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Electron Spin Resonance Studies on the Interaction between Liposomal Membrane and Triton X-100

Kazunori Anzai,[1a] Hideo Utsumi,[1b] Keizo Inoue, Shoshichi Nojima,[1] and Takao Kwan[1b]

Faculty of Pharmaceutical Sciences, The University of Tokyo[1]

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The interaction between Triton X-100 and phosphatidylcholine liposomes was studied by the spin labeling technique and turbidity measurement. The order parameter (S) of spin labeled stearic acid and the turbidity both decreased upon addition of Triton X-100. The effect of Triton X-100 depended on the species of phosphatidylcholine. In egg phosphatidylcholine liposomes, the effect of Triton X-100 was biphasic. In the range of low Triton/phospholipid molar ratios (below 0.4) there was a gradual decrease in the S value without any appreciable decrease in the turbidity. This suggests that Triton X-100 fluidizes the membrane without forming small fragments. Above a molar ratio of 0.4 there was a drastic decline in the turbidity, indicating that the membrane was solubilized. In dimyristoylphosphatidylcholine liposomes, biphasic behavior was not observed. The S value and the turbidity decreased noticeably upon addition of a little Triton X-100. The sensitivity of dimyristoylphosphatidylcholine liposomes to Triton X-100 was much greater than that of egg phosphatidylcholine liposomes. Phase separation between phosphatidylcholine-rich regions and Triton-rich regions may occur in view of the S values reached upon the addition of Triton X-100 to both phosphatidylcholine liposomes. This was also indicated by the spectral change of spin labeled phosphatidylcholine liposomes caused by Triton X-100.

Keywords——ESR; spin label; liposome; Triton X-100; solubilization

Introduction

Triton X-100, a non-ionic detergent, is a polydisperse preparation of *p,t*-octylphenoxypolyethoxyethanols containing an average of about 9.5 oxyethylene units per molecule. The average molecular weight is about 628 and the critical micellar concentration (CMC) is 0.2—0.3 mM. It is widely used in the field of biochemistry for the solubilization of membrane components.[2] However, the mechanism of the solubilization is not yet clearly understood. It is therefore important to investigate the effect of Triton X-100 on lipids, one of the main components of biological membranes. The lipids are considered to play important roles in maintaining the membrane structure and retaining cellular materials such as soluble proteins, nucleotides, and ions.

Using NMR spectroscopy, Ribeiro and Dennis[3] investigated the propetries of mixed micelles of Triton X-100 and several phosphatidylcholines. They postulated that at molar ratios above about 2: 1 Triton/phosphatidylcholine, all of the phosphatidylcholine formed mixed micelles, while at molar ratios below 1: 1, a bilayer structure was present.

1) Location: *Hongo 7-3-1, Bunkyo-ku, Tokyo, Japan*; *a*) Present address: *Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabedori 3-1, Mizuho-ku, Nagoya, 467 Japan*; *b*) Present address: *Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-cho, Tsukui-gun, Kanagawa, 199-01, Japan.*

2) A.H. Maddy and M.J. Dunn, "Biochemical Analysis of Membranes," John Wiley and Sons, Inc., New York, 1976, pp. 177—196.

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Inoue and Kitagawa[4] reported the effect of Triton X-100 on the permeability properties of liposomes. They showed that the sensitivity of the liposomes to Triton X-100 was affected by the lipid composition.

In the present work, the mechanism of the interaction between Triton X-100 and phospholipid membrane was studied using spin probes.

Experimental

Materials——Egg yolk phosphatidylcholine was extracted from egg yolk as described previously.[5] Dimyristoylphosphatidylcholine was synthesized according to Robles and van den Berg.[6] Phospholipids used in this study each showed a single spot on thin-layer chromatograms. Spin labeled stearic acids, 12-[N-oxyl-4',4'-dimethyloxazolidine]-stearic acid (12SLS) and 5-[N-oxyl-4',4'-dimethyloxazolidine]-stearic acid (5SLS), were synthesized by the methods of Waggoner *et al.*[8] and Jost *et al.*,[7] respectively. Spin labeled phosphatidylcholine (SL-PC) was synthesized according to Hubbell and McConnell.[9] Triton X-100, cholesterol, and other reagents were of commercial origin and were used without further purification.

Preparation of Liposomes——Multilamellar liposomes were prepared as described in a previous paper.[10] They were prepared in 0.15 M NaCl solution. The molar ratio of 12SLS to phospholipid was 1: 100. SL-PC unilamellar liposomes were prepared by sonication with a B-12 Sonifier at 0° under a nitrogen atmosphere.

ESR Measurement——Aliquots of about 40 μl of the preparations were transferred into a glass capillary tube (1.45—1.65 mm diameter, 75 mm length) and ESR spectra were obtained with a JEOL PE-1X machine (X-band, 100 kHz field modulation) at room temperature. The order parameter, designated as S, was estimated from the spectra. The quantity S is an index of the relative motional character or disorder of the membrane and is defined by

$$S = (A_{//} - A_{\perp})/(A_{zz} - (A_{xx} + A_{yy})/2)$$

where $A_{//}$ and $A_{\perp}$ are the parallel and perpendicular components of the hyperfine splitting (see Fig. 1). A_{zz} and $(A_{xx}+A_{yy})/2$ are motional averages of the nitroxide hyperfine tensors in the directions parallel and perpendicular to its long molecular axis, and are assumed to be 30.8 and 5.8 gauss, respectively.[9]

Determination of Turbidity of Liposomes——A liposome suspension containing 12 μmol of phosphatidylcholine was mixed with Triton X-100. The final volume of the reaction mixtures was always 4 ml. The turbidity was measured as optical density at 600 nm after 30 min. Under our experimental conditions the value did not change significantly during 10 hours.

Results and Discussion

Effect of Triton X-100 on the Order Parameter of 12SLS in Liposomes

A solution of Triton X-100 was added to the liposome suspesion and, after incubation for 30 min, ESR spectra were obtained. Three kinds of liposomes were assayed; egg phosphatidylcholine, egg phosphatidylcholine+cholesterol (33 mol %), and dimyristoylphosphatidylcholine liposomes. Typical ESR spectra are shown in Fig. 1, and the order parameter (S value) obtained from the ESR spectra are plotted against Trition X-100 concentration in Fig. 2. The S value observed in egg phosphatidylcholine liposomes was about 0.38, and did not change observably on treatment with Triton X-100 (data not shown).

The S value of liposomes composed of egg phosphatidylcholine+33 mole % of cholesterol was decreased by the addition of Triton X-100 and reached a plateau at 0.35 molar ratio of Triton/phosphatidylcholine. In the case of dimyristoylphosphatidylcholine liposomes, the S value was also decreased by the addition of Triton X-100. These results suggest that Triton X-100 could "fluidize" the liposomal membrane. It is noteworthy that this apparent

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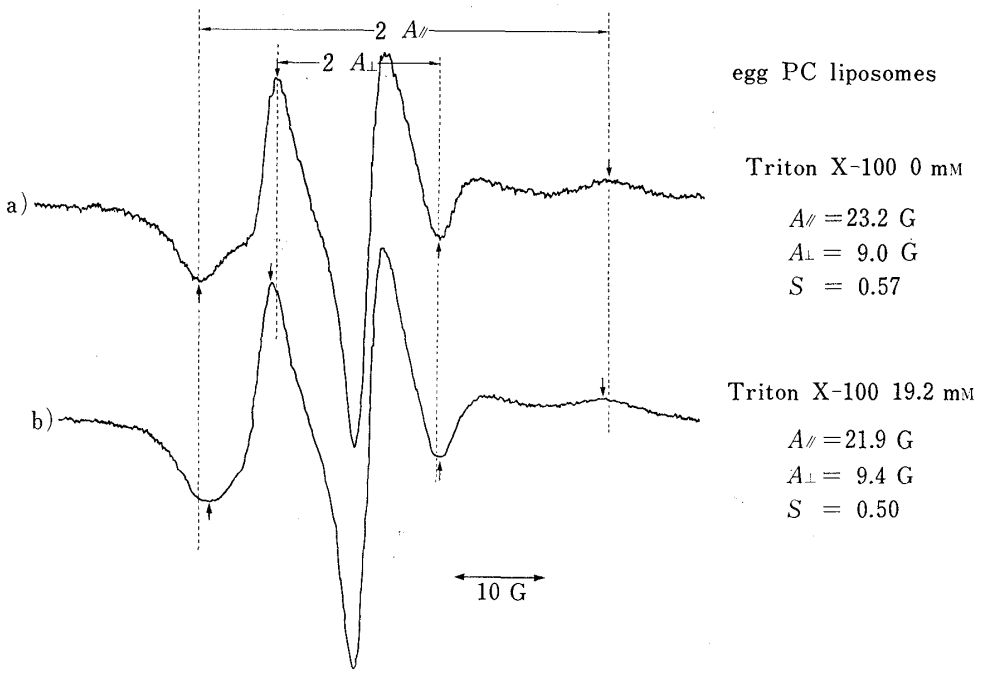

Fig. 1. Typical ESR Spectra of Spin Labeled Stearic Acid (12SLS) in Liposomes

The liposomes were composed of 66 mol % of egg phosphatidylcholine, 33 mol % of cholesterol, and 1 mol % of 12SLS. The concentration of egg phosphatidylcholine in the suspension was 40 mM. The spectrum (a) was obtained without Triton X-100 and (b) was obtained with 19.2 mM Triton X-100. The order parameter, S, was calculated using the equation described in the text.

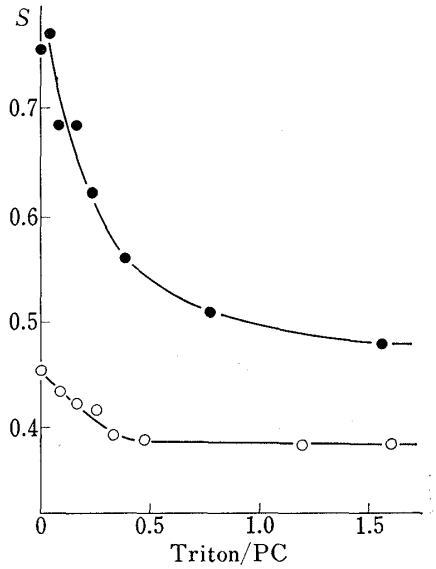

Fig. 2. Effects of Triton X-100 on the Order Parameters of Dimyristoylphosphatidylcholine Liposomes and Egg Phosphatidylcholine Liposomes

The liposomes were composed of 20 mM phosphatidylcholine and 0.2 mM 12SLS. Egg phosphatidylcholine liposomes contained 33 mol % of cholesterol. The spectra were obtained at room temperature. ○, egg phosphatidylcholine liposomes (at 17°); ●, dimyristoylphosphatidylcholine liposomes (at 14°).

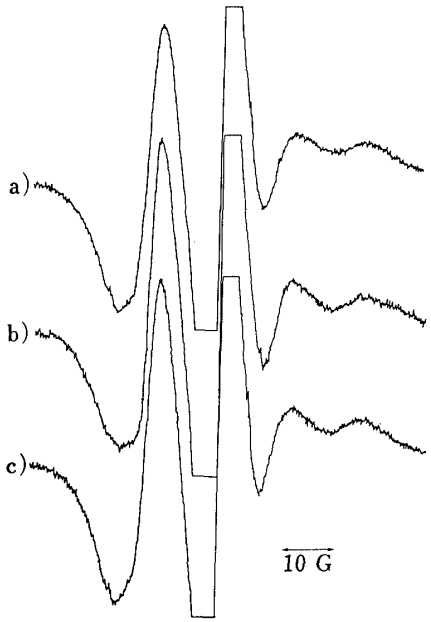

Fig. 3. ESR Spectra of Spin Probes in Triton Micelles

Three kinds of spin probes (12SLS, 5SLS, and SL-PC) were incubated with Triton micelles. The Triton concentration was 78 mM and the concentration of each probe was about 2% of that of the Triton micelles. (a) 12SLS; (b) 5SLS; (c) SL-PC.

"fluidizing" effect of the detergent was affected by the phospholipid composition of the membrane, since the decrease in the S value of dimyristoylphosphatidylcholine liposomes was much greater than that in egg phosphatidylcholine-cholesterol liposomes.

Apparent Order Parameter of Sipn Probes in Triton Micelles

12SLS, 5SLS, and SL-PC were used as spin probes. Each probe was mixed with 78 mM Triton solution and the ESR spectra were observed (Fig. 3). The S values obtained with each probe in Triton micelles were almost same (about 0.5). The value with 12SLS was larger than that observed with the same probe in egg phosphatidylcholine liposome (0.38). The similarity of the S values with different probes in Triton micelles indicates that the probes undergo isotropic motion, since it is well known that the S values of 12SLS and 5SLS, which undergo anistropic motion in a bilayer membrane, are completely different because of the difference of the position of the N-oxide group attached to the hydrocarbon chains.[9] The apparent large S values can be explained by slow motion of the probes in Triton micelles. It was reported that the positions of the inner and outer extrema in the spectrum might be affected by the long correlation time when the rate of probe motion decreased.[11] Under such conditions, the S value calculated from $A_{//}$ and $A_{\perp}$ will be overestimated; even when no order is present, inner and outer extrema can sometimes be obtained in the spectra as a result of slow motions. Hertz and Barenholz[12] reported that microviscosity of Triton micelles was much larger than that of cationic detergents such as cetyl trimethyl ammonium bromide. The slow motion of the probes may be due to the high viscosity of Triton micelles.

Heterogeneous Mixing of Triton X-100 and Phosphatidylcholine

Figure 2 shows that the S value of egg phosphatidylcholine-cholesterol liposomes without Triton X-100 was 0.45, which was smaller than that of Triton micelles (0.5), and that addition of Triton X-100 decreased this to a plateau value (0.4). If Triton X-100 and phosphatidylcholine were mixed homogeneously, the S value of the mixed micelles should reach a value between 0.45 and 0.5, depending on the Triton/phosphatidylcholine molar ratio, and 0.5 at infinite Triton concentration. Therefore, the result for egg phosphatidylcholine-cholesterol in Fig. 2 indicates heterogeneous mixing of Triton X-100 and egg phosphatidylcholine-cholesterol. The spin probes may remain in the region enriched with egg phosphatidylcholine-cholesterol, showing relatively constant S values even afetr mixing with large amounts of Triton X-100.

The S value of dimyristoylphosphatidylcholine liposomes was changed drastically by the addition of a little Triton X-100 and reached the same level as for Triton micelles. This result also suggets that Triton-rich regions may be separated from phosphatidylcholine-rich regions. Most spin probe molecules may exist in more fluid regions, that is, Triton-rich regions in this case.

Interaction between Spin Labeled Phosphatidylcholine Liposomes and Triton X-100

Various amounts of Triton X-100 were added to a suspension of sonicated SL-PC liposomes and the ESR spectra were measured. The spectrum of SL-PC liposomes before the addition of Triton X-100 showed a single broad resonance line due to the strong spin-spin exchange interaction of SL-PC (Fig. 4a). The addition of Triton X-100 at concentration below the CMC caused no significant change in the spectra (Fig. 4b, c), while at higher concentration above the CMC the line width (peak to peak) of the single broad resonance line gradually broadened

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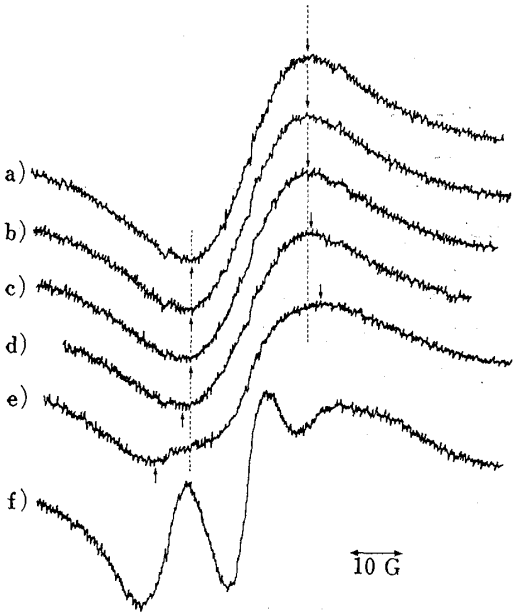

Fig. 4. ESR Spectra of Spin Labeled Phosphatidylcholine Liposomes

The final concentration of the spin labeled phosphatidylcholine was 2 mM. (a) without Triton X-100; (b) with 0.13 mM Triton X-100 (the Triton/phosphatidylcholine molar ratio was 0.06); (c) with 0.26 mM Triton X-100 (the molar ratio was 0.12); (d) with 0.36 mM Triton X-100 (the molar ratio was 0.18); (e) with 2.44 mM Triton X-100 (the molar ratio was 1.2); (f) with 9.7 mM Triton X-100 (the molar ratio was 4.8).

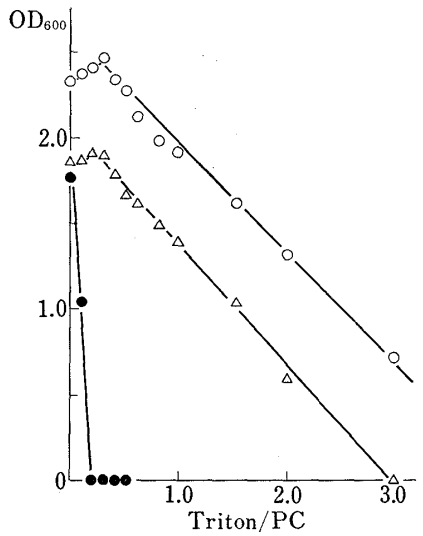

Fig. 5. Effects of Triton X-100 on the Turbidity of Dimyristoylphos phatidylcholine Liposomes and Egg Phosphatidylcholine Liposomes

The liposomes were prepared from 3 mM phosphatidylcholine. ○, egg phosphatidylcholine+ 33 mol % of cholesterol; △, egg phosphatidylcholine; ●, dimyristoylphosphatidylcholine.

(Fig. 4d, e). Further addition of Triton X-100 led to the appearance of three resonance lines superimposed on the broad line (Fig. 4f). It is noteworthy that the single broad resonance line still remained at a very high concentration of Triton X-100 (9.7 mM; the molar ratio of Triton X-100 and SL-PC was 4.8). A spectrum similar to that shown in Fig. 4f has been well characterized by several groups,[13] and was concluded to correlate with the existence of at least two distinct phases. The three resonance lines arise from spin labeled compounds sufficiently diluted with nonparamagnetic species and the other broad line is due to the aggregation of the spin labeled compounds. Therefore, the results shown in Fig. 4 indicate that some heterogeneous mixing may occur on the liposomal membranes and that Triton-rich regions and phosphatidylcholine-rich regions are separated to some extent.

Though no appreciable change was observed below the CMC, monomers of Triton X-100 should interact with the lipid bilayer, since glucose permeability was affected even below the CMC.[5] The amounts of Triton molecules which penetrate into the liposomal membrane might be small at below the CMC, having no significant influence on the spectra. Above the CMC, Triton micelles can also interact with liposomes. Due to this interaction the amounts of Triton X-100 in the membrane increase, leading to solubilization of the membrane. The ESR spectra shown in Fig. 4 are consistent with the solubilization of the membrane described below.

Effect of Triton X-100 on the Turbidity of Liposomes

The change in the turbidity was measured in order to study the solubilization of liposomes by Triton X-100 (Fig. 5). In egg phosphatidylcholine liposomes prepared in the presence and absence of cholesterol, the turbidity increased initially (small amounts of Triton X-100). Next (higher amounts of Triton X-100), the turbidity decreased gradually and linearly. The increase in the turbidity may indicate that the liposomes were swollen by small amounts of

Triton X-100 without being disrupted. Further incorporation of Triton X-100 causes fragmentation of the lipid bilayer, resulting in the decrease in the turbidity. According to Hertz and Barenholz,[12] this effect is defined as membrane solubilization. Increasing the Triton/phosphatidylcholine molar ratio above the turning point (0.3 in the case of egg phosphatidylcholine-cholesterol liposomes) may cause the formation of Triton phosphatidylcholine-cholesterol mixed micelles. The turning point of the turbidity corresponded well to the "critical" molar ratio of Triton/phosphatidylcholine determined by ESR study (about 0.35). Figure 5 also demonstrates that the solubilization of egg phosphatidylcholine liposomes required more Triton X-100 that that of dimyristoylphosphatidylcholine liposomes. In the case of dimyristoylphosphatidylcholine liposomes the turbidity decreased drastically in the range of low Triton concentration. The molar ratio of Triton X-100 to phosphatidylcholine at which the turbidity was fully decreased was 0.2.

In egg phosphatidylcholine-cholesterol liposomes Triton X-100 incorporated in the lower range of concentration may cause "fluidization" of the whole membrane without disrupting the liposomal structure. The presence of cholesterol did not have any significant influence on the sensitivity of egg phosphatidylcholine liposomes to Triton X-100. It can be concluded that the sensitivity of dimyristoylphosphatidylcholine liposomes to Triton X-100 was much greater than that of egg phosphatidylcholine liposomes. In dimyristoylphosphatidylcholine liposomes the Triton-rich regions might be more easily formed than in egg phosphatidylcholine liposomes. According to Yedgar *et al.*,[14] formation of Triton-rich regions with high curvature might cause fragmentation and solubilization of lipid membranes. In the case of egg phosphatidylcholine liposomes, Triton X-100 incorporated may have some tendency to be evenly distributed due to lateral diffusion. Thus, a much lower Triton concentration might be required to reach a local Triton concentration sufficient to cause the solubilization of dimyristoylphosphatidylcholine liposomes.

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