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Kinetic studies on the interaction of phosphatidylcholine liposomes with Triton X-100

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Sonicated unilamellar and large multilamellar liposome suspensions have been treated with the non-ionic detergent Triton X-100, and the subsequent changes in turbidity have been studied as a function of time. Sonicated liposome suspensions exhibit an increase in turbidity that takes place in two stages, a fast, low-amplitude one is completed in less than 100 ms, and a slow large-amplitude one occurs in 20–40 s. The first increase in turbidity is associated to detergent incorporation into the bilayer, and the second one, to vesicle fusion. The fast stage may be detected at all detergent concentrations, while the slow one is only seen above the critical micellar concentration of Triton X-100. Both processes may be interpreted in terms of first-order kinetics. Studies of the variation of k_{exp} with lipid and detergent concentration suggest a complex multi-step mechanism. In the case of multilamellar liposomes, a fast increase in turbidity is also seen after detergent addition, which is followed by a slow (20–60 s) decrease in turbidity and a very slow (up to 12 h) large scale decrease in turbidity. These processes do not conform to single-exponential patterns. The fast stage is also thought to reflect surfactant incorporation, while the decrease in turbidity is interpreted as bilayer solubilization starting with the outer bilayer (slow stage) and proceeding through the remaining ones (very slow stage).

Introduction

The interaction of detergents with phospholipid bilayers leads to the breakdown of lamellar structures and formation of lipid-detergent mixed micelles [1,2]. Previous studies from this laboratory [3,4] have shown that the behaviour of Triton X-100 and other commonly used detergents is different with multilamellar or with sonicated unilamellar vesicles. In the former case, solubilization is the only phenomenon observed, and the suspension turbidity decreases monotonically with surfactant addition. Small unilamellar liposomes, however, fuse together so that the turbidity of the suspension increases, reaching a maximum at equimolar phosphatidylcholine (PC) and Triton X-100 proportions; at this point, only multilamellar vesicles are seen, arising from fusion of the small ones. Increasing amounts of detergent fi-

Abbreviations: PC, phosphatidylcholine; k_{exp}, experimental rate constant.

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nally produce the solubilization of these multilamellar structures.

Various aspects of these processes are still poorly understood. One of them is the kinetics of vesicle fusion and bilayer solubilization. The aim of the present study is to explore this relatively unknown aspect, which can give light on the mechanisms of detergent–bilayer interaction. Phosphatidylcholine liposomes and Triton X-100 have been chosen since they are representative examples of membrane models and detergents, respectively, and because their interactions have been partially characterized already in our previous work [3,6]. Both vesicle fusion and solubilization may be easily followed as changes in turbidity, so that our kinetic studies have been directed towards variations of this parameter.

Information on kinetics of detergent effects is scarce. Some time ago it was shown that micelle formation and dissociation are fast reactions with rate constants higher than 10 s^{-1} [7], while movement of surfactant through lipid bilayers seems to be fairly slow, as judged from black lipid film measurements [8,9]. More recently Lichtenberg et al. [10] studied the solubilization of PC multilamellar liposomes by sodium deoxycholate, and showed that the process was a slow one, occurring through consecutive 'peeling-off' of PC bilayers. The incorporation of lysophosphatidylcholine into PC vesicles and lysophosphatidylcholine-induced vesicle fusion are relatively fast processes, whose kinetics have been studied by Elamrani and Blume [11]. In our case, all three processes, detergent incorporation, vesicle fusion and bilayer solubilization have been examined.

Materials and Methods

Egg-yolk PC was purified according to Singleton et al. [12]. Triton X-100 (Rohm and Haas) was from Sigma. Organic solvents were freshly redistilled before use. All other reagents were analytical grade.

Sonicated and multilamellar liposomes were prepared as described previously [3,4]. For slow kinetic studies or equilibrium measurements, aliquots of the liposome suspensions were treated with equal volumes of the appropriate detergent solution. Final phospholipid concentration was 1 mM, unless otherwise stated. Turbidity (as absorbance at 500 nm) was measured in a UV-2600 Beckman spectrophotometer at room temperature. In the case of measurements being carried out many hours after detergent addition, some tendency of the larger vesicles to aggregate and/or precipitate was observed; this was easily reversed by vortex mixing the samples prior to each measurement. Some experiments were carried out in which PC was mixed with dicetyl phosphate, at a 10 : 1 molar ratio, in order to obtain negatively charged bilayers; no aggregation nor precipitation was observed in this case, and solubilization occurred under the same conditions than in pure PC vesicles.

Fast processes were studied with an Aminco DW-2A dual-wavelength spectrophotometer equipped with an Amino-Morrow stopped-flow accessory. The surfactant and liposome suspensions were put in separate syringes and mixed in the optical cell. Turbidity was measured as ΔA (350–800 nm). Final PC concentration was also 1 mM, except when otherwise stated.

Results

Sonicated liposomes

It was known that addition of Triton X-100 produces an increase in turbidity [3]. The stopped-flow technique allows us to distinguish two phases or periods, a fast one and a slow one, in this process (Fig. 1). Within the first 10 ms after reagent mixing, there is a slight increase in turbidity ($\Delta A \approx 0.02$–0.03), reaching usually an apparent equilibrium after about 100 ms (Fig. 1A). However, this cannot account for the large increase in turbidity observed by us [3] and confirmed by other authors [13]. The large-scale turbidity increase is a relatively slow phenomenon, that is seen when the data collection period is extended to 10–60 s, as a ΔA of about 0.1–0.2 units (Fig. 1C). Choosing an intermediate time for data acquisition, we can see the transition between the fast and the slow process (Fig. 1B). Following the studies by Elamrani and Blume [11], we have attributed the fast process to detergent incorporation in the bilayers, and the slow one, to vesicle-vesicle fusion. After fusion had occurred, the system remained stable for hours.

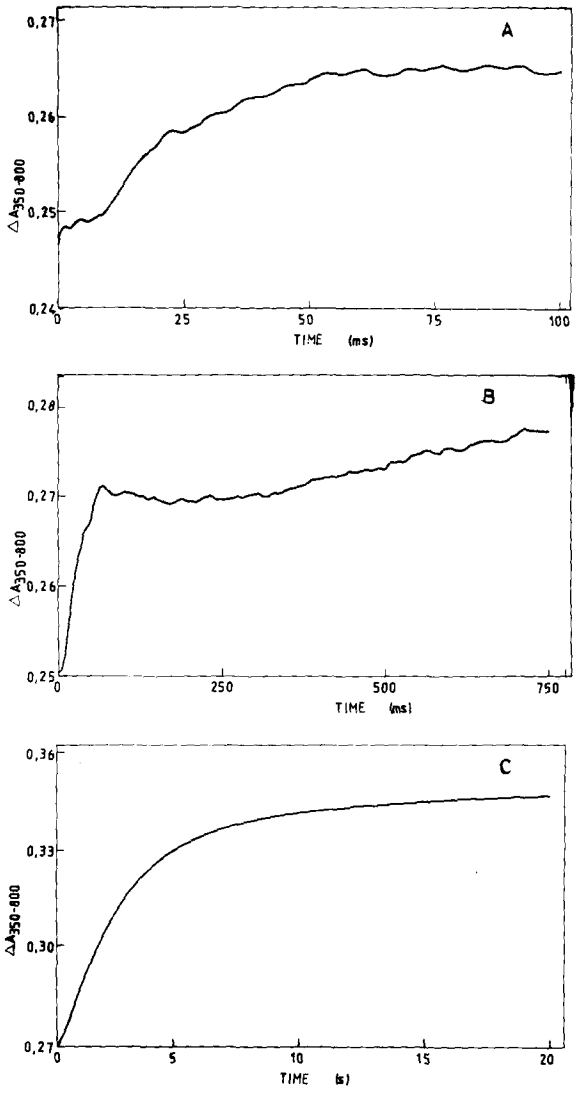


Fig. 1. Changes in turbidity of a 1 mM sonicated liposome suspension, after addition of an equimolar amount of Triton X-100, as a function of time. Data acquisition time: (A) 100 ms; (B) 2 s; (C) 20 s.

The results in Fig. 1 were obtained with equimolar proportions of PC and Triton X-100. This mixture is known to give rise to the maximum increase in turbidity in equilibrium measurements [3]. When the studies described in Fig. 1 are repeated with varying amounts of detergent, an increase in turbidity is always seen in the fast period as soon as some Triton X-100 is present. However, the slow period may consist either of an increase or of a decrease in turbidity, according to the lipid: detergent ratio (Fig. 2) as predicted from our earlier equilibrium measurements. These results support the interpretation of our studies in the terms proposed by Elamrani and Blume [11].

Both the fast and the slow increases in turbidity occurring in detergent-treated liposomes may be fitted to a single exponential. Fig. 3 shows an example of a fast process together with the semilogarithmic representation, according to Guggenheim [20]. The latter is linear as expected from an exponential curve. An experimental rate constant k_{exp} may be obtained from the slope of the straight line in Fig. 3B. The kinetics of Triton X-100 incorporation and vesicle fusion have been studied as a function of detergent and lipid concentration. When the experimental rate constants k_{exp} for the fast process (incorporation) are plotted versus detergent concentration (Fig. 4A) a curve is obtained, which intercepts the origin of the axes. The curve passes through zero detergent concentration, confirming our assertion that, as soon as some detergent is added, its incorporation to bilayers leads to a fast increase in turbidity. The corresponding values for k_{exp} of the slow process are shown in Fig. 4B. A threshold concentration of about 0.25 mM is observed, and this corresponds to the critical micellar concentration of

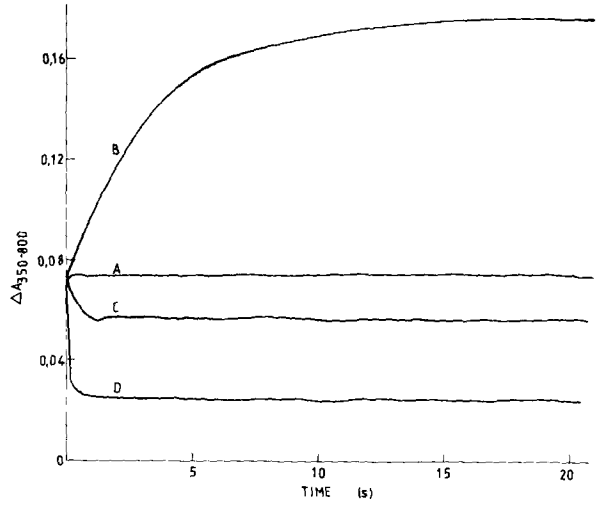


Fig. 2. Changes in turbidity of a 1 mM sonicated liposome suspension, after addition of varying amounts of Triton X-100 as a function of time. Final detergent concentrations (mM): (A) 0; (B) 1; (C) 2; (D) 4.

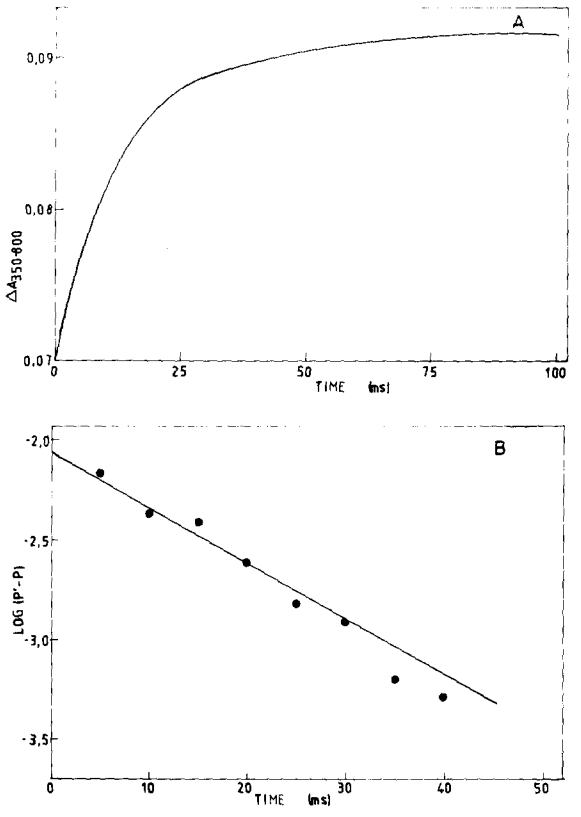


Fig. 3. (A) The fast increase in turbidity of a $5 \cdot 10^{-4}$ M sonicated liposome suspension upon addition of an equimolar amount of Triton X-100. (B) Guggenheim plot of the above exponential trace.

Triton X-100 [14] implying that below such concentration no vesicle fusion occurs, even if the detergent monomers are incorporated to the lipid bilayers.

The influence of lipid concentration was also examined, at a constant 1 mM Triton X-100 concentration (Fig. 5). Values of k_{exp} change with increasing Triton X-100 concentrations in a manner opposite to what was observed when lipid concentration was increased.

Multilamellar liposomes

Under most conditions, multilamellar liposomes interact with Triton X-100 in a fast and a slow stage, as in the case of the sonicated vesicles. However, the slow stage consists here of a decrease in turbidity. In addition, a third, very slow stage of decrease in turbidity is often found.

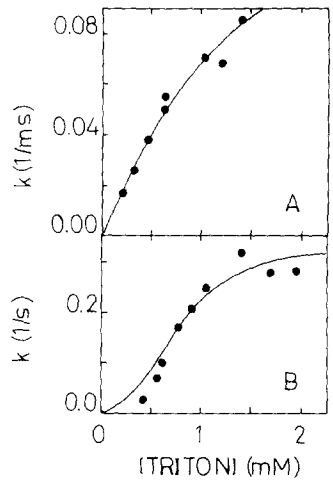


Fig. 4. Experimental rate constants of (A) the fast increase, and (B) the slow increase in turbidity of sonicated liposomes after addition of Triton X-100, as a function of detergent concentration. (●) Experimental results. Curve A is obtained from Eqn. 8 taking the values: $k = 0.28$ ms^{-1}; $K_{D1} = 2.2$ mM^{-1}; $K_D = 0.1$ mM^{-1}; $K = 12600$ mM^{-1}. Curve B is generated from Eqn. 13, taking the values: $k = 0.7$ s^{-1}; $K_1 = 12$ mM^{-1}; $K_2 = 0.7$ mM^{-1}; $m = 128$; $j = 64$.

The first, fast process is shown in Fig. 6A for a 1:1 PC/Triton X-100 molar ratio. It is in principle very similar to what found for sonicated liposomes. After about 0.5 s, turbidity starts decreasing (Fig. 6B) and continues doing so for the next 20–60 s (Fig. 6C). The effect of varying detergent

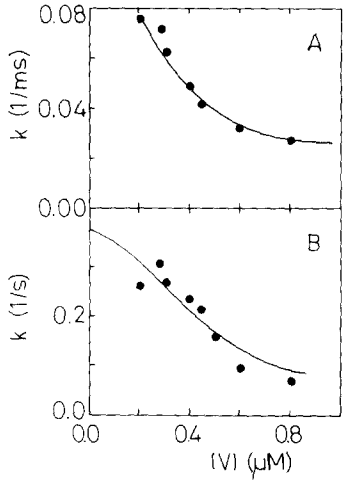


Fig. 5. As in Fig. 4, as a function of vesicle concentration. 2450 lipid molecules per vesicle are assumed [18].

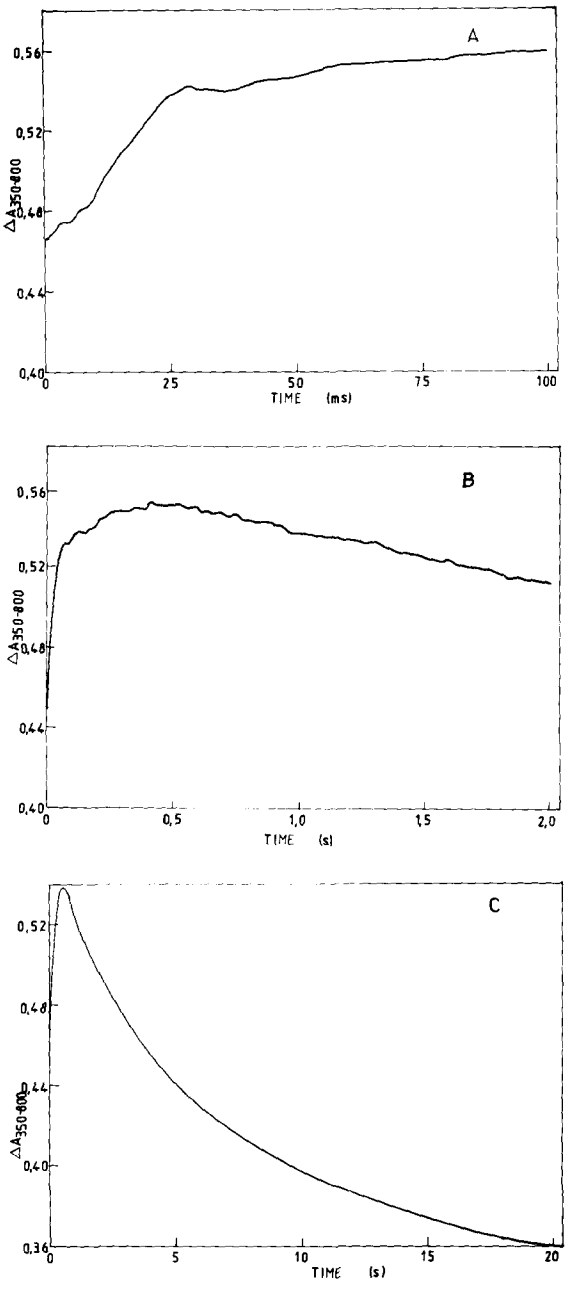


Fig. 6. Changes in turbidity of a 1 mM multilamellar liposome suspension, after addition of an equimolar amount of Triton X-100, as a function of time. Data acquisition time: (A) 100 ms; (B) 2 s; (C) 20 s.

concentrations on the slow process is shown in Fig. 7; the rate increases with increasing detergent concentrations. As in the case of sonicated vesicles, the fast component may be observed as soon as

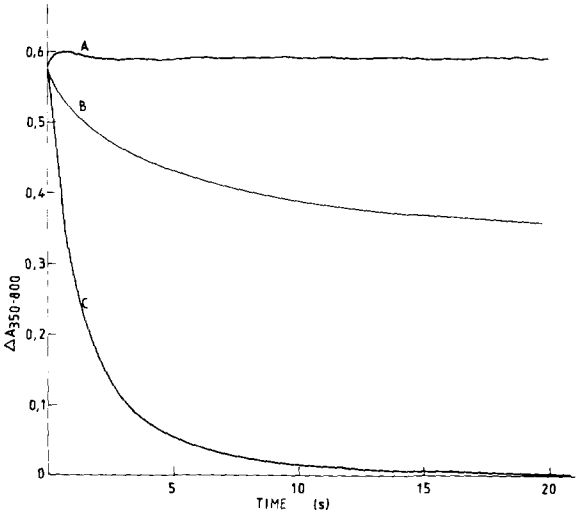


Fig. 7. Changes in turbidity of a 1 mM multilamellar liposome suspension, after addition of varying amounts of Triton X-100, as a function of time. Final detergent concentrations (mM): (A) 0.2; (B) 1; (C) 8.

some detergent is added; however, only Triton X-100 concentrations above the critical micellar concentration are able to produce bilayer solubilization. One important difference between the sonicated unilamellar vesicles and the multilamellar liposomes, is that, in the latter case, the turbidity plots as a function of time are usually not exponential. This property, which prevents further kinetic studies, is attributed to the difficulty of simple simultaneous interaction of the inner and outer bilayers with the detergent.

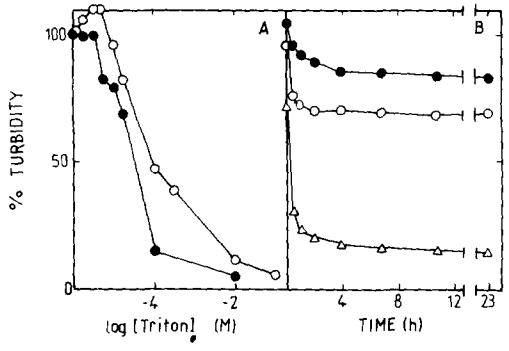


Fig. 8. (A) The turbidity of a 1 mM multilamellar liposome suspension treated with varying amounts of Triton X-100. (○) 5 min after detergent addition; (●) 24 h after detergent addition. (B) Changes in turbidity of some of the above mixtures as a function of time. Final surfactant concentrations: (●) $7.5 \cdot 10^{-4}$ M; (○) $1.25 \cdot 10^{-3}$ M; (△) $2 \cdot 10^{-3}$ M.

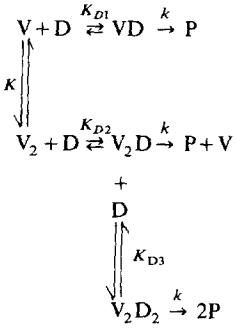
We have also monitored a third, very slow, process occurring when multilamellar liposomes are treated with Triton X-100, and consisting of a further decrease in turbidity. This may be seen by comparison of the turbidities of detergent-treated liposome suspensions 5 min and 24 h after detergent addition (Fig. 8A). No changes were apparent after 24 h. We have examined this slow decrease in turbidity as a function of time for a variety of detergent concentrations. Some results are shown in Fig. 8B. The curves are approximately exponential, with solubilization half-times of the order of hours. Again the rates seem to increase with detergent concentrations, but we have not found any simple relationship between both parameters.

Discussion

Fast stage

The increase in turbidity observed both in sonicated and non-sonicated vesicles within 100 ms after detergent addition (Figs. 1 and 6) is attributed to the incorporation of surfactant into the lipid bilayer. We follow in this case the argumentation of Elamrani and Blume [11] who studied the incorporation of lysophosphatidylcholine into PC bilayers. In summary, turbidity may increase because of vesicle shrinking or of increasing vesicle weight; in our case, it is generally accepted that the detergent is incorporated into the bilayers in the form of monomers (stage I of bilayer solubilization, Ref. 1); this increases vesicle weight with only a slight increase of vesicle radius, and thus turbidity increases. A quantitative relationship between vesicle size and specific turbidity has been developed [15] and was found to support the interpretation of Elamrani and Blume [11] of the fast increase in turbidity. Our increments are of the same order of magnitude as theirs, but much faster (about two orders of magnitude). We have shown that the interaction of Triton X-100 with lipid bilayers is very different from that of lysophosphatidylcholine [4,16], and this may explain the difference in timescale. In addition, the fact that the experimental rate constant has a value of zero in the absence of surfactant also supports the idea that the fast stage turbidity increase is due to the incorporation of detergent monomers.

We can analyze this process, at least for sonicated unilamellar vesicles, in terms of the following model that includes the possibility of vesicle association (as different from vesicle fusion), and detergent adsorption to (as different from detergent incorporation into) vesicles



where V represents either a detergent-free vesicle or a vesicle containing one or more incorporated detergent molecules. D is the detergent; VD a vesicle with one adsorbed surfactant monomer, and P, a vesicle containing one or more detergent molecules incorporated into the bilayer. Thus

$$K = \frac{[V_2]}{[V]^2} \tag{1}$$

$$K_{D1} = \frac{[VD]}{[V][D]}\text{; and } [VD] = K_{D1}[V][D] \tag{2}$$

$$K_{D2} = \frac{[V_2D]}{[V_2][D]} = 2K_D\text{; } [V_2D] = 2K_D[V_2][D] \tag{3}$$

$$K_{D3} = \frac{[V_2D_2]}{[V_2D][D]} = \tfrac{1}{2}K_D\text{;}$$

$$[V_2D_2] = \tfrac{1}{2}K_D[V_2D][D] = K_D^2[D]^2[V_2] \tag{4}$$

where K_D is the intrinsic (microscopic) constant [21].

It has been suggested [22] that two vesicles, when free of detergent may not aggregate in this timescale. If this were the case, it should be assumed that at least one of the two vesicles giving rise to the dimer V_2 in our kinetic scheme should contain one or more incorporated detergent molecules in order to facilitate interaction.

An important assumption of this model is that rapid equilibrium exists between the free and adsorbed detergent (equilibrium constants K, K_{D1}, K_{D2}, K_{D3}), whereas detergent incorporation into the bilayer is the rate limiting step of the process (rate constant k). It is also assumed that the equilibrium constant for the adsorption of detergent into a single vesicle is different from that of detergent absorption into a vesicle dimer. Since vesicle dimerization is not a cooperative phenomenon, the apparent equilibrium constants K_{D2} and K_{D3} are kinetically related to the microscopic constant K_D.

Under the rapid equilibrium assumption, a saturation function $\bar{Y}_D$ (i.e., the concentration of vesicular species containing adsorbed detergent molecules divided by the total concentration of vesicular species) may be defined for the reactive species leading to P, a vesicle containing one or more detergent molecules incorporated into the bilayer

$$\bar{Y}_D = \frac{[VD]+[V_2D]+2[V_2D_2]}{[V]+[VD]+2([V_2]+[V_2D]+[V_2D_2])} \tag{5}$$

Introducing the equations for the equilibrium constants, we obtain

$$\bar{Y}_D = \frac{K_{D1}[V][D]+(2K_D[D]+2K_D^2[D]^2)[V_2]}{[V](1+K_{D1}[D])+2(1+2K_D[D]+K^2[D]^2)[V_2]} \tag{6}$$

Substituting now for the dimerization constant K, and simplifying

$$\bar{Y}_D = \frac{K_{D1}[D]+(2K_D[D]+2K_D^2[D]^2)K[V]}{1+K_{D1}[D]+2K[V](1+2K_D[D]+K_D^2[D]^2)} \tag{7}$$

Thus, the apparent first-order rate constant for the production of P, a vesicle containing detergent molecules in its bilayer, is given by

$$k_{app} = k\cdot\bar{Y}_D = \frac{k\{K_{D1}[D]+(2K_D[D]+2K_D^2[D]^2)K[V]\}}{1+K_{D1}[D]+2K[V](1+2K_D[D]+K_D^2[D]^2)} \tag{8}$$

According to the limits of Eqn. 8, k_{app} should vary for vesicle (or lipid) concentration between zero and infinity just as shown in Fig. 5, provided that

$$\frac{K_{D1}}{1+K_{D1}[D]} > \frac{K_D}{1+K_D[D]}$$

On the other hand, k_{app} increases as a function of detergent concentration, according to Eqn. 8, but tends to reach a limiting value. This is also in agreement with the experimental findings (Fig. 4A).

In summary, our experimental results for the fast stage of increase in turbidity may be explained as the result of the absorption and later incorporation of detergent monomers to the phospholipid bilayers of vesicles, or vesicle dimers. It is evident from the above treatment that, once the P species (vesicle containing detergent molecules incorporated into the bilayer) is formed, it behaves in the same way as a detergent-free vesicle V, since it may absorb and incorporate a new molecule of detergent. The picture emerging from these experiments and calculations is different from the simple kinetic mechanism proposed by Elamrani and Blume [11] for the incorporation of lysophosphatidylcholine to lipid bilayers. Thus kinetic measurements show, in accordance with the structural data from our own [4,16] and other [17] laboratories, that the incorporation of Triton X-100 and lysophosphatidylcholine to lipid bilayers follow different patterns.

The interaction of Triton X-100 with bilayers in multilamellar liposomes is probably similar, but requires in addition to the processes described above, the flip-flop movement of detergent across the bilayers, and this accounts for the non-exponential shape of the 'turbidity vs. time' curves.

Slow stage

The slow stage of turbidity changes is very different in the sonicated and non-sonicated vesicles. In the first case, vesicle fusion leads to an increase in light scattering, whereas in the second, liposome solubilization makes the suspensions optically clear. Only the sonicated vesicles give rise to exponential turbidity changes in the timescales of seconds after detergent addition. Elamrani and Blume [11] found that lysophosphatidylcholine-induced fusion took place with half-times of

the order of minutes; in addition, the curves they observed were not exponential, and were analyzed in a somewhat crude way, estimating V_{max} values in each case. In our case, the Guggenheim plots were virtually linear. In general, these results were very similar to those found for the fast stage, with one important exception. The slow stage of turbidity increase occurs only at detergent concentrations above the critical micellar concentration of Triton X-100, 0.24 mM. This agrees with the proposal by Fendler and Fendler [17] that detergents exert their solubilizing power only above their critical micellar concentration.

A kinetic model has been developed for the slow stage of increase in turbidity, assuming that it is due to the aggregation and fusion of vesicles containing detergent molecules in their bilayers. For a fusion event to occur, it is essential that a vesicle contains at least enough detergent monomers to give rise to a structure that will destabilize the bilayer and then induce fusion. These 'destabilizing structures', as we have called them, could correspond, among others, to phospholipid-detergent mixed micelles embedded in the bilayer. Let j be the minimum number of detergent monomers to give rise to one such structure. We assume that fusion requires vesicle-vesicle contact through bilayer points containing destabilizing structures. Thus there will be a number of possible contacts not leading to fusion.

Let a vesicle have m interaction sites, of which n are occupied by destabilizing structures. The probability of forming a dimer through sites containing those structures will be n^2. Assuming that only these dimers will be able to fuse together, the probability of non-effective interactions will be $m^2 - n^2$. These n sites are given by the total number of detergent monomers h, incorporated into the vesicle bilayer during the fast stage, and by the minimum number of monomers j, required to form a destabilizing structure: $n = h/j$. h will also be the ratio between externally added detergent monomers and initial number of vesicles: $h = [D]/[V]$. Note that all the detergent is considered to be incorporated to the vesicles in the fast stage (this has been confirmed to a large extent by [^{3}H]Triton X-100 binding measurements), thus $n = [D]/j \cdot [V]$. The above assumptions are incorporated into the following kinetic scheme

$$\begin{array}{ccccc} & & \overset{nk_1}{\underset{k_{-1}}{\rightleftarrows}} & & \overset{k}{\rightarrow} \\ P_n & & (P_n)_2 & & Q \\ & (m-n)k_2 \Updownarrow k_{-2} & & & \\ & (P_n)_{2A} & & & \end{array}$$

where P_n represents vesicles each containing n destabilizing structures; $(P_n)_2$ refers to vesicles associated through destabilizing structures, and $(P_n)_{2A}$, to vesicles associated through points other than destabilizing structures. Q corresponds to two fused vesicles.

Taking an equilibrium approach, the observed equilibrium constants may be defined as follows

$$K = \frac{n^2 k_1}{k_{-1}} = \frac{[(P_n)_2]}{[P_n]^2} \tag{9}$$

$$K_A = \frac{(m^2 - n^2)k_2}{k_{-2}} = \frac{[(P_n)_{2A}]}{[P_n]^2} \tag{10}$$

A partition function may be defined for $(P_n)_2$

$$\bar{Y}_{(P_n)_2} = \frac{[(P_n)_2]}{[P_n] + 2\{[(P_n)_2] + [(P_n)_{2A}]\}} \tag{11}$$

Thus,

$$\bar{Y}_{(P_n)_2} = \frac{n^2 K_1 [P_n]}{1 + 2\{n^2 K_1 [P_n] + (m^2 - n^2) K_2 [P_n]\}} \tag{12}$$

where $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$. Introducing now the value of n, the number of interaction sites occupied by destabilizing structures and taking into account that, according to the mechanism proposed for the fast stage, $P_n \equiv V$ (i.e., that all vesicles contain an average number of n destabilizing structures).

$$k_{app} = \frac{k[D]^2 K_1}{j^2[V] + 2[D]^2 K_1 + 2m^2 K_2 j^2 [V]^2 - 2[D]^2 K_2} \tag{13}$$

Eqn. 13 explains sufficiently the variation of k_{app} with phospholipid (or, for that purpose, vesicle) concentration seen in Fig. 5B, where a computer simulation has been drawn, showing a good agreement with the experimental points. We have assumed an average figure of 2450 lipid molecules

per vesicle [18] and the minimum number of monomers required to form a destabilizing structure $j = 64$ [19]. k_{app} will vary sygmoidally as a function of detergent concentration, as seen in Fig. 4B. However, k_{app} may only have a physical meaning when at least j detergent monomers have been incorporated into a vesicle, i.e., when the number of vesicle interaction sites occupied by destabilizing structures $n > 1$. According to Fig. 4B, $n \approx 1$ at a Triton X-100 concentration near its critical micellar concentration.

Solubilization of multilamellar vesicles is not an exponential process and is discussed in more detail in the next paragraph.

Very slow stage

In the case of multilamellar liposomes, Triton X-100 produces solubilization, which is observed as a decrease in turbidity. This process occurs in a timescale of hours (Fig. 8). However, a pseudo-equilibrium appears to have been attained in a matter of seconds (Fig. 6C), in what we have called the slow stage. Our interpretation of the facts is that, as soon as detergent and liposome are mixed, detergent is incorporated to the outer bilayer(s) (fast stage) until the lamellar to micellar transition occurs (slow stage). It is conceivable that the solubilization of the individual bilayers follows exponential kinetics, but the observed changes in turbidity correspond actually to complex phenomena involving probably various bilayers at different stages of solubilization.

Once the outer bilayer(s) are converted into mixed micelles a pseudo equilibrium is observed in the timescale of seconds, and a much slower process is instaured, leading to the complete solubilization of the multilamellar vesicle in a few hours. In this process, the fast and slow stages of solubilization, described above, are repeated for each bilayer. The more lipid is solubilized, the more detergent is in the form of mixed micelles, the smaller the effective Triton X-100 concentration, and the slower the solubilization of the next bilayer. This is reflected in Fig. 8B.

A similar very slow solubilization of multilamellar liposomes was described by Lichtenberg et al. [10] for sodium deoxycholate. In our case, the timescale is nevertheless shorter, since our solubilization half-times are of the order of 1–4 h, i.e., one order of magnitude smaller than those found for sodium deosycholate. In conclusion, although the amplitude and timescale of the changes observed in our case are much smaller than in the case of bile acids, inconsistencies in the length of time between addition of Triton X-100 and experimental measurements may lead to irreproducible results, especially at detergent: lipid molar ratios between 1 and 4.

Acknowledgements

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