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Increase in Size of Sonicated Phospholipid Vesicles in the Presence of Detergents

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Summary. Triton X-100, sodium dodecylsulphate, sodium cholate, and β-octylglucoside increase the size of sonicated, but not of unsonicated, phospholipid vesicles above the T_c gel to liquid-crystalline transition temperature. Lysophosphatidylcholine or glyceryl monooleate do not increase liposome size under these circumstances. The observed phenomenon of vesicle growth is virtually unaffected by phospholipid composition, surface potential, calcium ions, EDTA, or albumin. The presence of cholesterol makes the vesicles more susceptible to detergent-induced growth.

The presence of detergents, even at concentrations much lower than required to observe any increase in vesicle size, favors the liberation of vesicle contents. These results may have implications concerning membrane fusion as well as the reconstitution of membrane proteins in the presence of detergents.

Key words liposomes · detergents · Triton X-100 · lysophosphatidylcholine · membrane fusion · membrane reconstitution

Introduction

Water-soluble amphiphiles (detergents) are commonly in use in membrane research (Helenius & Simons, 1975). However, several aspects of detergent action at sublytic concentrations are still poorly understood. The area of membrane-detergent interaction is interesting, among others, from two important points of view, namely, membrane reconstitution and membrane fusion. A wide range of detergents is used in the reconstitution of intrinsic membrane proteins (Helenius, McCaslin, Fries & Tanford, 1979), and the size of the resulting vesicles is often an important parameter to be considered (Prestegard & Fellmeth, 1974). On the other hand, from the earlier investigations on membrane fusion (Lucy, 1970), it was suggested that amphiphilic substances could be involved in the molecular mechanism of the process. A number of tensioactive chemicals have since been identified as potential fusogenic agents (Ahkong, Fisher, Tampion & Lucy, 1973; Howell & Lucy, 1969), and liposomes have been used as models for the study of cell fusion (Kantor & Prestegard, 1975; Papahadjopoulos, Poste & Schaefer, 1973; Prestegard & Fellmeth, 1974).

We have recently shown that the non-ionic detergent, Triton X-100 effectively promotes an increase in size of sonicated phosphatidylcholine vesicles at temperatures above the T_c transition temperature of the pure lipid (Alonso, Villena & Goñi, 1981). The aim of the present study was to extend our observations on detergent-induced vesicle growth to a wide variety of surfactants, some of which have proven useful for membrane solubilization and reconstitution, and others, such as lysophosphatidylcholine, are used in cell fusion studies.

Materials and Methods

Reagents

Egg-yolk phosphatidylcholine (EYL) was purified by column chromatography. 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC), dicetylphosphate (DCP), and stearylamine were purchased from Sigma (Poole, Dorset). Triton X-100, lot No. 68C 0281, was also from Sigma; sodium dodecylsulphate (SDS), lot No. 1233010, was obtained from BDH; cholic acid, lot No. 59C 0013, from Sigma, and octyl-β-D-glucopyranoside (octylglucoside) was lot No. 810012 from Calbiochem. Cholesterol was from Sigma, analytical reagent grade.

Liposome Preparation and Detergent Treatments

The lipid mixtures were prepared by mixing chloroform solutions of the pure lipids. A solution containing 11 μmol lipid, unless otherwise stated, was evaporated to dryness and the lipid resuspended in 5 ml 0.15 M NaCl, 0.0067 M phosphate buffer, pH 7.4. When required, the liposome suspension was sonicated in an MSE sonicator at 10–12 μm amplitude, for 20 min, at a temperature above the transition temperature of the mixture. Usually, this sonication treatment produced liposome suspensions containing mainly unilamellar vesicles; however, in the case of cholesterol-containing vesicles, longer sonication times, up to 60 min, were required. No

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lipid oxidation products were found after 60 min sonication under the above conditions.

Detergents were dissolved in the buffer mentioned above. Sodium cholate was prepared by neutralizing a cholic acid solution with aqueous NaOH. Detergents were added as required to aliquots of the vesicle suspensions. Final concentrations of detergent were different in each case, but lipids were kept at a final 1-mM concentration except when indicated. In all cases detergent treatments took place at room temperature for 30 min.

Analytical Techniques

Turbidity was measured against pure buffer in a Beckman DB-GT spectrophotometer at 500 nm. Release of vesicle contents (potassium chromate) was determined spectrophotometrically as absorbance at 410 nm minus absorbance at 500 nm, after separating the liberated from the entrapped chromate by filtering the detergent-treated liposome suspension through two Millipore filters, 0.22 and 0.025 μm in diameter, respectively (ref. Nos. GS WP 01300 and VS WP 01300) in series. The validity of this method was checked by Richards and Gardner (1978). In a previous paper (Alonso et al., 1981) we have shown that the method allows a distinction to be made between spontaneous and detergent-induced leakage. Differential scanning calorimetry (DSC) was performed in a Perkin-Elmer DSC-1 apparatus, as described by Chapman, Williams and Ladbrooke (1967).

Electron Microscopy Observations

Ultrastructural studies were carried out on freshly prepared samples as follows. A formvar-coated grid was put on top of a drop of liposome suspension for 3 min. After eliminating the excess liquid, the grid was transferred onto a drop of 3% ammonium molybdate and left for another 3 min. The excess liquid was again eliminated, and the grid observed under a Philips EM-300 electron microscope, at 80 kV.

Results

Turbidimetric and Ultrastructural Studies

It is generally accepted that the turbidity of a liposome suspension decreases upon addition of a detergent. This is indeed the case when nonsonicated phosphatidylcholine liposomes are treated with Triton X-100, SDS, sodium cholate, octylglucoside, or, to a smaller extent, lysolecithin. The situation is different when a sonicated liposome preparation is treated with a detergent. Figure 1*A* depicts the changes in turbidity of such a preparation in the presence of increasing concentrations of SDS: the turbidity of the suspension increases, reaching a maximum at a 0.075% SDS concentration (wt/vol), corresponding to about 2.4 detergent molecules per molecule of phospholipid; higher detergent concentrations lead to a rapid decrease in turbidity. The behavior of nonsonicated liposomes under similar conditions is included in the same figure for comparison. Essentially similar results were obtained with sonicated and nonsonicated liposome preparations in the presence of Triton X-100, sodium cholate, or octylglucoside, although the maximum turbidity was obtained at different detergent/lipid ratios in each case. The corresponding data are summarized in Table 1. No direct relationship exists between the critical micellar concentrations (CMC) of the various detergents and their respective concentrations giving maximum turbidity; however, both parameters appear to be always of the same order of magnitude.

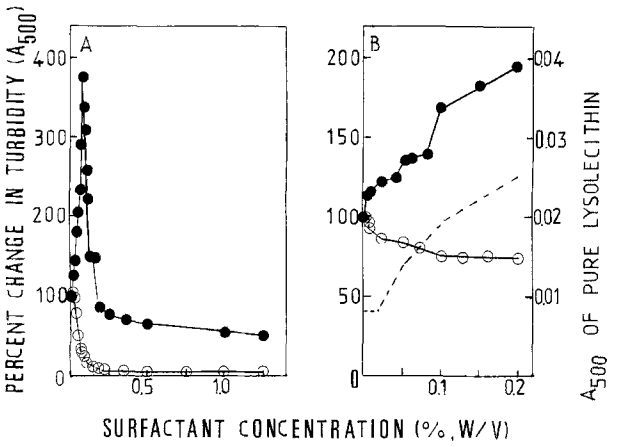

Fig. 1. The effect of surfactants on the turbidity of liposome suspensions. The turbidity (A_{500}) of sonicated (●) and nonsonicated (○) liposome suspensions was measured in the presence of various concentrations of detergents. (*A*): Sodium dodecylsulphate; (*B*): lysolecithin (dotted line: turbidity of pure lysolecithin). Initial absorbances were 0.048 for sonicated and 1.23 for nonsonicated liposomes

Table 1. Detergent concentrations and detergent/phospholipid ratios giving maximum turbidity when sonicated phosphatidylcholine liposomes are treated with various soluble amphiphiles

Detergent	CMC[a] (mM)	Detergent concentration		Detergent/phospholipid molar ratio
		(%, wt/vol)	(mM)	
Triton X-100	0.24	0.069	1.10	1:1
SDS	1.12	0.075	2.60	2.4:1
Sodium cholate	3.0	0.15	3.48	3.2:1
Octylglucoside	25.0	0.55	18.68	17:1

[a] Data taken from Helenius et al. (1979).

The situation is different when sonicated liposome preparations are treated with lysolecithin (Fig. 1*B*): it can be seen that the increase in turbidity has a smaller slope than in the preceding case and that it corresponds simply to the addition of increasing amounts of lysolecithin (dotted line). Similar observations were carried out using the fusogenic lipid glyceryl monooleate (monoolein) (Ahkong et al., 1973).

The detergent-induced decrease in turbidity of the nonsonicated vesicle suspensions is usually interpreted as a decrease in the number (or size) of such vesicles; in the case of the sonicated liposomes, the

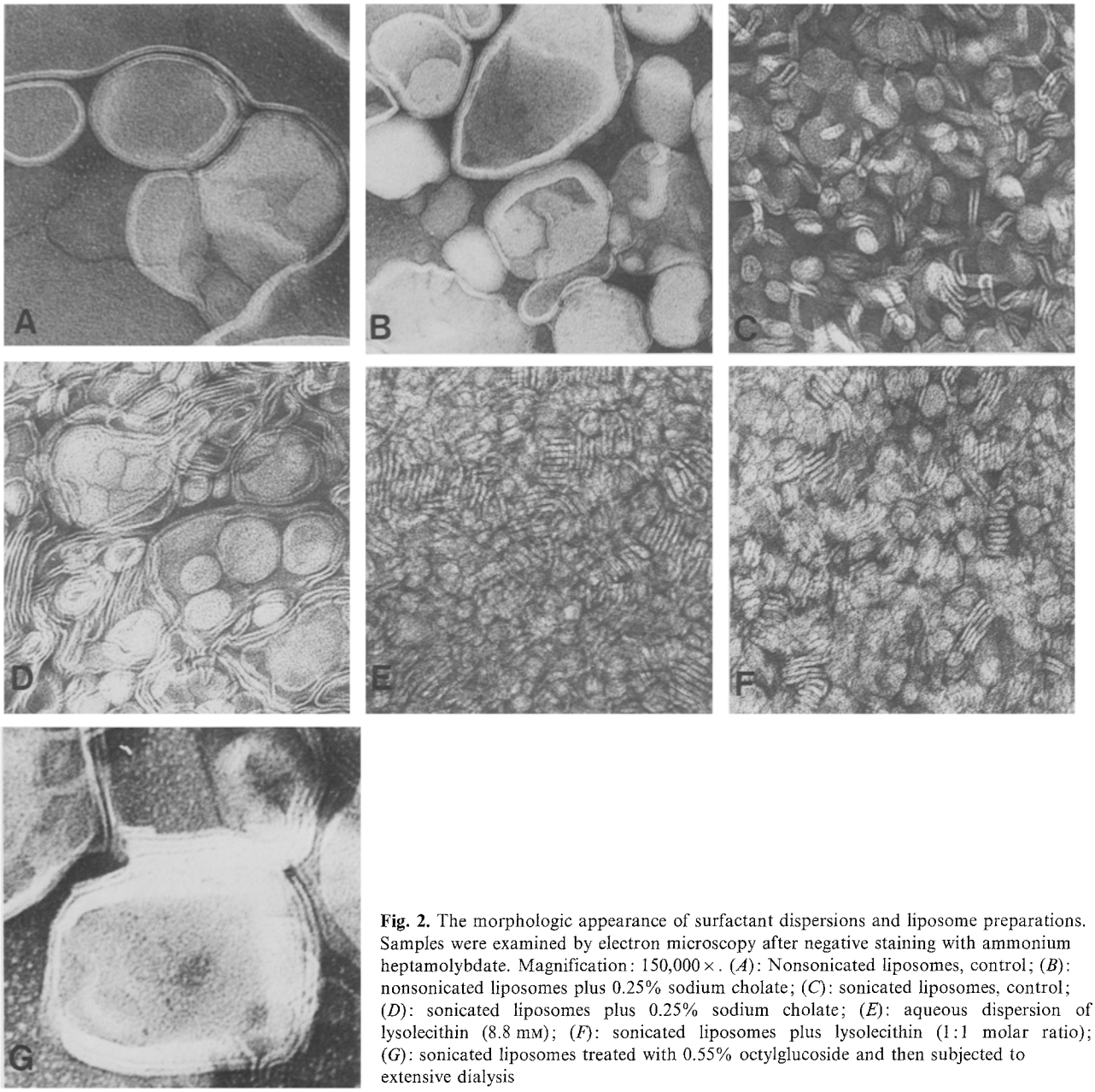

Fig. 2. The morphologic appearance of surfactant dispersions and liposome preparations. Samples were examined by electron microscopy after negative staining with ammonium heptamolybdate. Magnification: 150,000×. (*A*): Nonsonicated liposomes, control; (*B*): nonsonicated liposomes plus 0.25% sodium cholate; (*C*): sonicated liposomes, control; (*D*): sonicated liposomes plus 0.25% sodium cholate; (*E*): aqueous dispersion of lysolecithin (8.8 mM); (*F*): sonicated liposomes plus lysolecithin (1:1 molar ratio); (*G*): sonicated liposomes treated with 0.55% octylglucoside and then subjected to extensive dialysis

increase in turbidity can be due to vesicle aggregation or shrinking, or to an increase in the vesicle size, due to reorganization of the lipid material into larger structures. This hypothesis was tested by electron microscopy observations of the negatively-stained liposome suspensions. Their morphologic appearance is shown in Fig. 2. Untreated, nonsonicated liposomes consist of relatively large, multilayered structures (Fig. 2*A*); their appearance does not change much when detergents are added at concentrations reducing considerably the original turbidity, for instance, 0.25% sodium cholate (wt/vol) (Fig. 2*B*), although it can be said, on a semi-quantitative basis, that their number is decreased. On the other hand, sonicated phospholipid vesicles are much smaller, highly curved, and mostly single-shelled (Fig. 2*C*); the increase in turbidity of these is due to a considerable increase in size that takes place in the presence of certain detergents. Figure 2*D* shows a sonicated liposome preparation treated with 0.15% sodium cholate (wt/vol), i.e., the cholate concentration giving maximum turbidity. It can be seen that the newly-formed vesicles are much bigger, being comparable in size to the nonsonicated ones. Similar vesicles are ob-

tained when sodium cholate is substituted by Triton X-100, SDS, or octylglucoside. No aggregation or shrinking of the small, sonicated vesicles was seen that could be related to the detergent-induced increase in turbidity. Most, if not all, of the larger vesicles obtained after detergent treatment are multilamellar. However, we cannot rule out the existence of large, collapsed unilamellar vesicles in our samples.

Experiments were carried out in order to establish whether or not the large vesicles emerging from the detergent treatment were stable once the detergent had been removed. For that purpose, a sonicated liposome preparation was treated with the amount of octylglucoside giving peak turbidity. This particular detergent was chosen because of its high CMC and subsequent easy removal by dialysis. After 30 min treatment, the vesicles were dialyzed against 500 vols 0.15 M NaCl, 0.067 M phosphate, pH 7.4, for 24 hr, with six buffer changes. A new electron microscopic examination (Fig. 2*G*) revealed that the large vesicles had remained even after an extensive dialysis treatment, that has been shown to remove virtually all the detergent (Baron & Thompson, 1975). This observation also confirms that the large structures are originated by the detergent in suspension, and are not artifacts generated on the grid during drying.

The morphologic appearance of the mixtures of sonicated vesicles and lysolecithin is completely different. The pictures of the surfactant-treated vesicles (Fig. 2*F*) seem to be a mere juxtaposition of the pictures of pure lysolecithin (Fig. 2*E*) and of untreated vesicles (Fig. 2*C*).

Effect of Phospholipid Concentration and Nature. Surface Charge

The above-mentioned turbidity measurements were carried out at a 1 mM phospholipid concentration. A series of experiments was conducted by varying this value from 0.5 to 8 mM and studying the detergent concentration giving the maximum turbidity. Results with four different detergents are shown in Fig. 3. Linear plots are obtained in all cases.

The influence of phospholipid nature on the ability of the various detergents to produce vesicle growth was tested by treating vesicles prepared with different phospholipid mixtures of synthetic and natural origin with detergents. The turbidimetric and ultrastructural results obtained with different phospholipid mixtures are very similar to those obtained with pure phosphatidylcholine.

One of the consequences of varying the vesicle phospholipid composition may be the change in surface potential, which may in turn be important for the growth process. In order to assess the importance

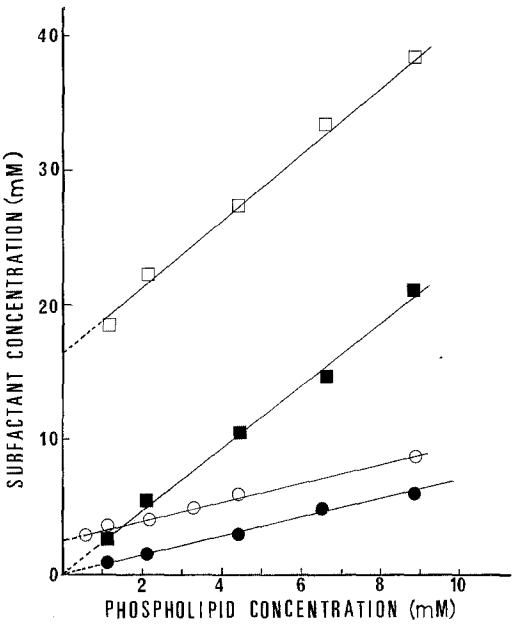

Fig. 3. The detergent concentrations giving maximum turbidity when added to sonicated liposome suspensions of varying phospholipid concentration. (●) triton X-100; (■) SDS; (○) sodium cholate; (□) octylglucoside

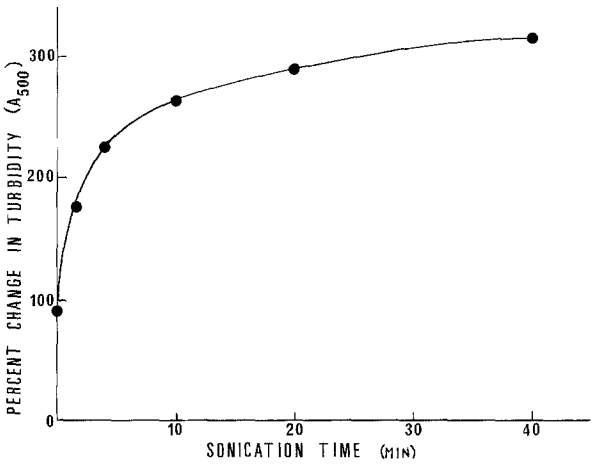

Fig. 4. The percent increase in turbidity of a liposome suspension (1.1 mM phospholipid), sonicated for various lengths of time, upon addition of 0.069% Triton X-100. Initial absorbances went from 1.06 (nonsonicated) to 0.011 (40 min sonication)

of this factor, sonicated and nonsonicated vesicles were prepared with pure EYL and various EYL/DCP and EYL/stearylamine mixtures, up to a 3:1 molar ratio. DCP and stearylamine are assumed, respectively, to charge the vesicle surface negatively and positively. The same detergent effects described in the preceding section were found in all cases.

The length or degree of unsaturation of the hydrocarbon side chain did not influence either the precise detergent/lipid ratio at which turbidity maxima were

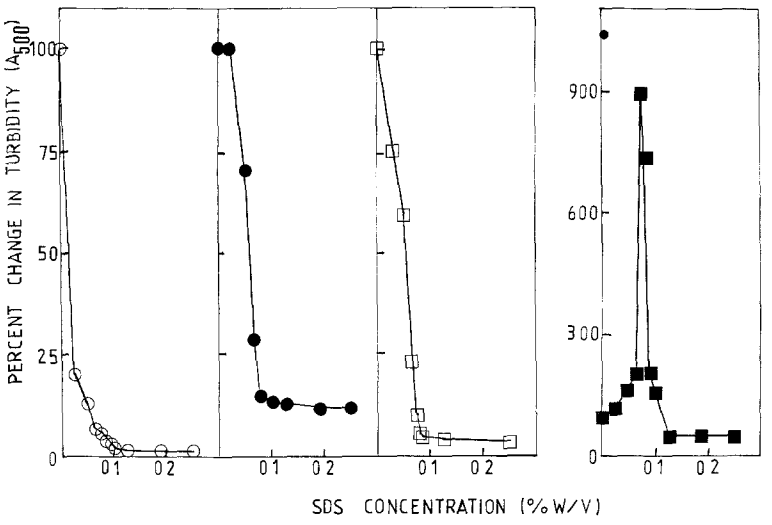

Fig. 5. The changes in turbidity (A_{500}) of sonicated (squares) and nonsonicated (circles) DMPC liposomes above (filled symbols) and below T_c (open symbols) in the presence of various SDS concentrations. Initial absorbances were, from left to right, 0.54, 0.42, 0.19 and 0.11

observed, as was shown by experiments in which sonicated vesicles of EYL, DMPC, DPPC, and mixtures of these with DCP were treated with Triton X-100 or SDS.

Influence of sonication and temperature

It has been shown in Fig. 1 that SDS produces an increase in turbidity of sonicated – but not of unsonicated – lecithin vesicles. The same was true of Triton X-100, sodium cholate, or octylglucoside. The effect of the extent of sonication on the increase in turbidity by Triton X-100 is shown in Fig. 4. All four detergents mentioned above behave in the same way, giving rise to hyperbolic curves that show that sonication of EYL/DCP mixtures for longer than 20 min has little or no effect. Under our conditions, this is the time required for most of the EYL/DCP vesicles to become unilamellar.

The influence on the process under study was checked by turbidimetric and ultrastructural observations of the action of detergents on vesicles made out of pure synthetic phospholipids whose main T_c transition temperatures were easily accessible. DMPC ($T_c = 23$ °C) and DPPC ($T_c = 41.5$ °C) were chosen for this purpose; however, DMPC was found to be more convenient in this respect. Figure 5 shows the changes in turbidity of sonicated and nonsonicated DMPC vesicles at temperatures well above and below T_c of the pure lipid, as a function of SDS concentration. It is clearly visible that only sonicated DMPC vesicles above the T_c transition temperature of the pure lipid increase the turbidity of the suspension when treated with SDS. Again, the same results were obtained with Triton X-100, sodium cholate, or octylglucoside. Electron microscopy observations revealed that the increase in turbidity corresponded indeed to an increase in vesicle size, although these preparations of fully-saturated phospholipids are not visualized as easily as the EYL vesicles by the negative staining method.

Effect of Ca^{++}, EDTA, and Albumin

Several studies on cell and vesicle fusion have pointed out the role of Ca^{++} in these processes (Wilschut, Duzgunes, Fraley & Papahadjopoulos, 1980), especially when negatively charged phospholipids are involved. However, our results are not immediately comparable to the previous work, since those authors were using pure acidic lipids and not mixtures of neutral and acidic ones. When sonicated and nonsonicated vesicles composed of EYL and DCP at a 10:1 molar ratio are treated with Triton X-100 in the presence of 1.8 mM Ca^{++} or 5.0 mM EDTA, the effect of the detergent is completely unaffected by these reagents. Sodium cholate or SDS react strongly with Ca^{++} and consequently have complex effects on the suspension turbidity at the concentrations under study.

Similarly, albumin has been used in media inducing cell fusion (Ahkong et al., 1973). Albumin competes with the lipids for the binding of some detergents, especially SDS, but, when used at a concentration of 6 mg/ml, has no further effect on the detergent-induced apparent fusion of sonicated EYL liposomes.

Effect of Cholesterol

Cholesterol is known to affect the fluidity and disorder of the lipid bilayers (Oldfield & Chapman, 1972). Sonicated and nonsonicated liposomes were prepared with pure phospholipids and molecular mixtures of EYL/cholesterol and DMPC/cholesterol at

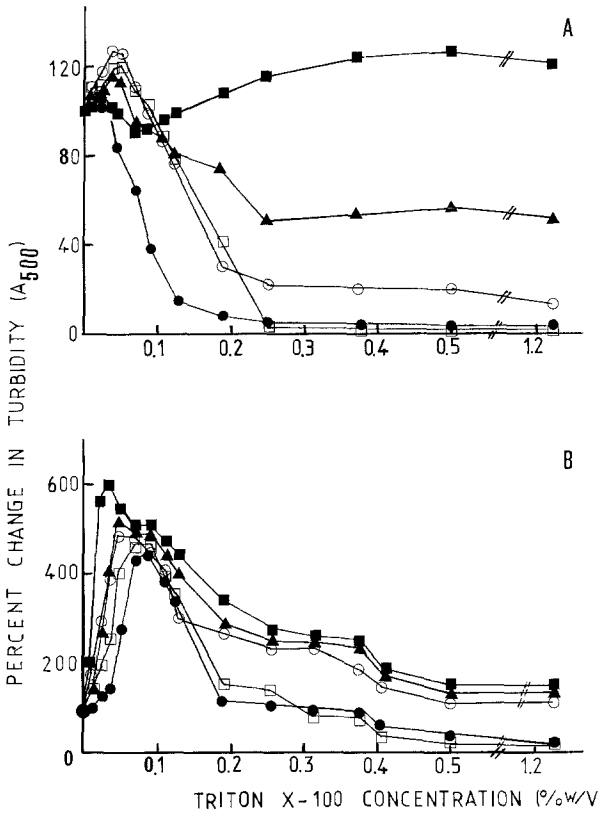

Fig. 6. The changes in turbidity (A_{500}) of liposomes prepared from different mixtures of EYL/cholesterol in the presence of various concentrations of Triton X-100. (*A*): Nonsonicated vesicles; (*B*): sonicated vesicles. Phospholipid/cholesterol molar ratios: (●) pure phospholipid; (□) 10:1; (○) 3:1; (▲) 2:1; (■) 1:1. Initial absorbances were, respectively (*A*): 1.2, 0.97, 0.92 and 1.0; and (*B*): 0.024, 0.034, 0.029, 0.019 and 0.022

molar ratios ranging from 10:1 to 1:1. Figure 6 summarizes our findings on the effects of Triton X-100 on EYL-cholesterol liposomes. It is clear that cholesterol makes more difficult the solubilization of nonsonicated vesicles by detergents (Fig. 6*A*). The same is true of the sonicated liposomes (Fig. 6*B*), but, in addition, it can be said that the presence of cholesterol makes the latter more susceptible to detergent-induced growth since, in the presence of cholesterol, this takes place at lower detergent concentrations and turbidity increases are bigger. Electron microscopy observations failed to reveal any significant difference between cholesterol-containing and cholesterol-free lipid vesicles when treated with detergents.

Liberation of Vesicle Contents

The physiological role of membrane fusion as it happens in cell membranes requires that the vesicle contents are not spilled out during the fusion process. In order to assess the validity of liposomes as models for membrane fusion, it is imperative to determine whether the increase in size is accompanied by the liberation of vesicle contents or not. In our previous study (Alonso et al., 1981) we found that Triton X-100 produced nearly complete vesicle depletion at concentrations much smaller than required for vesicle fusion to occur, so we were not able to say whether the detergent-induced process should be considered as "fusion" or as "lysis and reassembly" of the liposomes. We have extended our observations to other detergents (Table 2), measuring the amount of potassium chromate liberated by the vesicles at the lowest and highest detergent concentrations used in our studies. The lowest concentration is always lower (three to twenty times) than the detergent concentration giving peak turbidity; the highest concentration has been selected in such a way that no further chromate liberation should be reasonably expected by increasing detergent concentrations. Considerable variability is observed in these results, due to intrinsic characteristics of the experimental technique. However, it can be seen that – as previously shown for Triton X-100 – SDS, sodium cholate, and octylglucoside liberate most of the vesicle contents already at the lowest concentrations studied. In general, Triton X-100, SDS, and sodium cholate are less gentle than octylglucoside; the latter promoted vesicle growth at higher concentrations than the others (Table 2). Lysolecithin differs from the other detergents in that it causes the release of sonicated, but not of unsonicated vesicle contents; this is in accord with the slight effect of this detergent on liposome turbidity (Fig. 1*B*).

Table 2. Potassium chromate liberated from sonicated and nonsonicated EYL/DCP (10:1) vesicles by the action of detergents

Detergent	$K_2Cr_2O_4$ liberated (arbitrary units)	
	Sonicated vesicles	Nonsonicated vesicles
0.025% Triton X-100	85±31.2 (6)	197±64.2 (6)
2.5% Triton X-100	137±26.3 (6)	225±54.2 (6)
0.025% SDS	86±24.1 (6)	170±40.0 (5)
2.0% SDS	155±39.5 (6)	200±27.1 (5)
0.025% Sodium cholate	120±24.5 (4)	151±31.5 (5)
2.0% Sodium cholate	125±28.0 (4)	230±37.4 (5)
0.025% Octylglucoside	97±12.4 (4)	84±43.3 (5)
5.0% Octylglucoside	141±17.5 (3)	145±44.1 (5)
0.002% Lysolecithin	156±37.7 (5)	18±28.3 (13)
0.2% Lysolecithin	167±62.5 (5)	−10±26.6 (13)

[a] Liposome suspensions were treated with various amounts of surfactants and left to equilibrate for 30 min. The suspensions were then filtered and the filtrate, containing the extravesicular chromate, was measured spectrophotometrically (*see* Methods).

Figures correspond to mean values ±SD. Number of experiments is indicated in parentheses.

Discussion

The results summarized in the present paper show that some detergents (Triton X-100, SDS, sodium cholate, or octylglucoside) interact with sonicated phospholipid vesicles, inducing an important increase in the size of the same, whereas other amphipathic molecules, such as lysolecithin or glycerylmonooleate, do not produce such effects. These results make a discussion on the nature and mechanism of vesicle growth pertinent, as well as a discussion of its possible biological implications.

Some confusion arises from the fact that vesicle growth and vesicle fusion are often used as synonyms. However, not every increase in size of a lipid vesicle can be accurately termed vesicle fusion. Papahadjopoulos, Hui, Vail and Poste (1976) distinguished between fusion of entire vesicles and diffusion of free lipid molecules from one vesicle to another; the latter mechanism was also described by Martin and McDonald (1976). More recently, Gingell and Ginsberg (1978) have brought about the distinction between "true fusion" and "cracking and reannealing" of phopholipid vesicles, the difference being that in true fusion the vesicle contents are not spilled out in the process, as they are not in physiological instances of membrane fusion. Unfortunately, we cannot distinguish in our system between exchange diffusion, fusion, or lysis and reassembly. The distinction between fusion and exchange diffusion was made by means of differential scanning calorimetry (DSC) observations (Papahadjopoulos et al., 1976); however, our sonicated vesicles give very broad transitions, and these are made still broader by the detergents (A. Alonso, *unpublished data*), so that DSC observations do not provide clear information in our system. Mason, Lane, Miller and Bangham (1980) have published narrow transition endotherms of sonicated liposomes; this is probably due to their mild bath sonication procedure. It is interesting in this respect that the increases in vesicle size in their preparations are more modest than in ours. Furthermore, as detergents increase the permeability of the vesicles at concentrations smaller than required to induce fusion (Table 2), the vesicle contents (potassium chromate) are spilled out independently of whether an increase in size takes place or not; as a consequence, we cannot distinguish either between true fusion and cracking and reannealing of the lipid vesicles.

Despite these problems, our data provide a basis for a hypothetical interpretation that accounts for most if not all the observed phenomena. We consider that a mechanism of exchange diffusion is highly improbable in our case, as in any circumstance where all the vesicles have a similar composition, since it would not give rise to a net transfer of lipids, and that it can only be invoked where two different populations of vesicles (for instance, DMPC and DPPC) are mixed. In our opinion, the essential character that makes the vesicles susceptible to fusion by detergents is the "metastability" (Gershfeld, 1978) originated by the packing faults of the sonicated vesicles above T_c (Lawaczek, Kainasho & Chan, 1976). Only sonicated vesicles above T_c fuse in the presence of detergents (Fig. 5). The packing faults, together with the high curvature of these vesicles, will originate a considerable lateral strain. The adsorption of the hydrocarbon part of a detergent into a bilayer will lead to an increase in its tension (Gruen & Haydon, 1980) to the point that the detergent will act as a "wedge" (Haydon & Taylor, 1963), disrupting the vesicle structure, and thus liberating it from the imposed lateral strain. However, these "open vesicles" are highly unstable, since the paraffin chain-water contact is thermodynamically very unfavorable. Thus, various open vesicles will show a natural tendency to join together, giving rise to larger, strain-free vesicles. We should not forget the tendency of the detergent to bring about a lamellar-micellar phase transition in the lipid bilayer and to solubilize some phospholipids in the form of mixed micelles (Helenius & Simons, 1975). This is indeed its main effect when interacting with unsonicated, strain-free vesicles, or liposomes below T_c (Fig. 5).

In the present state of knowledge, it is difficult to establish the relationship between our observations of increased liposome sizes and the physiologically relevant cases of membrane fusion. In favor of some common mechanism is the fact that soluble amphiphiles have often been involved in cell fusion (Howell & Lucy, 1969; Lucy, 1970) as well as vesicle growth (Hunt, 1980; Kantor & Prestegard, 1975) processes. However, not all cell fusogens were effective in promoting vesicle growth. Lysolecithin and glyceryl monooleate are well-known fusogens in cell membrane systems (Ahkong et al., 1973; Lucy, 1970). Howell et al. (1973) proved that both fusogenic lipids were able to bring about different structural modifications in sonicated lecithin bilayers, according to negative-staining electron microscopy observations, but did not show any increase in size of the lipid vesicles treated with such detergents. We have confirmed these observations. Calorimetric studies also failed to show phosphatidylcholine liposome "fusion" induced by lysolecithin (Papahadjopoulos et al., 1976).

The relevance of our results to the field of membrane solubilization and reconstitution is clearer. First, we have established that a wide range of com-

monly used detergents cause vesicle growth at sublytic concentrations. When reconstitution techniques are in use, detergents are often mixed at those concentrations with sonicated phospholipid vesicles (Gómez-Fernández et al., 1980); in other cases, the initial detergent concentrations are in the lytic range, but are decreased at some stage during a dialysis procedure (Hesketh et al., 1976). The result will be the formation of large, multilamellar vesicles containing the reconstituted protein; this may be satisfactory sometimes, whereas it may prove inconvenient in other instances; our results suggest that this process of vesicle growth during reconstitution could be regulated, i.e., favored by using one of the detergents inducing size increase, maintaining the mixture at a temperature above T_c, sonicating the lipid vesicles carefully before adding the protein, etc.; or, similarly, avoided by taking the opposite measures. In addition, the structure of the recombinant vesicle may be important in relation to the assay of intrinsic enzymes. These enzymes often translocate some metabolite between the inner and outer liposome regions. In the case of a multi-shelled vesicle, as are often formed through the action of detergents, the initial velocity measurements will reflect mainly the behavior of the enzyme molecules in the outer layers; however, the inner space actually available for these molecules is very small, and consequently their activity will rapidly slow down, even in the presence of an excess of the assumedly limiting substrate, due to saturation (or depletion) of the inner space, thus giving rise to serious artifacts in the corresponding kinetic studies.

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