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Surfactant-induced release of liposomal contents. A survey of methods and results

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A systematic approach to the phenomenon of surfactant-dependent release of liposomal contents has been attempted. A variety of methods have been comparatively studied. The influence of the size of the entrapped molecule, nature of the surfactant, composition of bilayers and sonication of liposomes have been considered separately. In order to compare different results, a parameter has been defined, R_{50}, as the phospholipid/surfactant mole ratio producing 50% release of the entrapped solute. This parameter appears to be, to a large extent, independent of time and liposome concentration. Surfactant-induced release of liposomal contents does not occur as a result of breakdown of phospholipid bilayers, but is rather a different phenomenon, occurring at detergent concentrations substantially lower (2–5-times) than solubilization. The required amount of surfactant appears to increase with the size of the entrapped solute. R_{50} depends clearly on the nature of the soluble amphiphile, but there is no obvious relationship with its critical micellar concentration. Liberation of vesicle content also depends on bilayer composition: phospholipids have various effects on the stability of the membrane, while the hydrophobic peptide, gramicidin A, appears to have little influence. Cholesterol is interesting, since at equimolar proportions with phosphatidylcholine, it decreases the stability of bilayer towards Triton X-100, while increasing it in the presence of cholate. Sonication also exerts an influence on the surfactant-dependent release of vesicle contents; it appears to decrease the bilayer stability, so that lower detergent concentrations are required to liberate the entrapped solutes. Finally, it should be noted that, although the decrease in self-quenching of 6-carboxyfluorescein is a convenient method for the study of solute liberation, glucose release, as detected by enzymatic methods, may be more reliable for accurate measurements.

Introduction

Liposomes, i.e., phospholipid vesicles, have been found to be of extensive value as biological membrane models [1]. More recently, they have also been considered as potential tools for the controlled release of drugs, DNA transfer or as red blood cell substitutes [2]. For such applications, liposomes are important mainly because of their contents. In these cases, liposomes are often required to act as tight containers of a given mole-

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; PE, phosphatidylethanolamine; R_{50}, phospholipid/surfactant mole ratio producing 50% release of trapped solute from liposomes; S_{50}, phospholipid/surfactant mole ratio producing 50% solubilization of liposomal phospholipids; SDS, sodium dodecyl sulphate.

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cule (generally in aqueous solution), and to release their content under pre-stated conditions. Liposome tightness is impaired by their interaction with amphiphilic molecules, particularly when the vesicles are delivered into physiological fluids. Bearing in mind this new range of liposome applications, it is important to consider the manner in which liposomal content is released in the presence of foreign molecules that may damage the phospholipid bilayer. In the present study, we examined the liberation of vesicle content by a variety of surfactants, since these reagents effectively perturb the bilayer architecture [3,4]. Although of synthetic origin, they may be considered as models for the many natural amphiphiles to be found in cellular and extracellular fluids. In addition, surfactants offer the advantage that their interaction with model and cell membranes is already well characterized [3,4].

Our study includes a survey of the various methods that have been proposed for the study of vesicle content liberation. Moreover, the method of liposome preparation, the nature of the surfactant and of the entrapped molecule and the phospholipid composition of the bilayers have been considered separately. These results are interpreted in the light of our previous work on liposome–surfactant interaction [5–8].

Materials and Methods

Egg-yolk phosphatidylcholine was purified according to Singleton et al. [9]. Dimyristoylphosphatidylethanolamine and all other lipids were purchased from Sigma. Triton X-100, sodium dodecyl sulphate (SDS) and sodium cholate were also from Sigma; β-octylglucoside was obtained from Calbiochem; egg-yolk lysophosphatidylcholine was from Lipid Products; CHAPS was from Boehringer and lauryldimethylamine oxide was from Fluka. Gramicidin A was provided by Koch-Light; dextran ($M_r = 40\,000$) was provided by Merck and 6-carboxyfluorescein was provided by Eastman-Kodak. Milli Q (Millipore) water was used in these experiments.

Liposomes were prepared as follows. The required amounts of lipid (or lipid and polypeptide) in organic solution were mixed and dried in a round bottom flask, evaporated under vacuum and left overnight under reduced pressure in order to remove solvent traces. Multilamellar vesicles were formed by resuspending the dry lipid in an aqueous solution of the molecule to be entrapped; unless otherwise stated, liposome formation took place at 37°C and the aqueous solution was buffered with 10 mM Tris-HCl (pH 7.4). Small unilamellar vesicles were obtained by sonicating a multilamellar vesicle suspension in an MSE sonicator for 30 min, at 10-s intervals, with a 10–12 μm amplitude. The sonicated suspension was then centrifuged at $13\,800 \times g$ for 10 min in order to remove probe debris. All liposomes were allowed to equilibrate for at least 1 h at room temperature.

Various methods were used in order to remove the non-entrapped solute from the liposomal suspension: centrifugation under various conditions, dialysis, gel filtration, etc. For multilamellar vesicles, the method which was by far the most convenient and efficient was to wash them three times by centrifugation in an Eppendorf centrifuge at $13\,800 \times g$ for 3 min, followed by resuspension in an isotonic buffer free from the entrapped solute. In the case of sonicated liposomes, the suspension was passed through a $20 \times$ 1-cm Sephadex column G-50 and eluted with an isotonic buffer free from the entrapped solute. The final phospholipid concentration was 2 mM in all cases.

Aliquots from the liposomal suspensions were treated with equal volumes of the appropriate surfactant solutions in order to obtain the desired lipid/surfactant ratios. Equilibration was allowed to occur for 30 min at room temperature. It is known that in these systems complete equilibrium may take several hours [16,17]; however, after such lengthy periods of time, detergent-induced release of liposomal contents would be masked by the concomitant spontaneous diffusion of solutes outside the vesicles. Also, a substantial part of the detergent effect has taken place after 30 min, and this should allow the comparison of different systems, all of them subjected to the surfactant action for the same time. Nevertheless, in strict terms, all our results should be considered as expressing the percent liposomal contents released in 30 min. After detergent treatment, solute release (as percent liberation) could be measured in some cases on the surfactant-treated liposome suspension,

without any further procedure. When this was not the case, the external aqueous phase had to be separated from the vesicular phase (in these cases always consisting of multilamellar vesicles). Separation was carried out either by centrifugation at 150 000 × *g* for 1 h at 4° C, or by filtration through Millipore GSWP 02500 filters, 0.22 μm pore diameter.

Liberation of 6-carboxylfluorescein was assayed according to Weinstein et al. [10]; chloride was assayed through the formation of a mercury complex [27] using Bio-Mérieux kits, phosphorus was measured according to Bartlett [11], dextran was measured by the anthrone reaction [12] and sodium ions were measured by atomic absorption spectrophotometry, using a 2380 Perkin Elmer instrument. Glucose release was determined as follows: aliquots of the surfactant-treated suspensions (10 μl) were assayed for glucose using the glucose oxidase plus peroxidase method, with phenol and amino-4-antipyrine as the colour reagent [28]. Liposomally entrapped glucose could not be used as substrate by glucose oxidase under our conditions. Release was expressed as a percentage, 100% being the amount of sugar liberated in the presence of excess (10^{-2} M) Triton X-100. Phospholipid solubilization by detergents was measured as described previously [7,8]: surfactant-treated liposome suspensions were filtered under reduced pressure through Millipore GSWP 02500 filters, 0.22 μm pore diameter, and lipid P was assayed in the filtrates according to Bartlett [11]. It was checked that the filtrates contained no vesicular material. Results were expressed as percent solubilization with respect to the original amount of lipid P in the sample.

Results and Discussion

Methodological aspects

The general procedure to assess the effects of surfactants on the release of liposomal contents consists of treating a suspension of phospholipid vesicles, loaded with the appropriate solute, with increasing amounts of detergents and measuring the proportions of liberated solute. A simple example is shown in Fig. 1: the liberation of 6-carboxyfluorescein by Triton X-100, a commonly used non-ionic detergent [3,4]. The fluorescent dye

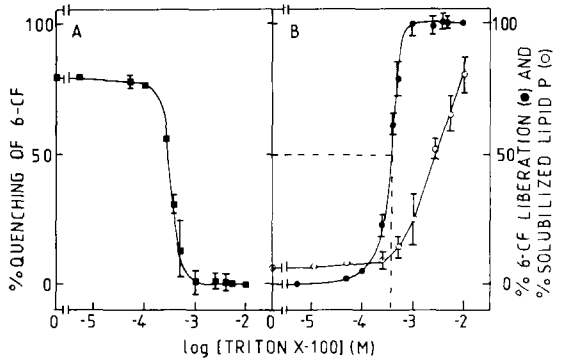

Fig. 1. The use of 6-carboxyfluorescein (6-CF) to detect release of liposomal contents. (A) The percent self-quenching of 6-carboxyfluorescein [10], originally entrapped in multilamellar vesicles, after treatment with the surfactant Triton X-100, as a function of surfactant concentration. (B) (●) percent release of 6-carboxyfluorescein from liposomes as a function of Triton X-100 concentration (data derived from the self-quenching measurements in A). The dotted lines indicate the procedure to obtain the R_{50} parameter. (○) Percent solubilization of liposomal phospholipids, as a function of detergent concentration.

is released and its dilution leads to a decrease in self-quenching of its fluorescent emission [10] (Fig. 1A); the self-quenching data can be converted into percent liberated dye (Fig. 1B), for which a surfactant concentration producing maximum dye release (100%) must be determined previously. 6-Carboxyfluorescein is useful because no separation of liposomes from the external aqueous phase is required. The general pattern observed in our studies is very similar to the results by Schubert et al. [13,14] who studied the cholate-induced release of liposomal contents. In order to compare results obtained under different conditions, a simple parameter is required, independent from the nature or concentration of reagents. Throughout this work, we have used R_{50}, defined as the lipid/surfactant mole ratio producing 50% release of liposomal contents (Fig. 1B). A similar parameter has been used in our previous work on phospholipid solubilization by detergents [7,15]. Note that, in all cases, our lipid/surfactant mole ratios refer to total amount of surfactant, i.e., membrane-bound plus free in solution [4]. For the experiment depicted in Fig. 1, $R_{50} = 2.9$; under the same conditions, 50% lipid solubilization takes place at an approx. 0.6 lipid/detergent mole ratio [7,8]. Thus, solute liberation occurs at surfactant

concentrations much below those producing phospholipid solubilization, as can also be seen in Fig. 1B. This is a general phenomenon, not limited to Triton X-100 or egg phosphatidylcholine bilayers, as well be discussed below.

When multilamellar vesicles are treated with surfactant solutions, complete equilibration takes place only after a long period, in the order of hours, because of the many concentric phospholipid bilayers that participate in this event [16,17]. At least in the case of Triton X-100, our previous studies have shown that most of the detergent effects have already taken place 30 min after surfactant addition [17]. Moreover, the parameter R_{50} appears to be virtually time insensitive (Table I, Nos. 1–3). Consequently, all our measurements were carried out 30 min after adding the soluble amphiphile, as stated in Materials and Methods.

With most entrapped solutes, release can only be measured after the detergent-treated vesicles have been separated from the external aqueous phase. This is usually achieved either by ultracentrifugation or by filtration. In order to test the validity of the various procedures, we have studied the liberation of glucose by the glucose oxidase method, a procedure that may be carried out either with or without liposome separation. The results using ultracentrifugation, filtration or no separation procedure are shown in Table I, (Nos. 4–6). No difference can be seen in either case, indicating that there is no a priori method of choice, but rather the decision can be made on the basis of experimental convenience in each case.

Another point of methodological interest, especially in view of comparing results from different laboratories, is whether the surfactant effects depend qualitatively or not on phospholipid concentration. We have studied the release of 6-carboxyfluorescein from multilamellar liposomes in the presence of Triton X-100, at phospholipid concentrations ranging between $5 \cdot 10^{-4}$ and $5 \cdot 10^{-3}$ M. The same qualitative effects are found in all cases; Fig. 2 demonstrates the linear relationship between egg-yolk phosphatidylcholine concentration and total surfactant concentration producing 50% release. This linear relationship confirms that the R_{50} value of 2.9 given above for 1 mM phospholipid is valid within a large range of phospholipid concentrations. This suggests strongly that R_{50} or similar parameters can be applied to compare data from different sources.

TABLE I

THE RELEASE OF LIPOSOMAL CONTENT BY THE NON-IONIC DETERGENT TRITON X-100

R_{50} phospholipid/surfactant mole ratio producing 50% solute release (see Fig. 1). R_{50} values are obtained from curves constructed on experimental points representing average values of three independent measurements. Liposomes were derived from egg-yolk phosphatidylcholine. The final phospholipid concentration was 1 mM in all cases. See text for experimental details.

No.	Solute	R_{50}	Observations
1	6-carboxy-fluorescein	2.8	30 s after surfactant addition
2	6-carboxy-fluorescein	2.9	30 min after surfactant addition
3	6-carboxy-fluorescein	2.9	11 h after surfactant addition
4	glucose	1.7	ultracentrifugation
5	glucose	1.8	filtration
6	glucose	1.8	no separation
7	dextran	1.2	
8	P_i	3.8	
9	Cl^-	2.4	
10	Na^+	3.7	
11	6-carboxy-fluorescein	2.6	sonicated vesicles
12	glucose	3.2	sonicated vesicles

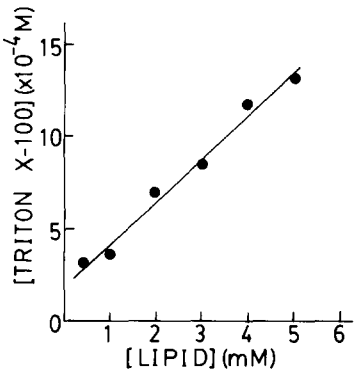

Fig. 2. The influence of liposomal phospholipid concentration on the surfactant-dependent release of vesicle contents. The Triton X-100 concentrations releasing 50% of liposomal content are plotted as a function of vesicle phospholipid concentration.

It is conceivable that mixed detergent/phospholipid micelles formed from the outer bilayer of a liposome might release detergent again to the subsequently exposed bilayer, causing vesiculation of the remaining phospholipid, thus explaining the delay in the solubilization process as compared to the solute release process. Our ^{31}P-NMR data [29] suggest that this is the case, at least at low detergent concentrations (phospholipid/detergent ≈ 7). However, the phenomenon is not quantitatively important and, in fact, we do not detect any release of liposomal contents at these low surfactant concentrations.

Release of different solutes

It is apparent from the above results, concerning the release of glucose or 6-carboxyfluorescein (Table I, Nos. 1–6), that the amount of detergent required to produce 50% liberation is different in each case, being more for glucose. The issue of a 'selective permeability' being induced by surfactants, across lipid bilayers has been addressed by others in previous studies. Schlieper and De Robertis [18], using black lipid films, established a scale of Triton X-100-induced permeability for cations, decreasing in the order $Rb^+ > K^+ > Na^+ > Li^+$, thus suggesting that cations with smaller hydrated ionic radii could move more readily across the bilayer. This is in agreement with the studies of Schubert et al. [13,14] who found a direct relationship between the size of the entrapped molecules and the amount of cholate required to produce vesicle leakage. We have confirmed the latter observations by comparing the release of glucose and its polymer dextran: about 50% more Triton X-100 is required to liberate the larger molecule (Table I, Nos. 6 and 7). In general, the fact that the required amount of detergent increases with the size of the solute to be released is an indication that leakage is different in nature from bilayer solubilization, in agreement with the observation that membrane solubilization requires higher surfactant concentration than release of vesicle contents (Fig. 1B). On the other hand, our preliminary studies on the release of various inorganic ions (Table I, Nos. 8–10) indicate the more complex nature of this phenomenon, and the need for further experimentation.

Studies with different surfactants

The results described above have been obtained with Triton X-100, as a representative example of a detergent widely used in biochemistry. The next step consists of extending those results to a variety of surfactants. For that purpose, we have studied the release of 6-carboxyfluorescein and phospholipid solubilization from egg-yolk phosphatidylcholine liposomes in the presence of a series of well-known detergents. The corresponding results are summarized in Table II; two main conclusions can be drawn. First, the amount of detergent required to produce solute leakage varies widely with the nature of the surfactant molecule. Second, the amount of detergent required to produce lipid solubilization is always 2–5-times higher than that required to release the liposomal content. Our results confirm and extend previous studies from this and other laboratories. R_{50} values of around 0.32 (i.e., the same as ours for glucose) may be estimated for raffinose liberation in the presence of cholate from the results published by Schubert et al. [13,14]. O'Connor et al. [20], studying the effects of cholate on sonicated liposomes, as well as Bangham and Lea [21], using Triton X-100 and

TABLE II

THE RELEASE OF 6-CARBOXYFLUORESCEIN FROM EGG-YOLK PHOSPHATIDYLCHOLINE LIPOSOMES BY DIFFERENT SURFACTANTS

R_{50}-phospholipid/surfactant mole ratio producing 50% 6-carboxyfluorescein release (see Fig. 1). S_{50} = phospholipid/surfactant mole ratio producing 50% phospholipid solubilization. R_{50} and S_{50} values are obtained from curves constructed on experimental points representing average values of three independent measurements. Final phospholipid concentration was 1 mM in all cases. See text for experimental details.

Surfactant	Critical (mM) micellar concentration [a]	R_{50}	S_{50}
Triton X-100	0.24	2.9	0.6
SDS	1.33	0.83	0.4
Sodium cholate	3.0	0.32	0.14
Octylglucoside	25	0.09	0.05
CHAPS	4.1	0.86	0.45
Lauryldimethylamine oxide	2.4	1.3	0.29
Lysophosphatidylcholine	0.02–0.2	< 0.1	< 0.1

[a] Values for surfactant in 0.1 M NaCl [19].

SDS on black lipid films, have observed significant increases in bilayer permeability below the critical micellar concentration of the surfactants, thereby proposing that detergent monomers are being incorporated into the bilayer. This fully agrees with our results shown in Fig. 2 and Table II, as well as with our previous studies on the interaction of Triton X-100 with phosphatidylcholine bilayers [7]. Gallo et al. [22] have also found that lysophosphatidylcholine increases bilayer permeability; the present data indicate, however, that the synthetic surfactants are much more effective for this purpose. The peculiarities of the interaction of lysophosphatidylcholine as compared to other detergents, with phospholipid bilayers, have been explored elsewhere [23].

The influence of bilayer composition

The leakage from liposomes containing molecules other than phosphatidylcholine in their bilayers was also examined and the results are shown in Table III. The presence of cholesterol seems to affect bilayer permeability and solubilization at high concentrations, i.e., at near equimolar phospholipid/sterol ratios. At the 1 : 1 ratio, solubilization is made more difficult, in accordance with our previous studies [8], but release of liposomal contents is easier, i.e., requires less detergent. This is interesting since it is known that bilayers containing 1 : 1 phosphatidylcholine/cholesterol are less permeable than those of pure phosphatidylcholine [24]; our data show that, in addition, they are more unstable with respect to Triton X-100. The issue of the influence of cholesterol on surfactant-induced permeability of bila[illegible]rs has been addressed by other authors. Inoue and Kitagawa [25], studying changes in permeability of liposomes in the presence of Triton X-100, found that phosphatidylcholine/cholesterol bilayers at 1.5 : 1 mole ratio behaved very much like those of pure phospholipid, while those at a 1 : 1 ratio were slightly less sensitive to the detergent. Schubert et al. [13] have described an increased stability of liposomes containing cholesterol (2 : 1 ratio) towards cholate-induced leakage. The same effect is found by O'Connor et al. [20] using sonicated vesicles. In order to clarify this point, multilamellar liposomes composed of phosphatidylcholine/cholesterol (1 : 1 mole ratio) were treated with Triton X-100 or sodium cholate at various concentrations; 6-carboxyfluorescein liberation and phospholipid solubilization were studied. The re-

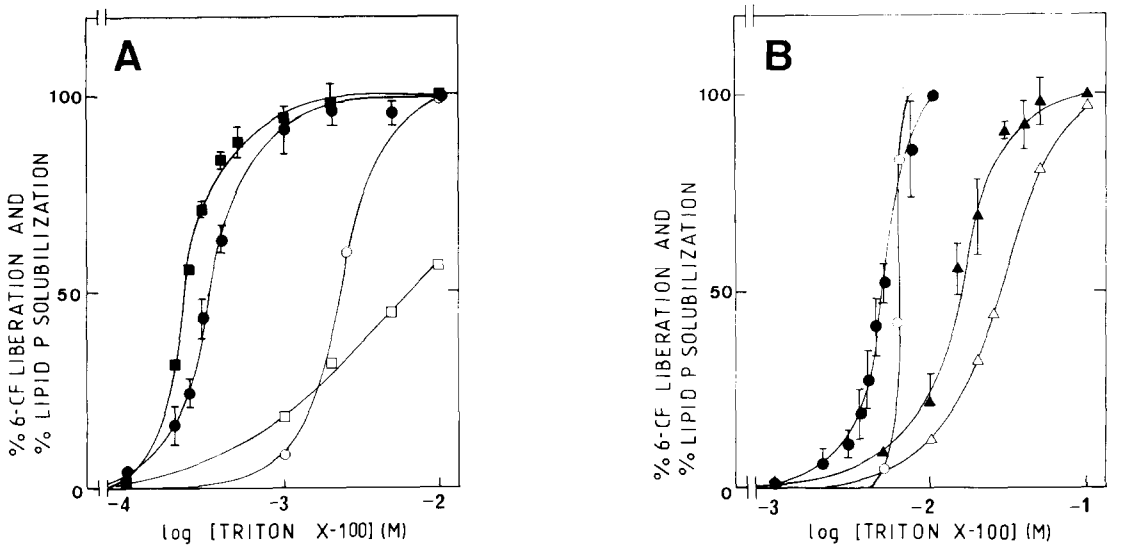

Fig. 3. The influence of cholesterol on the surfactant-dependent release of 6-carboxyfluorescein (6-CF) and solubilization of phospholipid from multilamellar liposomes. (A) Experiments in the presence of Triton X-100. Circles, pure egg-yolk phosphatidylcholine; squares, equimolar mixture phosphatidylcholine/cholesterol. Filled symbols, 6-carboxyfluorescein release; open symbols/solubilization. (B) Experiments in the presence of sodium cholate. Circles, pure phosphatidylcholine; triangles, equimolar mixture phosphatidylcholine/cholesterol. Filled symbols 6-carboxyfluorescein release; open symbols, solubilization.

sults are depicted in Fig. 3: cholesterol makes the liposomes more resistant to detergent solubilization and to cholate-induced leakage. However, they are made more prone to liberate their contents in the presence of Triton X-100. The reason for this discrepancy with the results of Inoue and Kitagawa [25] remains to be established.

The presence of gramicidin A, a hydrophobic polypeptide that has been used as a model for intrinsic membrane proteins [30], alters but slightly the sensitivity of egg-yolk phosphatidylcholine bilayers towards Triton X-100, rendering them in need of larger amounts of surfactant for leakage or solubilization (Table III). This is attributed to the competition between phospholipids and gramicidin A for the binding of detergent [3]. Finally, the effect of mixing PE, or its methylated derivatives, to egg-yolk phosphatidylcholine (at 75 mol% phosphatidylcholine) was found to be relatively small (Table III), their destabilizing capacities increasing in the order PE < *N*-methylPE < *N*,*N*-dimethylPE. This is an indication of the necessity of exploring the properties of bilayers formed by phospholipid mixtures in order to understand the behaviour of cell membranes.

TABLE III

THE RELEASE OF 6-CARBOXYFLUORESCEIN FROM LIPOSOMES OF VARYING COMPOSITIONS BY TRITON X-100

R_{50} = phospholipid/surfactant mole ratio producing 50% 6-carboxylfluorescein release (see Fig. 1). S_{50}-phospholipid/surfactant mole ratio producing 50% phospholipid solubilization. R_{50} and S_{50} values are obtained from curves constructed on experimental points representing average values of 2–3 independent measurements. Final egg-yolk phosphatidylcholine concentration was 1 mM in all cases. See text for experimental details.

Bilayer composition (mole ratios)	R_{50}	S_{50}
Pure egg-yolk PC	2.9	0.6
PC/ cholesterol (3 : 1)	2.8	0.5
PC/ cholesterol (1 : 1)	4.0	0.18
PC/ gramicidin A (50 : 1)	3.2	0.56
PC/ gramicidin A (25 : 1)	2.8	0.29
PC/ gramicidin A (10 : 1)	2.4	0.28
PC/ PE (3 : 1)	2.5	0.50
PC/ *N*-methylPE	3.2	0.67
PC/ *N*,*N*-dimethylPE	4.0	0.80

The stability of sonicated liposomes

Sonicated unilamellar vesicles have been described as metastable structures [26]. It is, thus, worthwhile exploring their stability towards soluble amphiphiles in comparison with the multilamellar vesicles used in the above experiments. The release of 6-carboxyfluorescein and glucose from sonicated liposomes has been measured in the presence of Triton X-100 and the results are given in Table I, Nos. 11 and 12 and in Fig. 4. Liberation of glucose from sonicated vesicles requires substantially less detergent than in the case of multilamellar liposomes, in agreement with Inoue and Kitagawa [25]. The opposite is found with 6-carboxyfluorescein. It should be noted that, also with multilamellar liposomes (Table I, Nos. 1–6), considerably different R_{50} values are consistently found for glucose and 6-carboxyfluorescein, although both molecules are similar in size. Various reasons may be suggested to explain this difference: (a) release of 6-carboxyfluorescein is measured only indirectly, through a decrease in self quenching; (b) 6-carboxyfluorescein is an ionic compound, that may interact with the phospholipid polar headgroups; (c) 6-carboxyfluorescein is relatively non-polar, and may also interact with the bilayer hydrophobic matrix. All three reasons point to the fact that, despite its convenience, the 6-carboxyfluorescein method may be less reliable than that of glucose release.

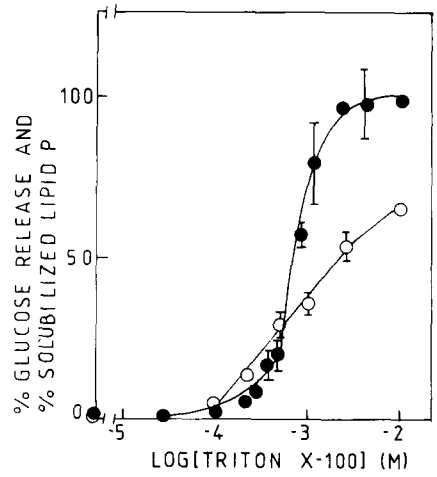

Fig. 4. The release of glucose (filled symbols) and phospholipid solubilization (open symbols) from sonicated unilamellar vesicles of egg-yolk phosphatidylcholine, as a function of Triton X-100 concentration.

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