Clearly the most important limiting factor in the rate of TX 100 removal by Bio-Beads SM_2 is the availability of the free bead surface because the accessibility of TX 100 to the pores of the bead is hampered by the prior binding of other detergent molecules.

In addition, Fig. 5 also shows that adsorption of TX 100 by the Bio-Beads occurs more quickly from a solution not containing phospholipids (closed symbols) whatever the initial detergent concentration. The delayed uptake rate of TX 100 in the presence of phospholipids can be accounted for by many reasons: (i) only unbound detergent interacts with the hydrophobic beads *; (ii) removal of detergent associated with lipids is less efficient and is limited by the rate of exchange between the two detergent pools (bound and free) and/or (iii) prior binding of phospholipids to the pores of the beads gives rise to a delay in detergent removal (see below).

Phospholipid binding to Bio-Beads SM_2

To obtain efficient liposome reconstitution it is important to minimize the losses of phospholipids during detergent removal by Bio-Beads SM_2. In this connection, phospholipid adsorption onto hydrophobic resins has sometimes been reported [12,14]. Here, experiments were thus conducted to determine the extent of phospholipid adsorption. For this purpose untreated liposomes or detergent-phospholipid mixtures were suspended with Bio-Beads and the phospholipid concentration of the solutions was kinetically analyzed. The results reported in Fig. 6 and Table I lead to a number of interesting observations. First, adsorption of phospholipid by the Bio-Beads occurs in a short time. As shown in the inset of Fig. 6, plateau values were always reached in about 5 min whatever the initial detergent-to-phospholipid ratios, phospholipid concentrations and/or Bio-Bead concentrations. Second, as illustrated in Fig. 6, the amount of lipid bound to the beads increased linearly with the bead concentrations. Third, the amount of phospholipid adsorbed depended upon the initial detergent-to-phospholipid ratio. From the results reported in Table I, it can be seen that in the absence of detergent, about 0.9 mg phospholipid was adsorbed onto 1 g of Bio-Beads. This amount increased to 1.2 mg when liposomes were pretreated with solubilizing amounts of TX 100 and to 2.4 mg in the presence of an excess of detergent. It is interesting to note that although the adsorptive capacity of Bio-Beads towards phospholipid is very low (100–200-times lower than for TX 100) our results point out the need to use high

* Our previous studies (Paternostre et al. [15] indicated that in the presence of phospholipids the detergent aqueous monomer concentration was significantly lower than the critical micelle concentration of the detergent determined in aqueous solution (0.12 mg TX 100/ml in the presence of 4 mg/ml lipid as compared to 0.2 mg TX 100/ml in aqueous solution as deduced from Fig. 2). Therefore in the presence of lipids the concentration of monomeric detergent, i.e., the detergent pool that interacts with Bio-Beads is about 1.5-times lower than in the absence of lipid. This difference is even more important when taking into account for the observation that in the absence of lipids pure TX 100 micelles are also adsorbed, thus contributing to the free detergent pool that interacts with the beads. On the other hand mixed-phospholipid-TX 100 micelles adsorption is very limited as indicated by the very low phospholipid adsorption. Under these conditions TX 100 adsorption may occur through dissociation of micelles into monomers.

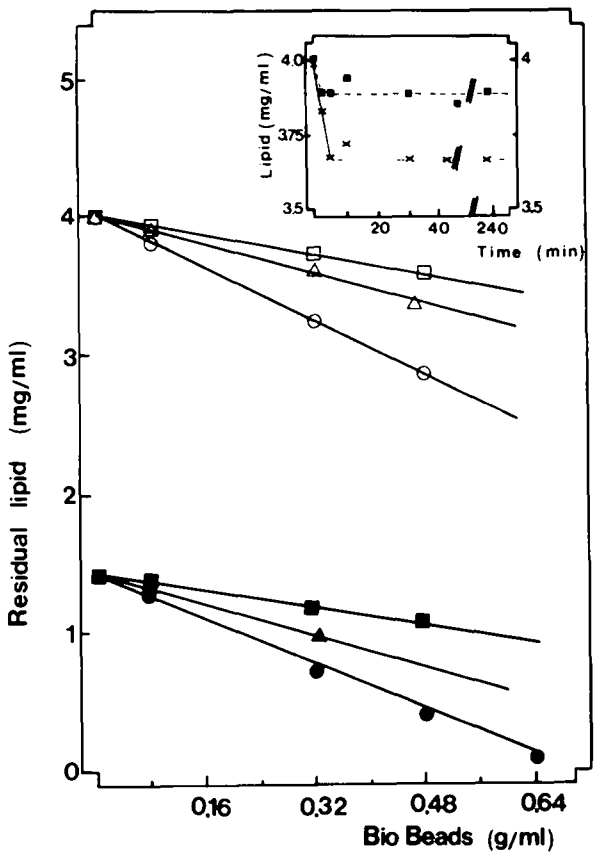

Fig. 6. Phospholipid binding to Bio-Beads SM_2. Liposomes containing [^{14}C]PC were prepared by reverse-phase evaporation, resuspended at 4 (open symbols) or 1.4 (closed symbols) mg lipid/ml and then treated with different proportions of Triton X-100: 0 (□,■), 8 (△,▲) and 16 (○,●) mg TX 100/ml. Final volume 1 ml. These solutions were then mixed over a period of 1 h with the indicated amount of Bio-Beads SM_2 and supernatants analyzed for their radioactivity. (Inset) Kinetics of phospholipid binding to Bio-Beads SM_2. Liposomes (4 mg lipid/ml) were solubilized in the presence of 8 mg TX 100/ml and mixed with 80 (■) or 320 (×) mg beads/ml.

phospholipid concentrations and low Bio-Bead-to-phospholipid ratios in order to minimize loss of lipids during reconstitution experiments. A reasonable explanation for the increase in phospholipid absorption with increasing detergent to phospholipid ratio would be to consider the reduction in size of the mixed micelles associated with increasing detergent concentrations [15]. Mixed micelles with decreasing size could enter more easily the resin pores.

Basic procedure for TX 100 mediated liposome reconstitution

On the basis of all the results presented above a reconstitution procedure was developed providing a reproducible way to achieve unilamellar, relatively large and impermeant liposomes starting from micellar phospholipid-TX 100 solutions. The principles of the procedure are as follows: (i) enough detergent must be added for complete lipid solubilization; (ii) initial Bio-Bead concentration must be adjusted to promote micellar-to-lamellar transition in not less than about 3 h; (iii) after such transition, Bio-Bead concentration can be increased to accelerate detergent removal; (iv) final Bio-Bead concentration must never exceed about 50-times the lipid concentration (w/w).

Turbidity measurements provide a convenient way to monitor such reconstitution (Fig. 7). Here lipids were resuspended at the desired concentration (reconstitution experiments have been performed between 2 and 16 mg/ml lipid without significant change) and TX 100 was added under vortex mixing at the concentration needed for complete solubilization. After 5–10 min of incubation (time of detergent equilibration was complete within few minutes), a few Bio-Beads were added (bead-to-detergent weight ratio of about 10) allowing slow detergent removal over a period of 3 h. At this stage the micellar-to-lamellar transition was complete as indicated by the constant optical density of the solution and by the average detergent-to-phospholipid weight ratio of 0.5 (0.64 mol/mol) reached. Then a second portion of fresh beads was added for fast removal of residual detergent: 2 h were generally sufficient to remove all the detergent. Among the parameters exerting a predominant influence on the results of the reconstitution (temperature, detergent to Bio-Beads ratio, and Bio-Bead concentration) we believe that the most important is the low bead-to-detergent ratio used in the first step of reconstitution: this is essential, both to

TABLE I

Characterization of phospholipid binding to Bio-Beads SM_2

The experimental conditions are described in the legends of Fig. 6.

Lipid (mg/ml)	Triton X-100 (mg/ml)	Triton X-100/lipid ratio (mol/mol)	Bio-Beads SM_2 (g/ml)	Phospholipid adsorptive capacity (mg lipid/g beads) (±0.1)
4	0	0	0.32	1
4	1.6	0.5	0.08	0.9
4	1.6	0.5	0.32	0.85
4	8	2.5	0.08	1.2
4	8	2.5	0.32	1.2
4	16	5	0.08	2.25
4	16	5	0.32	2.3
4	16	5	0.48	2.2
1.4	0	0	0.08	0.8
1.4	0	0	0.32	0.9
1.4	2.8	2.5	0.08	1
1.4	2.8	2.5	0.32	1.2
1.4	5.6	5	0.08	2.4
1.4	5.6	5	0.32	2.6

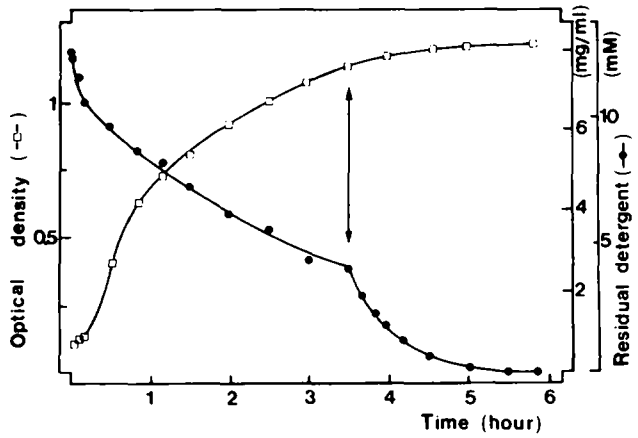

Fig. 7. Triton X-100 mediated reconstitution of liposomes using Bio-Beads SM_2 as detergent removing agent. Liposomes prepared by reverse evaporation were resuspended at 4 mg lipid/ml in a buffer 110 mM K_2SO_4, 20 mM Pipes (pH 7.2) and solubilized by 8 mg/ml TX 100 and 1 μg [^{3}H] TX 100. Then Bio-Beads (80 mg/ml wet beads) were added to the clear solution to remove detergent. Aliquots from the supernatant were removed at various time intervals for determination of TX 100 concentration (●) and optical density (□). The arrow indicated when additional fresh beads (80 mg/ml) were added. Turbidity of phospholipid-detergent mixtures was measured at 400 nm.

avoid phospholipid losses (see Table I) and to allow slow detergent removal during the micellar-to-lamellar transition, thus giving homogeneous and relatively large liposome preparations as demonstrated by the following electron microscopy study.

The size and unilamellarity of liposomes reconstituted by the method described above have been analyzed by freeze-fracture electron microscopy. Independently of the initial Bio-Bead concentrations used in this study (40 to 800 mg Beads/ml) reconstituted liposomes consisted of a unilamellar vesicle population (data not shown). However the sizes of the vesicles formed upon TX 100 removal drastically depended upon the rate of detergent elimination (Fig. 8): at increased detergent removal rate, liposomes become smaller. At the slowest rate of detergent removal analyzed, the vesicles obtained consist of a fairly homogeneous unilamellar vesicle population with a mean diameter of about 150 nm (Panel A) while at higher detergent removal rates small unilamellar vesicles are formed with mean diameters around 50 nm (Panels B and C in Fig. 8). However, calculation of phospholipid distribution (assuming an area per phospholipid molecule of 70 Å^2) indicate that the small liposomes comprise only 3–5% of total phospholipid molecules.

Ionic permeability

Since liposomes are mainly useful for transport studies we investigated some characteristics of the passive permeability of the liposomes prepared by the batch procedure using Bio-Beads SM_2 as TX 100-removing agent.

Proton fluxes generated by external acid pulses were monitored using the fluorescence of the pH-sensitive probe pyranine trapped inside liposomes [20]. Fig. 9A shows a typical experiment in which concentrated H_2SO_4 is injected in a reconstituted liposome suspension in order to lower the external pH from 7 to 6.5. A biphasic decrease in fluorescence intensity was observed: an initial rapid component that comprised about 5% of the total responses and which was attributable to small amount of external pyranine [20]; a slow kinetic component which reflected the internal pH decrease

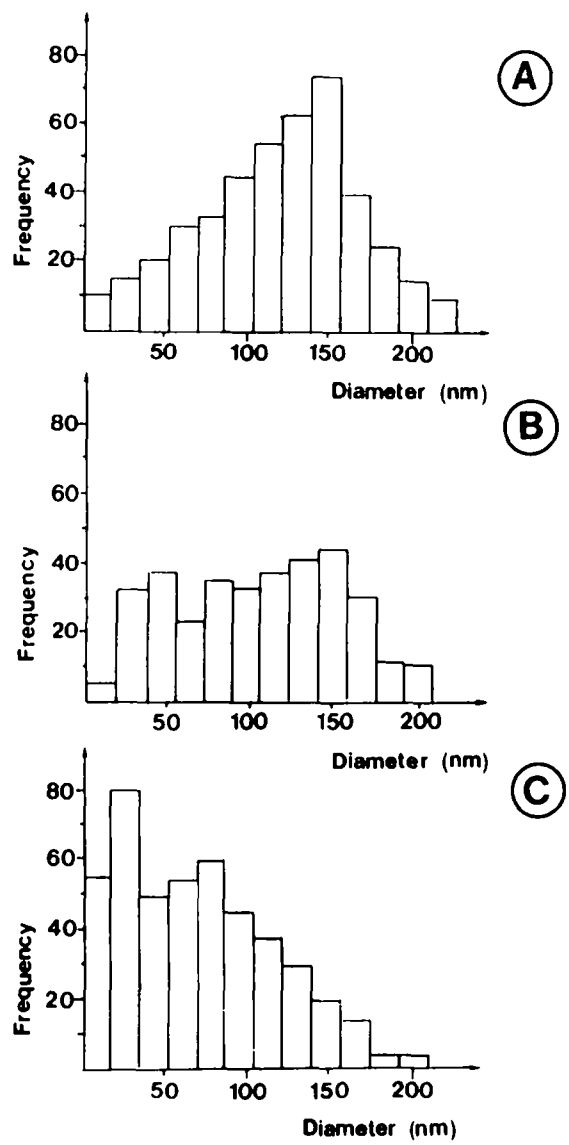

Fig. 8. Size distribution histograms of reconstituted liposomes. Liposomes prepared by reverse-phase evaporation were solubilized as described in the legend of Fig. 7 and treated by different amounts of beads. After total detergent elimination the samples were analyzed by freeze-fracture electron microscopy. Size distribution histograms of liposomes obtained after detergent removal by 80 (Panel A), 200 (Panel B) and 800 (Panel C) mg beads/ml. Bars are 16.66 nm wide.

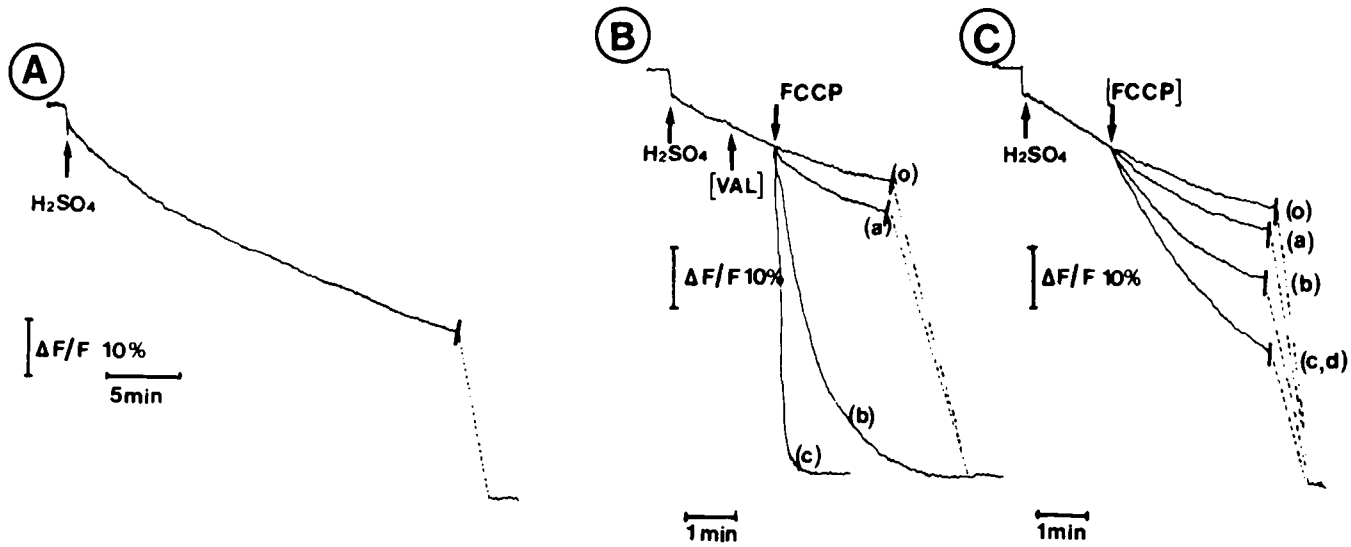

Fig. 9. Ionic permeability of reconstituted liposomes. Liposomes were prepared by the batch procedure as described in the legend of Fig. 6 except that the buffer initially contained 200 μM pyranine. After detergent removal, external pyranine was eliminated by passage through a Sephadex G-25 column. Fluorescence responses of pyranine trapped inside the reconstituted liposomes were analyzed after a 0.5 pH unit acid pulse (Panel A) as described under Materials and Methods. Final phospholipid concentration in the cuvette: 300 μg lipid/ml. Panels B and C: effect of valinomycin and FCCP upon the fluorescence response of entrapped pyranine after a 0.5 pH unit H_2SO_4 pulse. (B) First addition: 0 (o), 0.01 (a), 0.025 (b), 0.05 (c) μM valinomycin; second addition: 0.1 μM FCCP. (C) First addition: 0 (o), 0.0025 (a), 0.01 (b), 0.1 (c), 0.2 (d) μM FCCP.

due to passive H^+ diffusion into the liposomes along the pH gradient. Thus, H^+ equilibration through TX 100-reconstituted liposomes appears to be a rather slow process in K_2SO_4 medium ($t_{1/2} \approx 20$ min). Addition of valinomycin had no effect upon the rate of H^+ equilibration (Fig. 9B) indicating that this rate is governed by the unrestricted H^+ diffusion across the membrane and is not limited by K^+ counterion diffusion. A low permeability coefficient of $2.5 \cdot 10^{-5}$ cm · s^{-1} can be calculated from Fig. 9A. On the contrary addition of the protonophore FCCP to the liposomes after the 0.5 pH unit acid pulse accelerated five fold the slow kinetic component of the fluorescence response (Fig. 9C). In the presence of FCCP, H^+ permeability became limited by K^+ diffusion. Finally, it should be stressed that in the presence of Cl^- in the medium (HCl instead of

TABLE II

Triton X-100-mediated reconstitutions of different membrane proteins

Purple membrane (100 μg protein), Sarcoplasmic reticulum vesicles (100 μg protein) or purified chloroplast ATPase (60 μg protein) were first solubilized in 80 μl of a 10% stock solution of Triton X-100 and then added to 1 ml suspensions of liposomes prepared by reverse-phase evaporation (4 mg lipid/ml). Detergent was removed from the clear solutions by Bio-Beads SM_2 as described in the legends of Fig. 7 and the activities of the different reconstituted systems were analyzed. Light-induced proton movements of bacteriorhodopsin proteoliposomes (110 mM K_2SO_4, 20 mM Pipes (pH 7.2)) were assayed by the pH meter and [^{14}C]methylamine distribution as described earlier [16,20] in the presence of 1 μM valinomycin. Ca^{2+} uptake by Ca^{2+}-ATPase proteoliposomes (110 mM KCl, 10 mM Pipes (pH 7.2)) were measured in the absence of oxalate using Murexide to monitor external Ca^{2+} concentration. ATP synthesis by H^+-ATPase proteoliposomes (40 mM succinate, 20 mM Tricine, 5 mM NaH_2PO_4 (pH 8.0)) were measured using the pH jump technique [28] either after 15 s incubation in the basic medium (ATP yield) or from quenched flow experiments (ATP turn over).

Reconstituted system	activity	
Bacteriorhodopsin [a]	Initial rate	2200 nequiv. H^+/min per mg protein
(proton pump of	Total H^+ extent	3550 nequiv. H^+/mg protein
Halobacterium Halobium)	Δ pH	1.7
Ca^{2+}-ATPase [b]	Initial rate	1.5 μmol Ca^{2+}/min per mg protein
(Ca^{2+} pump of sarcoplasmic	Total Ca^{2+} extent	1.5 μmol Ca^{2+}/mg protein
reticulum)	$[Ca^{2+}]_{int}$	10 mM
H^+-ATPase [c]	ATP yield	150 mol ATP/mol protein
(ATP synthetase of spinach chloroplasts)	ATP turn over	30 mol ATP/s per mol protein

[a] Rigaud et al. [16].
[b] Lévy, D., Seigneuret, M. and Rigaud, J.L. (article in preparation).
[c] Richard, P., Rigaud, J.L., Gräber, P. (article submitted).

H_2SO_4, and/or KCl instead of K_2SO_4) a 10-fold increase in the initial rate of H^+ equilibration was found (data not shown) suggesting, that there exists a particular mechanism for electrically silent codiffusion of H^+ and Cl^- as demonstrated previously for liposomes prepared by reverse-phase evaporation [20].

Membrane protein reconstitution

The reproducible preparation of liposomes of well defined size, with a relative large internal volume and low ionic permeability was a necessary objective for their use in membrane protein reconstitution. We thus applied the 'batch procedure' described above to the reconstitution of integral membrane proteins. As representative examples, bacteriorhodopsin from *Halobacterium Halobium* [26], Ca^{2+}-ATPase from sarcoplasmic reticulum [27] and H^+-ATPase from spinach chloroplasts [28] were reconstituted by detergent removal from TX 100-lipid-protein micelles using the procedure described in Fig. 7. As opposed to detergent and phospholipid binding we have found no significant bacteriorhodopsin or Ca^{2+}-ATPase adsorption onto Bio-Beads SM_2 by direct measurement of protein concentration or using radioactive labelled proteins (data not shown; see also Ref. 21). In all three cases, about 100% incorporation of the protein into liposomes was obtained. However, since this investigation is not concerned with reconstitution per se, we only reported here activities of the different reconstituted proteoliposomes (Table II). Detailed studies of H^+-ATPase (Richard, P., Rigaud, J.L. and Graber, P., (article submitted)) and of Ca^{2+}-ATPase (Lévy, D., Seigneuret, M. and Rigaud, J.L., article in preparation) will be published elsewhere. The main conclusion to be drawn from Table II is that fairly high activities were observed for three different proteins, thus proving the efficiency and general validity of the procedure in creating highly functional proteoliposomes.

Discussion

The most common methods for Triton X-100 removal from mixed detergent lipid micelles rely on the original procedure by Holloway [10], i.e., adsorption of the non-ionic detergent onto Bio-Beads SM_2, a neutral porous styrene-divinylbenzene copolymer. This method has been extended to other hydrophobic resins such as Amberlite XAD_2, to other detergents such as $C_{12}E_8$ or octyl glucoside and the original batch procedure replaced by passage through small columns filled with beads [11,12,14,29]. From all the reports in the literature, sometimes conflicting, severe disadvantages have been pointed out: (i) Detergent is still present in the reconstituted liposomes which may affect theirs properties. This has been related to the fact that copolymer beads only adsorb the micellar species and not the monomer [7,10], to a slow flip-flop of the detergent across the bilayer (Ueno et al. [14] contradicted by Le Maire et al., [30]) and/or to the difficulty of removing the high and low molecular weight components of TX 100 [13]. (ii) Heterogeneous liposome or proteoliposome preparations are sometimes formed and related to the rate of detergent removal [7,8] or the proper initial lipid-detergent dispersion [31]. (iii) Large losses of lipids have been observed by Philipot et al. [12] and Ueno et al. [14] but not by other authors [6,8,32]. The incentive for studying in details the adsorption of TX 100 onto Bio-Beads SM_2 was partly provided by the contradictory nature of these results and partly by the fact that this detergent is one of the most suitable detergents for solubilizing and purifying membrane proteins [1,2]. We therefore believe that the results and methods developed here are important for membrane protein reconstitution studies mediated by Triton X-100.

The data reported in this paper allow us to identify several parameters exerting a predominant influence on the rate of TX 100 adsorption onto Bio-Beads SM_2: they include the amount and source of detergent, the amount of phospholipid, the amount of Bio-Beads and the temperature of the incubation medium. One striking feature of our results is the demonstration that a critical factor in determining detergent adsorption is the availability of the free bead surface. Indeed the data reported in Figs. 1 and 5 indicate that the rates of TX 100 removal were strongly dependent on and linearly related to the amount of Bio-Beads present in the solution, even in experimental conditions where the bead-to-detergent weight ratios were higher than the adsorptive capacity of the beads. Clearly, the accessibility of the detergent to the pores of the beads is hampered by the prefixation of the detergent molecules, and consequently, the key factor in determining TX 100 adsorption is the bead-to-detergent ratio. In addition, the mechanism by which TX 100 is adsorbed onto the beads was shown to be critically affected by the commercial source of the detergent (Fig. 3) and the temperature of the experiment (Fig. 4). In this respect, the elimination of detergent from a 9 mg/ml TX 100 solution with a bead-to-detergent ratio twice as high as the adsorptive capacity of the beads was complete within 30 min at 37 °C whereas it took over 5 h at 5 °C. All these findings may clarify the conflicting observations made in previous studies in which all these kinetics-influencing parameters have not been emphasized and where TX 100 removal was only related to the maximal adsorptive capacity of the beads, i.e., to the equilibrium aspect of detergent adsorption.

Concerning the mechanisms by which TX 100 is adsorbed onto Bio-Beads SM_2, the experiments reported in this paper demonstrate definitely that monomeric as well as micellar detergent are adsorbed onto the polystyrene beads. Furthermore, the dependence of

the initial adsorption rates on the total detergent concentration depicted in the inset of Fig. 2 indicates that monomers were adsorbed more efficiently than micelles. The obvious consequence of these observations is that it is possible to remove with Bio-Beads virtually all TX 100. Indeed our data show that whatever the initial experimental conditions (i.e., initial detergent concentration, temperature, detergent source) TX 100 removal by Bio-Beads SM_2 was almost complete. Importantly the present conclusions also apply when the polystyrene beads are used for detergent removal in the presence of phospholipids (Fig. 5). From the point of view of TX 100-mediated liposomes reconstitution, this procedure leads to vesicles containing in any case less than 7 TX 100/100 lipids. The presence of residual detergent at the levels found here may be also the result of detergent molecules trapped inside the vesicles: during reconstitution very small portions of the beads which tightly bind Triton X-100 may become trapped within the liposomes during their formation resulting in detergent sequestration inside the newly formed liposomes [13,30]. Implication of the almost complete detergent removal is the relatively low ionic permeability of the liposomes reconstituted by the batch procedure described in this paper. Observed H^+ permeabilities were in the range of 10^{-5} cm · s^{-1}, which is larger than those of liposomes prepared by reverse-phase evaporation [20] but still much smaller than those of other liposomal preparations [33].

The one observation which constitutes an unavoidable disadvantage in the use of Bio-Beads SM_2 in reconstitution experiments is that lipids are adsorbed by the beads. However, our data indicate that the adsorptive capacity towards phospholipids is very small compared to that of TX 100 (2 mg lipids per g beads as against to 185 mg TX 100 per g beads). Thus we would like to emphasize that, in reconstitution experiments using Bio-Beads SM_2 as TX 100 removal agent, lipid loss can be minimized (or neglected) if one uses high phospholipid concentrations or if one does not try to remove as much as detergent as possible in a single incubation time with a large excess of beads.

Beside providing new informations concerning the way by which TX 100 may adsorb to Bio-Beads SM_2, we believe that the most important benefit of our systematic study is the finding that the reconstitution method described in this paper provides a convenient and reproducible way for preparing large, unilamellar and fairly homogeneous preparation of liposomes with no significant amount of residual detergent. The main difference between the previously described methods for TX 100-mediated liposome reconstitution and the one described in this work resides in the way by which TX 100 is removed by adsorbent beads: the batch procedure with successive bead additions allows relatively slow and constant detergent removal which is essential and prevents the formation of heterogeneous liposomal systems. In this context, 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 liposomes are formed by rapid detergent removal, larger liposomes are formed by slow removal. These observations can be interpreted in the light of a model proposed by Lasic et col. (for a review, see Ref. 34) for vesicle formation by detergent-depletion techniques: three steps occur in the overall process, namely, micellar equilibration (micellar growth by fusion or phospholipid exchange), vesiculation (bilayer closure) and post-vesiculization size growth (due to the residual detergent in the formed vesicle). In general slower detergent depletion produces larger vesicles because micelle fusion [34], lipid exchange [35] and postvesiculization [14] are not instantaneous processes.

Finally, we would like to point out a more technical feature that emerges from this study and which is specifically related to membrane protein reconstitution into liposomes: it is that as pointed out in a recent workshop on membrane protein reconstitution [36], the rate of detergent removal is a key factor for final protein orientation, lipid-protein ratio homogeneity and liposomal morphology. Obviously no one strategy is likely to serve equally well for all membrane proteins. For example rapid dilution of rhodopsin-lipid-octyl glucoside micelles leads to more compositionally homogeneous preparations than does the slow detergent removal by dialysis [37]. On the contrary there are many examples in which slow detergent removal was found better appropriate than a rapid one [38,39]. Thus, useful detergent-mediated reconstitution procedures must be well suited for varying and controlling the rate of detergent removal: on the basis of our results, this is clearly the case for the batch procedure using Bio-Beads SM_2 as TX 100-removing agent. Additionally, although the results described here have been mainly dealing with reconstitution experiments starting from completely solubilized material, these are immediately transposable to reconstitution experiments by 'direct protein incorporation' using low detergent concentrations [16].

In conclusion, we would once again stress that the method of preparation described in this paper satisfies all the criteria that make the procedure well suited for membrane reconstitution. Our preliminary reports on H^+-ATPase and Ca^{2+}-ATPase reconstitutions together with our previous systematic study on bacteriorhodopsin demonstrate the high efficiency of the proteoliposomes reconstituted by this procedure. In this connection detailed studies of the reconstitution processes with other detergents which are adsorbed onto Bio-Beads (i.e., octyl glucoside, sodium cholate and $C_{12}E_8$) and with different membrane proteins are currently in progress in our laboratory and are likely to result in the

formulation of a set of general principles for reconstitution experiments.

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References

1 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.
2 Møller, J.V., Le Maire, M. and Andersen, J.P. (1986) Prog. Protein–Lipid Interact. 2, 147–196.
3 Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A. (1980) Biochim. Biophys. Acta 599, 699–714.
4 Chiesi, M., Peterson, S.W. and Acuto, O. (1978) Arch. Biochem. Biophys. 189, 132–136.
5 Jackson, R.L., Verkleij, A.J., Van Zoelen, E.J.J., Lane, L.K., Schwartz, A. and Van Deenen, L.L.M. (1980) Arch. Biochem. Biophys. 200, 269–278.
6 Lüdi, H., Oetliker, H., Brodbeck, U., Ott, P., Schwendimann, B. and Fulpius, B.W. (1983) J. Membr. Biol. 74, 75–84.
7 Van Dijck, P.W.M. and Van Dam, K. (1982) Methods Enzymol. 88, 17–25.
8 Wolosin, J.M. (1980) Biochem. J. 189, 35–44.
9 Allen, T.A., Romans, A.Y., Kerecret, H. and Sergest, J.P. (1980) Biochim. Biophys. Acta 601, 328–342.
10 Holloway, P.W. (1973) Anal. Biochem. 53, 304–308.
11 Cheetham, P.S.J. (1979) Anal. Biochem. 92, 447–452.
12 Philippot, J., Mutaftschiev, S. and Liautard, J.P. (1983) Biochim. Biophys. Acta 734, 137–143.
13 Scheule, R.K. and Gaffney, B.J. (1981) Anal. Biochem. 117, 61–66.
14 Ueno, M., Tanford, C. and Reynolds, J.A. (1984) Biochemistry 23, 3070–3076.
15 Paternostre, M.T., Roux, M. and Rigaud, J.L. (1988) Biochemistry 27, 2668–2676.
16 Rigaud, J.L., Paternostre, M.T. and Bluzat, A. (1988) Biochemistry 27, 2677–2688.
17 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53–57.
18 Allgyer, T. and Wells, M.A. (1979) Biochemistry 18, 5348–5353.
19 Rigaud, J.L., Bluzat, A. and Büschlen, S. (1983) Biochem. Biophys. Res. Commun. 111, 373–382.
20 Seigneuret, M. and Rigaud, J.L. (1986) Biochemistry 25, 6716–6722.
21 Moriyama, R., Nakashima, H., Makimo, S. and Koga, S. (1984) Anal. Biochem. 139, 292–297.
22 Crook, E.H., Fordyce, D.B. and Trebbi, G.F. (1963) J. Phys. Chem. 67, 1987–1994.
23 Egan, R.W., Jones, M.A. and Lehninger, A.L. (1976) J. Biol. Chem. 251, 4442–4447.
24 Ashani, Y. and Catravas, G.N. (1980) Anal. Biochem. 109, 55–62.
25 Lichtenberg, D. (1985) Biochim. Biophys. Acta 821, 470–478.
26 Dencher, N.A. (1985) Photochem. Photobiol. 38, 753–767.
27 Tanford, C. (1984) Crit. Rev. Biochem. 17, 123–151.
28 Gräber, P. (1987) Bioelectrochemistry II (Milazzo, G. and Blanks, M., eds.), pp. 379–429, Plenum Pub. Corp, New York.
29 Krämer, R. and Heberger, C. (1986) Biochim. Biophys. Acta 863, 289–296.
30 Le Maire, M., Møller, J.V. and Champeil, P. (1987) Biochemistry 26, 4803–4810.
31 Keenan, A.K., Gal, A. and Levitzki, A. (1982) Biochem. Biophys. Res. Commun. 105, 615–623.
32 Cornelius, F. and Skou, J.C. (1984) Biochim. Biophys. Acta 772, 357–373.
33 Deamer, D.W. (1987) J. Bioenerg. Biomembr. 19, 457–479.
34 Lasic, D.D. (1988) Biochem. J. 256, 1–11.
35 Almog, S., Kushnir, T., Nir, S. and Lichtenberg, D. (1986) Biochemistry 25, 2597–2605.
36 Silvius, J.R. and Allen, T.M. (1989) Biophys. J. 55, 207–208.
37 Jackson, R.L. and Litman, B.J. (1985) Biochim. Biophys. Acta 812, 369–376.
38 Dierks, T. and Krämer, R. (1988) Biochim. Biophys. Acta 937, 112–126.
39 Eytan, G.D. (1982) Biochim. Biophys. Acta 694, 185–202.