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The influence of membrane composition on the solubilizing effects of Triton X-100

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Multilamellar liposomes containing pure phosphatidylcholine (PC) or mixtures of PC with cholesterol, cholesteryl palmitate, β-carotene, cardiolipin, phosphatidylethanolamine or gramicidin A have been treated with the detergent Triton X-100. Solubilization has been monitored as a decrease in turbidity of the liposome suspension, and also by determination of bilayer components in the solubilized fraction. The same solubilization pattern is found for unsaturated (egg yolk) or saturated (dimyristoyl) PC. Similar results are also found when dimyristoyl PC is solubilized above or below its gel-to-fluid transition temperature. Cholesterol solubilizes in parallel with PC; gramicidin A is solubilized preferentially to this phospholipid and the non-polar lipids cholesteryl palmitate or β-carotene remain insoluble at detergent concentrations producing complete PC solubilization. Addition of cardiolipin or phosphatidylethanolamine does not seem to alter the general pattern of PC solubilization. Phosphatidylethanolamine is less soluble than PC, while cardiolipin solubilizes at the same detergent concentrations than PC. These results are considered in relation to previous studies with natural membranes.

Introduction

Many studies have been devoted to the understanding of the principles governing membrane solubilization by detergents (see Refs. 1 and 2 for a review). However, several aspects of the solubilization of phospholipid bilayers have not been systematically studied to date. One of these poorly understood areas is the effect of bilayer chemical composition on the solubilization process [2], and the joint problem of selective solubilization of membrane components by detergents.

A good number of pertinent observations has been published. In some cases, there is a selective solubilization of integral proteins, which are extracted at detergent concentrations not producing phospholipid solubilization [3–8]. Membrane lipids can also be selectively extracted, selectivity being dependent on the nature of both membrane and detergent [8–14]. Cholesterol, sphingomyelin and glycolipids are left behind in the residue when erythrocyte membranes are treated with Triton X-100 [9,10]. The non-ionic detergents seem to solubilize phosphatidylcholine (PC) better than sphingomyelin [13], and Triton X-100 extracts choline-containing phospholipids better than amino phospholipids from the Semliki Forest virus membrane [11]. It is also of interest that cholesterol esters and triacylglycerols are in general poorly solubilized [15–17]. Finally, the parallel selective solubilization of a given phospholipid and a pro-

Abbreviations: PC, phosphatidylcholine, PE, phosphatidylethanolamine

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tein has been observed occasionally, e.g. cardiolipin and ATPase from inner mitochondrial membranes treated with Triton X-100 [14].

One obvious approach to clarify this problem is the use of membranes with carefully controlled chemical compositions. The present study deals precisely with this kind of structure, namely phosphatidylcholine liposomes, containing other polar lipids (cholesterol, other phospholipids) or nonpolar lipids (cholesteryl palmitate, α-carotene) or else the intrinsic polypeptide gramicidin A. The detergent chosen was Triton X-100, partly because of its wide use [1,2], partly because of our previous experience with it [14,18–21].

Materials and Methods

Egg-yolk PC was purified according to Singleton et al. [22]. Unless otherwise stated, this phospholipid was used throughout this work when PC was required. Dimyristoyl PC (synthetic) was obtained from Sigma and used without further purification. Cardiolipin from bovine heart and phosphatidylethanolamine (PE), type V, from *Escherichia coli,* cholesterol, cholesteryl palmitate and β-carotene were obtained from Sigma. Gramicidin A was from Koch-Light. Triton X-100 (Rohm and Haas) was also purchased from Sigma. Organic solvents were freshly redistilled before use. All other reagents were analytical grade.

Multilamellar liposomes were prepared as follows. The required amount of lipids (or lipid and polypeptide) in organic solution were mixed and dried in a round bottom flask, and evacuated for at least 2 h. Liposome formation took place at 37 °C, in a 50 mM Tris-HCl buffer (pH 8.0). Liposome structure was checked for the different lipid mixtures by negative-staining electron microscopy, as described previously [19] Multilamellar vesicles were observed in all cases (data not shown); no differences in liposome structure were seen that could be attributed to variations in bilayer composition. Aliquots from the liposome suspension were treated with equal volumes of the appropriate Triton X-100 solution in the same buffer, in order to obtain the various PC: detergent molar ratios. Final PC concentration was 1 mM in all cases After detergent addition, equilibration was allowed to occur for 30 min. Although we have shown [20] that complete equilibrium is not reached in these systems before several hours, a substantial part of the detergent effect has taken place after 30 min, especially for purposes of comparison between different bilayer compositions, all of them subjected to the surfactant action for the same time.

Turbidity measurements (as absorbance at 500 nm) were carried out in a double-beam UV-5260 Beckman spectrophotometer, against a blank of pure buffer. Solubilization was also monitored by filtration as follows: surfactant-treated liposome suspensions were transferred to the wells of a Millipore filtration device. Negative pressure (approx. 20 kPa) was applied to the system and the suspensions were passed through GSWP 02500 Millipore filters, 0.25 μm pore diameter. The filtrates were collected and analyzed for lipid phosphorus. No phospholipid was found in filtrates from untreated liposome suspensions. Lipid phosphorus was determined as described by Bartlett [23]. Cholesterol was quantitated by a method based on the cholesterol oxidase reaction [24] and cholesteryl palmitate was determined colorimetrically by the Liebermann-Burchard reaction. α-Carotene was measured by its absorbance at 440 nm, against a standard curve. Gramicidin A was determined by the dimethylaminobenzaldehyde method, as described elsewhere [25]. Cardiolipin was determined, in the presence of PC, as lipid phosphorus, after thin-layer chromatographic separation of both lipids. PE in the presence of PC was determined by amino group titration [26].

Results

Solubilization of pure PC

Solubilization of pure egg PC liposomes, according to both filtration and turbidity methods, occurs as shown in Fig. 1A. 50% solubilization takes place at approx. 0.6 lipid : detergent mole ratio. The influence of the physical state of the bilayer on its solubilization by detergent is not adequately established. In order to clarify this point, the solubilization of dimyristoyl PC (T_c = 23 °C) was studied at 4 °C, in the gel state, and at 37 °C, in the fluid phase. The reagents and the filtration device were equilibrated for various hours

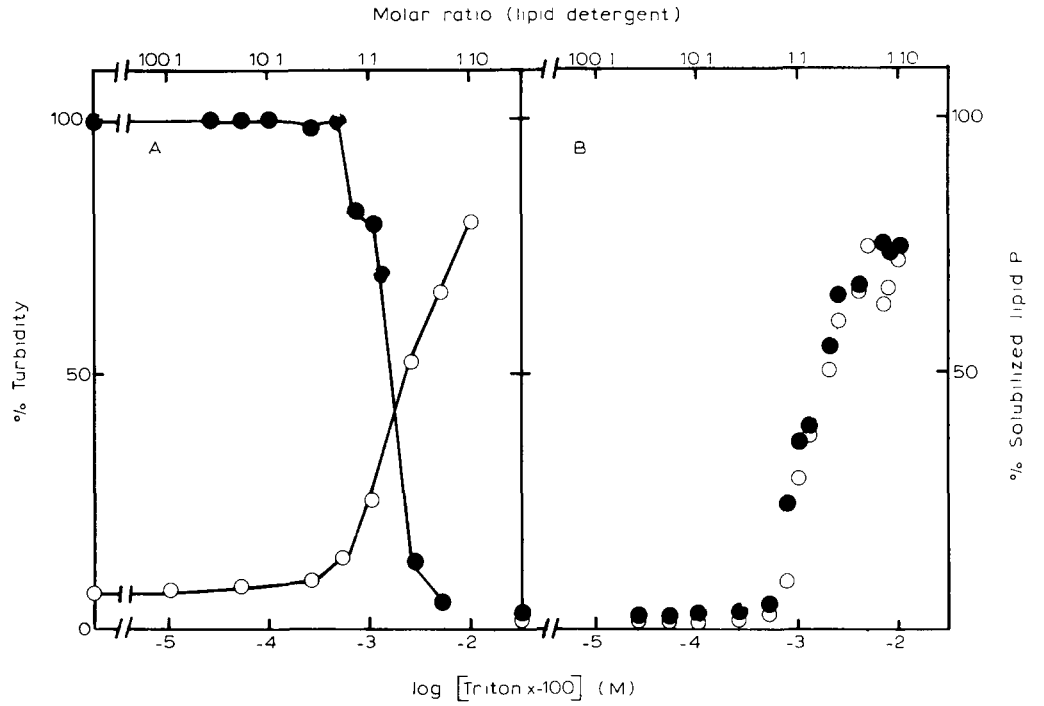

Fig 1 Solubilization of multilamellar phosphatidylcholine liposomes by Triton X-100 (A) Egg-yolk PC ●, Percent variation in turbidity, ○, percent solubilized lipid P (B) Dimyristoyl PC ●, Percent solubilized lipid P at 37 °C (in the fluid state), ○, percent solubilized lipid P at 4 °C (in the gel state). Average values of three independent experiments

in rooms acclimated to the appropriate temperature, and the whole solubilization and filtration process took place in the same acclimated rooms. The results in Fig. 1 indicate no difference between the solubilization patterns of liposomes in the gel and fluid states when treated with Triton X-100. No difference between dimyristoyl and egg PC is found either.

Cholesterol

Sterols are the main non fatty-acid containing lipids in membranes; cholesterol is a representative example of this group of molecules. Various egg-yolk PC : cholesterol mixtures, between 10 : 1 and 1 : 1 mole ratios, have been examined for their behaviour in the presence of Triton X-100. Our results are summarized in Fig. 2. Cholesterol and PC show almost identical patterns of solubilization at all PC : cholesterol molar ratios. Therefore, there is no preferential solubilization of any of these lipids in the system under study. There is a tendency towards a decreased solubilizing power when the cholesterol proportion in the bilayer is increased, but the effect is felt both on the phospholipid and the sterol.

The pattern of decrease in turbidity and PC solubilization in the 10 : 1 mixture (Fig. 2A) is virtually identical to that of pure PC (Fig. 1A). A similar change in turbidity is observed in the 3 : 1 PC : cholesterol system (Fig. 2B), but at a 1 : 1 ratio, turbidity decreases but slightly (Fig. 2C) This is partly due to the incomplete solubilization of this mixture, and may also be explained assuming that micelles containing high amounts of cholesterol display different scattering properties than those composed of PC and surfactant exclusively.

Gramicidin A

The basic matrix upon which biomembranes are built consists of a double layer of phospholipids; the so-called integral proteins are embedded in this bilayer. Gramicidin A is a hydrophobic pentadecapeptide that has been used as a model for integral proteins [25]. Solubilization of egg PC bilayers containing various proportions of grami-

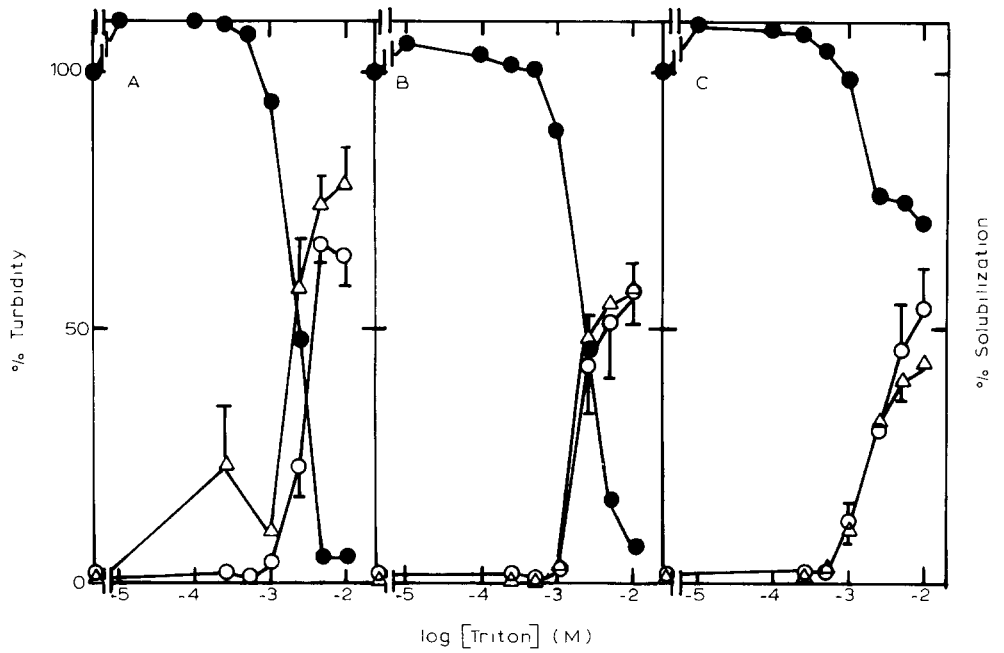

Fig 2 Solubilization of multilamellar liposomes, containing various proportions of egg-yolk phosphatidylcholine and cholesterol, by Triton X-100 PC cholesterol molar ratios, (A) 10 1, (B) 3 1, (C) 1 1 ●, Percent variation in turbidity, ○, percent solubilized lipid P, △, percent solubilized non-PC component Average values ± S E ($n = 3$)

cidin A is shown in Fig. 3. Changes in turbidity are not very different from those seen in pure PC, only at a very high polypeptide concentrations (PC · gramicidin A 5 : 1, corresponding to a 32% polypeptide by weight) the suspensions do not become optically clear even at the highest detergent concentrations tested. This is due to the low level of PC solubilization in these systems (Fig. 3C). A marked increase in turbidity is seen in the 5 · 1 mixture at 10^{-3} M Triton X-100. This is attributed to detergent-induced vesicle fusion [19]: we have shown (in sonicated vesicles) that the intensity of this fenomenon is dependent on the proportion of gramicidin A in the bilayer [27].

Contrary to what was seen with cholesterol, gramicidin A is solubilized better than PC in all cases: 100% of the polypeptide comes into solution at or near $5 \cdot 10^{-3}$ M detergent, while 70% phospholipid is solubilized at most under these conditions. Phospholipid solubilization is similar at 25 : 1 and 10 : 1 PC : gramicidin A ratios, but decreases rapidly at higher polypeptide proportions. The 7.5 : 1 mixture is already similar to the 5 · 1 system from the point of view of low PC solubilization (data not shown). This may be due to a suspected change from lamellar to hexagonal phase at these high gramicidin A concentrations [28].

Non-polar lipids

The solubilization effects of Triton X-100 on PC bilayers containing non-polar lipids were studied on mixtures of that phospholipid with either cholesteryl palmitate or β-carotene. They are commonly found representatives, respectively, of the cholesteryl esters and the carotenoids, two important lipid groups, frequently found in membranes. Both cholesteryl palmitate (Fig. 4) and β-carotene (Fig. 5) remain totally insoluble at the highest Triton X-100 concentrations tested, while PC solubilization does not seem to be affected by their presence. The insoluble lipids that remain in suspension produce a turbid appearance even at high detergent concentrations. For cholesteryl palmitate, the turbidity at 10^{-2} M Triton X-100 increases with increasing proportions of the non-polar lipid (Fig. 4A and B). At a 5 · 1 PC. cholesteryl palmitate ratio (data not shown) the turbidity of the suspension containing 10^{-2} M surfactant is 28% of the control, an intermediate

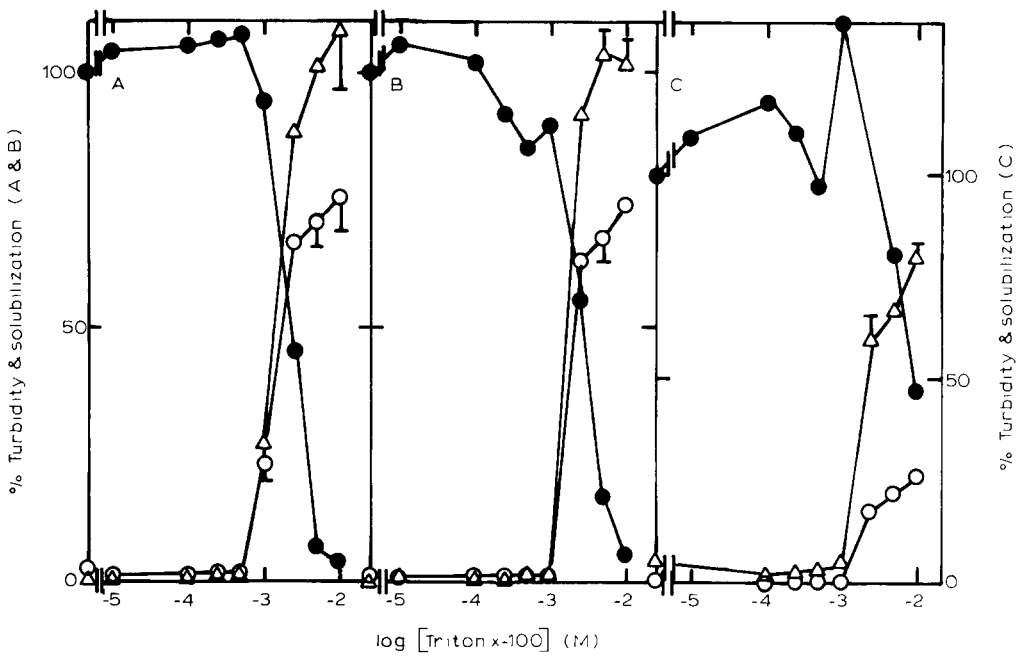

Fig 3 Solubilization of multilamellar liposomes, containing various proportions of PC and gramidicin A, by Triton X-100 PC gramicidin A molar ratios, (A) 25 · 1, (B) 10 . 1, (C) 5 1 Symbols as in Fig 2 Average values ± S E ($n = 3$)

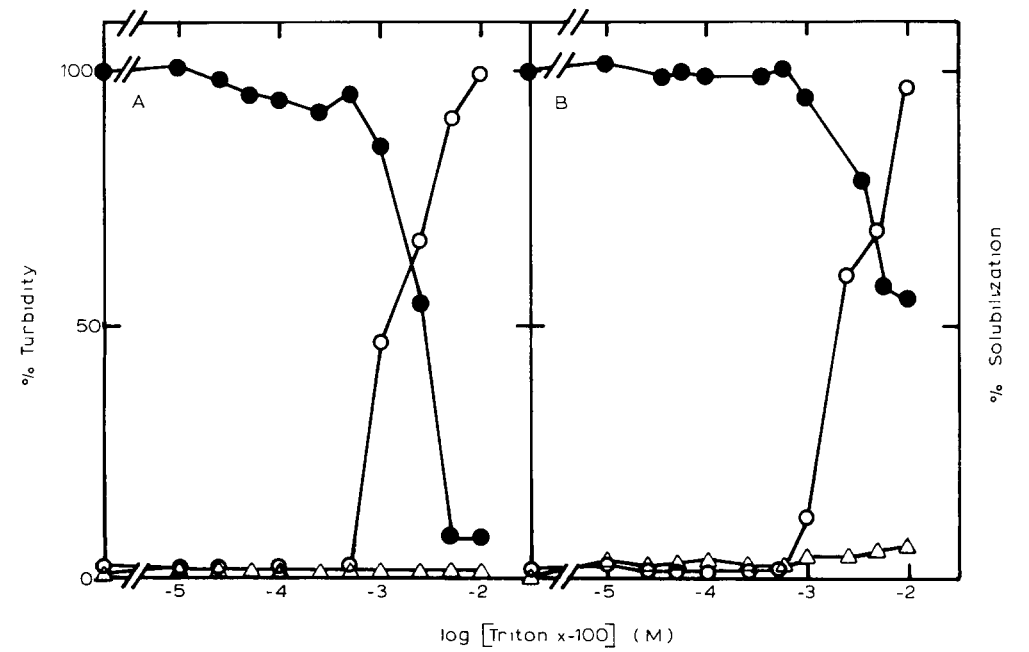

Fig. 4 Solubilization of multilamellar liposomes, containing various proportions of PC and cholesteryl palmitate, by Triton X-100 PC cholesteryl palmitate molar ratios, (A) 25 1, (B) 2 1. Symbols as in Fig. 2 Average values ± S.E. ($n = 3$).

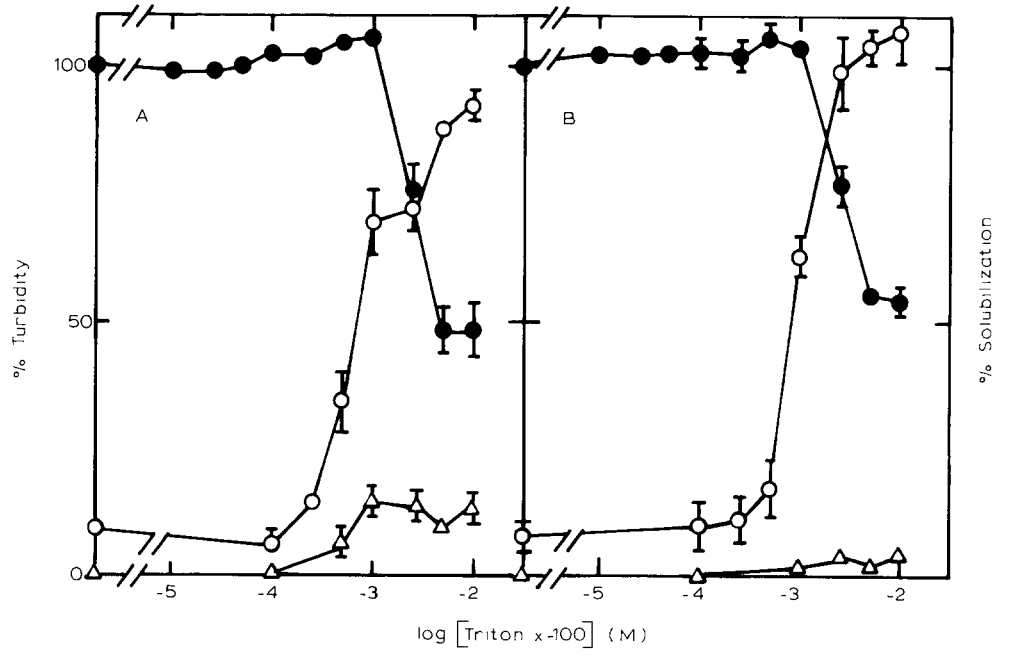

Fig 5 Solubilization of multilamellar liposomes, containing various proportions of PC and β-carotene, by Triton X-100 PC β-carotene molar ratios, (A) 25 1, 5 1 Symbols as in Fig 2 Average values ± S E ($n = 3$)

value between those in Fig. 4. This gradual effect on turbidity is not seen with β-carotene (Fig. 5).

Other phospholipids

As a first step towards the understanding of the solubilization of natural membranes, mixtures of PC with other phospholipids were prepared Cardiolipin and PE were chosen, respectively, as representative of the negatively-charged and the amino phospholipids.

Various mixtures containing PC and cardiolipin in proportions from 10 : 1 to 1 . 1 molar ratios were studied. They all behaved in a very similar way in the presence of surfactant. The case of the 1 . 1 mixture is shown as a representative example (Fig. 6). Both phospholipids follow identical solubilization patterns. Neither the decrease in turbidity nor the solubilization of total lipid P are significantly different from those seen with PC alone (Fig. 1). We may conclude that the presence of cardiolipin does not affect the bulk solubilization of PC bilayers.

The case of PE is different, as seen from the turbidity measurements (Fig. 7). Although the behaviour of the 10 : 1 PC : PE mixtures does not differ from pure PC (Fig. 7A), increasing proportions of PE make the suspension less clear at high detergent concentrations (Fig. 7B and C). When the solubilized lipid P is taken into consideration (Fig. 8), the presence of PE alters the solubilization pattern for total phospholipid In particular, PE appears to be less readily solubilized than PC under all circumstances. With PE, as was the case with cholesteryl palmitate, very high turbidities are observed even under conditions of substantial solubilization of lipid P (Figs. 7, 8). Our results suggest that, in this case, the high turbidity is mostly due to the presence of non-solubilized PE in suspension.

Discussion

The results in this paper demonstrate, using simple experimental models, that not all membrane components are equally solubilized by Triton X-100. We shall discus first the selective solubilization of non-phospholipid components, and then the effect of phospholipid mixtures. It should be noted that, according to extensive previous experience [31,32], all membrane components

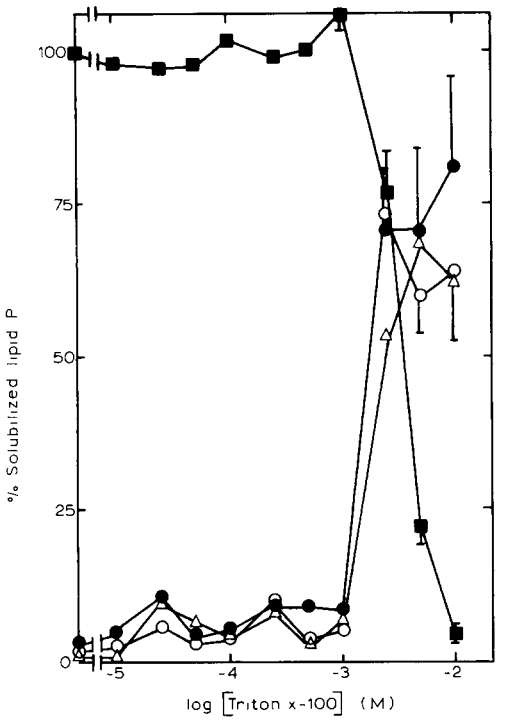

Fig 6. Solubilization of multilamellar liposomes, containing equimolar proportions of PC and cardiolipin, by Triton X-100. ■, Percent variation in turbidity, ●, percent solubilized lipid P, ○, percent solubilized PC, △, percent solubilized cardiolipin. Average values ± S E ($n = 3$)

tested give rise to, or are incorporated into, lipid bilayers under our experimental conditions. As a preliminary step, we have examined the influence

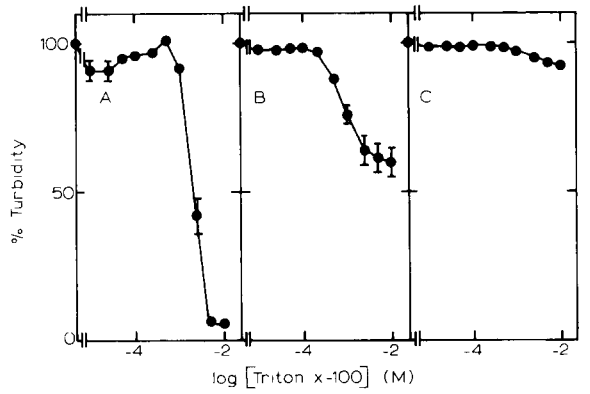

Fig 7 Percent changes in turbidity produced by Triton X-100 in suspensions of multilamellar liposomes containing various proportions of PC and PE. PC: PE molar ratios, (A) 10 1, (B) 3 1, (C) 1 1. Average values ± S E ($n = 3$)

of fatty acid composition and physical state of the lipids on the solubilization process, and found no effect whatsoever. There have been suggestions that the temperature at which phospholipid is solubilized may be critical with saturated phospholipids [2]. Fu and Laughlin [33] found differences in the interaction of zwitterionic surfactants with saturated PC bilayers in the fluid and gel states. However, in our case, after taking extensive precautions to ensure a good thermal equilibration of the system, the solubilization pattern of dimyristoyl PC liposomes by Triton X-100 is similar above and below T_c (Fig. 1).

Among the membrane components other than phospholipids, we have seen examples of substances that are solubilized with more, equal or less difficulty than PC. In fact, PC is used in these studies as an internal standard, so that many results may be conveniently given in relation to this standard. Non-polar lipids seem to be almost totally unable to form mixed micelles with Triton X-100 under the conditions of our study (Figs. 4, 5). This does not imply that they will not be solubilized at higher detergent: lipid ratios. The reason for the preferential solubilization of PC over cholesteryl palmitate or β-carotene may be in the amphiphilic nature of the PC molecule, that may favour mixed micelle formation. Another important factor may be length of the nonpolar lipid molecules, about twice that of PC; this may make difficult the incorporation of such substances into the mixed micelles. Other authors have already described that phospholipids and free cholesterol are more easily solubilized from plasma low density lipoprotein by deoxycholate [15], sodium dodecyl sulphate [15], 1-anilino-8-naphthalene-sulphonate [16] and decyl sulphate [17] than are the cholesterol esters and triacylglycerols.

In the opposite extreme, we find gramicidin A, which is more easily solubilized than PC (Fig. 3). Similar examples have been found in native membranes; in the solubilization of retinal rod outer segments disks by octyl glucoside, membrane-bound rhodopsin is solubilized prior to phospholipids [3], and other examples are also known [4–8]. A simple explanation for this phenomenon may be that intrinsic proteins are able to induce cooperative binding of detergent; this has been shown for some proteins, such as cytochrome b_5

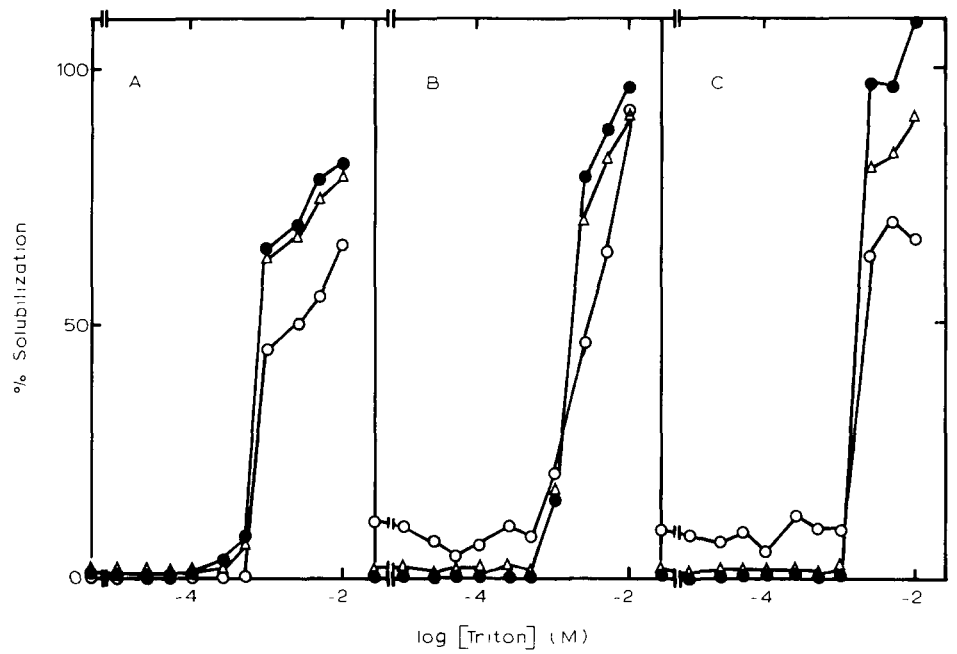

Fig 8 Solubilization of multilamellar liposomes, containing various proportions of PC and PE, by Triton X-100 PC PE molar ratios, (A) 10 1, (B) 3 1, (C) 1 1 △, Percent solubilized lipid P, ●, percent solubilized PC, ○, percent solubilized PE Average values ± S E. (n = 3)

[29] and Ca^{2+}-ATPase [30]. Independent measurements of detergent binding to gramicidin A are required to test this hypothesis, that would also explain the low PC solubilization in the high gramicidin A containing mixtures; the polypeptide would, if that were the case, sequester most of the available surfactant, thus competing favourably with PC for the detergent.

Our results for the PC : cholesterol mixtures (Fig. 2) show no preferential solubilization of either component. Cholesterol is an amphiphile, of about the same length as PC, and these two factors would assimilate the sterol to the phospholipid for solubilization purposes. These results do not support previous observations with Triton X-100 [9] or other detergents [8,10], according to which cholesterol is negatively discriminated in favour of phospholipids. However, it is not always clear from these studies whether free or total (i.e. free plus esterified) cholesterol has been measured in the non-solubilized residue. In addition, results obtained from natural systems should be interpreted with caution, in view of the complexities of the starting material. Moreover, studies involving other natural systems do not detect any discrimination between PC and cholesterol [15–17].

The effect of other phospholipids on the solubilization of PC bilayers seems to be highly dependent on the nature of the second phospholipid. Cardiolipin appears to have no effect (Fig. 6), while PE exerts a marked influence (Figs. 7, 8). Turbidity measurements may not be reliable in this case, because of unequal contributions to the suspension turbidity from the various phospholipid classes. Upon examination of the solubilization behaviour of individual phospholipids, it appears that PE is solubilized less easier than PC, and that PC and cardiolipin follow closely similar solubilization patterns (Figs. 6, 8). The molecular basis for these differences remains to be established.

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