ESR Study of Membrane Perturbation and the Lysis of Liposomes Induced by Chlorpromazine

Yasuko MORIMOTO,* Mikio HOSOKAWA, Hiroteru SAYO, and Yoshikazu TAKEUCHI

Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Arise, Ikawadani-cho, Nishi-ku, Kobe 651–21, Japan. Received May 24, 1993

The mechanism of interaction between chlorpromazine (CPZ) and artificial lipid membranes, negatively or positively charged liposomes was studied by an electron spin resonance (ESR) technique. Analysis by a 5-doxyl stearic acid (DS) spin probe indicated that regardless of the electric charge of liposomes, CPZ disordered the hydrophobic region near the surface of lipid membranes at CPZ/lipid >1. In the same CPZ/lipid range, the lysis of liposomes was observed, and it was considered that the formation of the CPZ/lipid mixed micelles as a consequence of this collapse of liposomes would probably lead to the disordering of the 5-DS reporting region. As to the middle portion of the fatty acyl chains reported by the 12-DS spin probe, the membrane disordering action of CPZ was only detected with the negatively charged liposomes at a ratio of CPZ/lipid <1, but no membrane alteration was observed with the positively charged liposomes, regardless of the concentration range of CPZ used. In comparing these two opposite results, it is conceivable that the disordering at the 12-DS reporting region was probably induced by the cationized CPZ which would enter into the hydrocarbon-polar interface, leading to an expansion of the space between the hydrocarbon chains at this area. Also, a strong influence of CPZ on the innermost portion of the lipid bilayers was observed with both the negatively and positively charged liposomes, as reported by the 16-DS probe. This perturbing action presumably occurred when undissociated CPZ molecules penetrated into the center of the bilayers (lipid core), which could have resulted in a reduction of hydrophobic interactions of the lipid molecules.

Keywords chlorpromazine (CPZ); liposome; electron spin resonance (ESR); hydrocarbon-polar interface; lipid core; liposome lysis

It is known that amphipathic drugs such as chlorpromazine (CPZ) protect erythrocytes against hypotonic hemolysis, but elicit a detergent like hemolysis.[1] The hemolytic mechanism has been investigated in the interest of elucidating drug action on biomembranes. Seeman and Weinstain[1a] reported that CPZ-induced hemolysis was associated with the release of K^+ ions from erythrocyte membranes. Lieber *et al.*[2] reported that CPZ induced holes which would have a diameter of approximately 14 Å, and in turn caused colloid osmotic hemolysis. Such a disordering effect of CPZ on the biomembrane may be result from the interaction of CPZ with the membrane lipids. It was also reported that the lipid bilayers were severely fluidized by CPZ in cases of both biological and artificial membranes.[3] In our previous study,[4] using Fourier transform infrared (FT-IR) spectroscopy, we showed that CPZ induced the conformational alteration of lipid fatty acyl chains of artificial membranes (liposomes) consisting of phosphatidylcholine (PC), dicetylphosphate (DCP) and cholesterol (CHL) in a ratio of 1/0.1/0.75. As to the mechanism of the binding of CPZ with membranes and the localization of CPZ in the lipid bilayers, Romer and Bickel[5] have suggested that CPZ bound to the polar surface of the membrane and concurrently penetrated into the inner hydrophobic region of the bilayers by using two fluorescence probes, perylene and 8-anilino-1-naphthalenesulfonate (ANS), which could be located in the interior and near the surface of the membrane, respectively. Zachowski and Durand[6] determined the partition of CPZ between the lipidic phase and the aqueous medium, and clarified the biphasic nature of the binding of CPZ with both artificial and biological membranes, *i.e.*, there are two types of interactions of CPZ with membranes, depending on CPZ concentration. They explained that the partition process of CPZ to the hydrophobic phase and the electrostatic association of CPZ to the membrane surface would be related to membrane stabilization and cell lysis.

The aim of our present study is to examine in detail the behavior of CPZ at the surface, middle and innermost portion of the artificial membranes by changing the CPZ/lipid ratio, taking into consideration the above findings. The mechanism of action of CPZ on membrane lysis was also pursued in relation to the localization of CPZ molecules in the bilayers. For this purpose, we determined the mobility of each of three different electron spin resonance (ESR) spin probes in positively or negatively charged liposomes in the absence or presence of CPZ.

Experimental

Materials CPZ HCl was purchased from Wako Pure Chemical Industries, Ltd. DCP, stearylamine (SA) and CHL were purchased from Nacalai Tesque, Inc. L-α-Phosphatidylcholine (PC) from egg yolk (99%), which contained the following fatty acyl components: 33% palmitoyl (C16 : 0), 13% stearyl (C18 : 0), 31% oleyl (C18 : 1), 15% linoleyl (C18 : 2) and 8% others, was purchased from Sigma Chemical Co. 5-, 12- and 16-doxyl stearic acids (DS) were purchased from Aldrich Chemical Co. These chemicals and other reagents used in this experiment were of analytical grade.

Liposome Preparation Liposomes were prepared as described previously,[4] except that an ESR spin probe dissolved in $CHCl_3$ was added to the lipid mixtures. Two milliliters of PC (10 mg/ml) containing $CHCl_3$ solution, and 10 ml of $CHCl_3$ solution, in which both the DCP (0.147 mg/ml) and CHL (0.782 mg/ml) were dissolved, were mixed in a 50 ml volume round bottom flask. In preparing the positively charged liposomes, SA (0.0725 mg/ml) was used instead of DCP. The required volume (92.3 μl) of spin probes (1.25 mg/ml) containing $CHCl_3$ solution was then added to the lipid mixture. The obtained mixture was dried to a thin lipid film on the wall of the round bottom flask under reduced pressure in a rotary evaporator. Just before ESR measurement, the film

was hydrated in 2 ml of 0.154 M NaCl solution (saline solution) and dispersed by vortexing it to the prepared liposomes. The obtained liposomes contained a spin probe at a concentration of 0.6 mol/mol% of total lipids (PC/CHL/DCP or SA = 1/0.75/0.1). When the liposomes were treated with CPZ, 20 μl of 0.2 M CPZ solution, which was adjusted to the saline solution by adding NaCl, and 20 μl of the saline solution were added to 80 μl of the prepared liposome suspension. This prepared sample contained 33.3 mM of CPZ, which was two times the total lipid concentration in the liposome suspension. A desired CPZ concentration in the liposome sample was prepared by changing the volume ratio of 0.2 M CPZ solution to the saline solution.

ESR Measurements JEOL JES FE-1X spectrometer was used to determine ESR spectra at ambient temperature (21—23 °C). Instrumental parameters were optimized to avoid artificial broadening. The conditions for measurement were as follows: microwave power, 20 mW for 5-DS or 10 mW for 12- and 16-DS; microwave frequency, 9.429 GHz; magnetic field, 3330 ± 50 G; response, 1 × 1000; response time, 0.3 s; modulation, 100 kHz; modulation amplitude, 1 G for 5- and 12-DS or 0.5 G for 16-DS. Order parameters were calculated from hyperfine splitting tensors according to the method described by Hubbell and McConnell.[7] The parallel and perpendicular components of the hyperfine splitting tensors, $T'_{//}$ and $T'_{\perp}$, were derived from the spectra as indicated in Fig. 2a, and the order parameter, S, was calculated by the following equations.

$$S=\frac{T'_{//}-T'_{\perp}}{T_{zz}-(T_{xx}+T_{yy})/2}\cdot\frac{a_N}{a'_N}$$

$$a'_N=(T'_{//}+2T'_{\perp})/3$$

$$a_N=(T_{xx}+T_{yy}+T_{zz})/3$$

The values of a_N and a'_N are isotropic interactions of nitroxide radicals on solvent polarity. The elements of the hyperfine tensors in the principal directions, T_{xx}, T_{yy} and T_{zz}, were taken from Gaffney and McConnell.[8] The behavior of 16-DS was analyzed using a parameter of $T'_{//}$.[9]

Gel Filtration The liposome samples without an ESR spin probe were prepared by the method described in the section Liposome Preparations. An aliquot of 500 μl of prepared sample was applied to a column of sephadex G-50 (300 mm length × 16 mm diameter). The column was eluted with 0.154 M NaCl solution at an elution rate of 2.9 ml/min. The eluted sample was fractionated and collected every 2 min. The absorbance at 253 nm (CPZ) and 450 nm (turbidity) for each sample was determined.

Critical Micelle Concentration (cmc) Determination The cmc value for CPZ in isotonic saline solution was determined according to the fluorescence method of Luxnat and Galla.[10] This method is based on the theory that aggregates of CPZ molecules are spectroscopically different from the monomer in solution. The fluorescence spectra of CPZ in 0.154 M NaCl solution were taken as a function of CPZ concentrations with a fluorescence spectrometer, Hitachi Fluorescence Spectrophotometer F-3000. Excitation spectra were measured at a fixed emission at 450 nm. From the obtained data, the cmc value was calculated as the starting point at which a step-like increase in the excitation maximum occurred.

Zeta Potential Measurement The lipid film for liposomes, prepared as described above, was hydrated in 0.0154 M NaCl solution (saline/10) and dispersed. An aliquot of this liposome suspension (400 μl) was diluted with 5600 μl of saline/10. This sample contained lipids at a concentration of 1.67 mM. When the liposomes were treated with CPZ, a required volume of 0.02 M CPZ solution (osmotically equivalent to saline/10) was replaced with saline/10. These prepared samples were then assayed to measure the zeta potential using Face's Zepom meter (Kyowa Kaimenkagaku Co., Ltd.).

Results and Discussion

Electric Charges and the Behavior of Liposomes in the Presence of CPZ Liposomes consisting of either PC/CHL/DCP or PC/CHL/SA used in this experiment were characterized by measuring the zata potential. The zeta potentials for the PC/CHL/DCP and for the PC/CHL/SA were −29.5 ± 2.7 and +47.7 ± 3.9, respectively, indicating that the DCP-liposomes can be characterized as negatively charged liposomes, and SA-liposomes as positively charged. When CPZ was added to the negatively charged liposomes at CPZ/lipid = 0.4, the zata potential turned to +39.2 ± 2.0 (Fig. 1, ●). This experiment demonstrated that the entry of cationized CPZ molecules into liposomes induced a change in the surface electric charges. Under such circumstances, it was conceivable that cationized CPZ molecules penetrated into the polar-hydrocarbon interface of the liposomes and protruded their $N(CH_3)_2$ groups into the water phase. The further addition of CPZ, up to two times as much as the lipids, resulted in a more positive electrophoretic mobility of the liposomes, but the change in surface charges was not remarkable. This phenomenon suggests that there is a limit to the amount of the cationized CPZ which can penetrate into the polar-hydrocarbon interface. In the case of the positively charged liposomes, CPZ did not cause any remarkable increase in the zeta potential of the liposomes in comparison to the negatively charged liposomes (Fig. 1, ○). This means that the electric repulsion probably interfered with the entry of the cationized CPZ into the positively charged liposomes.

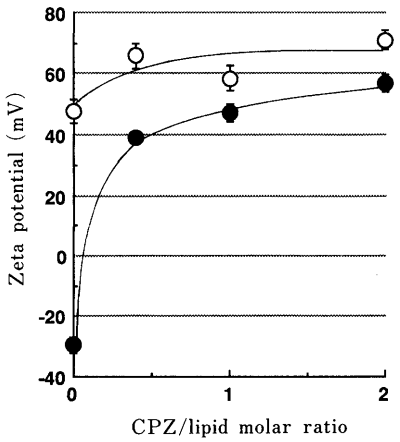

Fig. 1. Effect of CPZ on the Zeta Potential for the Liposomes Consisting of PC/CHL/DCP (●); PC/CHL/SA (○), in a Ratio of 1 : 0.75 : 0.1

Measurements for 20 particles (liposomes) in each suspension were averaged.

Determination of ESR Parameters ESR spectra of 5-, 12- or 16-DS in PC : CHL : DCP (1 : 0.75 : 0.1) liposomes (negatively charged liposomes), treated with or without CPZ at 22 °C, are shown in Figs. 2—4. From these spectra, order parameters for 5- or 12-DS in liposome membranes were calculated with each sample to evaluate the degree of membrane perturbation at the sites where these two probes localized. As for 16-DS probes, which report the molecular mobility of hydrophobic chains at the innermost portion, the line shape of the ESR spectra for this probe in the liposomes treated with CPZ was not similar to that without CPZ. The liposomes treated with CPZ gave sharp, isotropic spectra, while the control liposomes produced a broad, anisotropic one. As such, neither the order parameter or the rotational correlation times could be calculated under all of the conditions we tested. Therefore, a hyperfine splitting tensor, $T'_{//}$, which was easily obtained from the width of external peaks of the spectra, was instead introduced to evaluate the effect of CPZ on the molecular motion of hydrophobic chains. The calculated parameters were plotted against the molar ratio of CPZ present in the

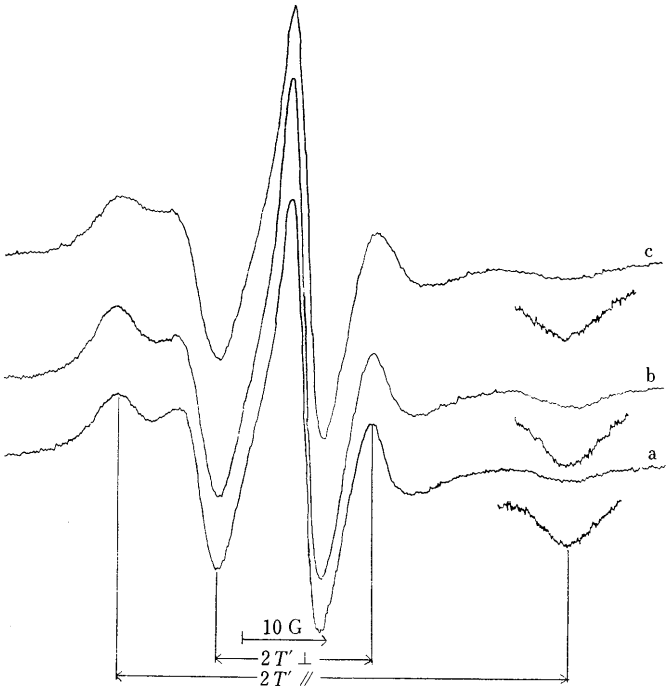

Fig. 2. ESR Spectra of 5-DS in the PC/CHL/DCP (1:0.75:0.1)-Liposomes

a, untreated with CPZ; b, c treated with CPZ when the CPZ/lipid ratio=1, or 2, respectively.

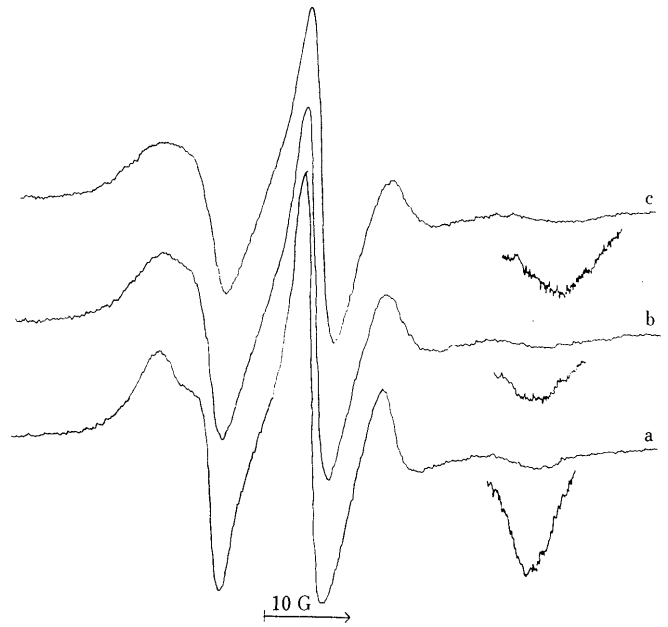

Fig. 3. ESR Spectra of 12-DS in the PC/CHL/DCP (1:0.75:0.1)-Liposomes

a, untreated with CPZ; b, c treated with CPZ when the CPZ/lipid ratio=0.6, or 2, respectively.

liposome suspension, and the results are shown in Fig. 5. For positively charged liposomes consisting of PC, CHL and SA, the same parameters were used to evaluate the environment of each probe in the liposomes.

CPZ Action on the Negatively Charged Liposomes at a Ratio of CPZ/Lipid $\leqq 1$ When CPZ was added to the

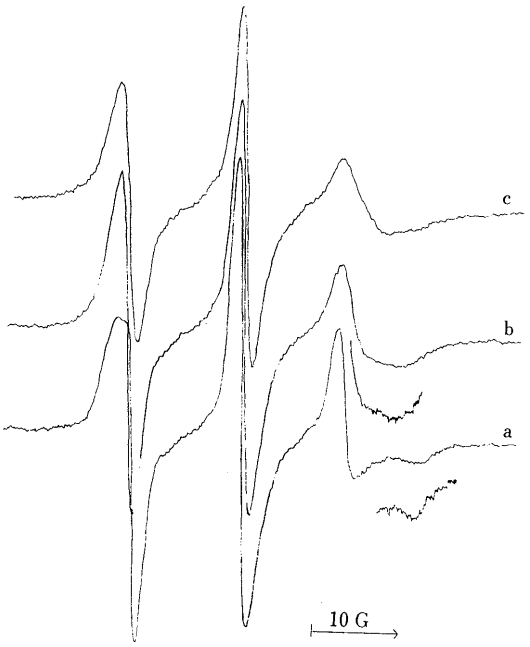

Fig. 4. ESR Spectra of 16-DS in the PC/CHL/DCP (1:0.75:0.1)-Liposomes

a, untreated with CPZ; b, c treated with CPZ when the CPZ/lipid ratio=0.2, or 2, respectively.

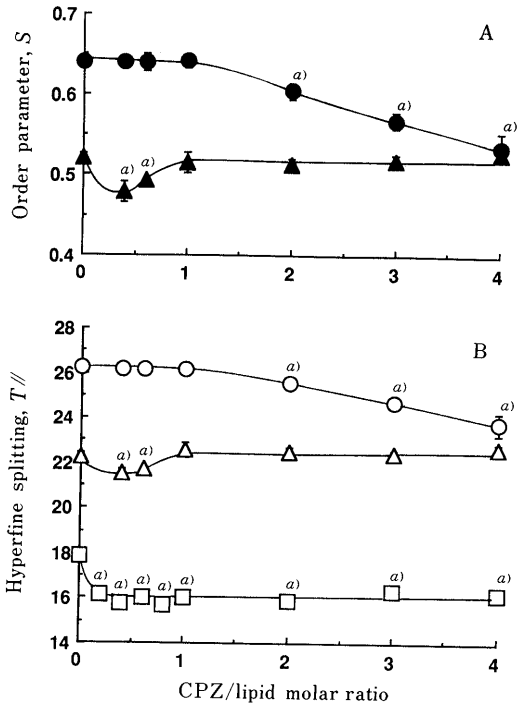

Fig. 5. Effect of CPZ on (A) Order Parameters for 5-DS (●) or 12-DS (▲); (B) Hyperfine Splitting for 5-DS (○), 12-DS (△) or 16-DS (□), in the PC/CHL/DCP (1:0.75:0.1)-Liposomes at 22 °C

a) Significantly different from the control (without CPZ) at $p<0.001$.

5-DS labeled liposome suspension up to an equimolar ratio of CPZ to lipid (CPZ/lipid $\leqq 1$), membrane fluidity near the surface probed by 5-DS spin probe was hardly changed (Fig. 5A, ●). As to the middle portion of fatty acyl chains, where 12-DS spin probe was reporting, the disordering

action was maximal at a CPZ/lipid ratio = 0.4. Further addition of CPZ enfeebled the membrane perturbing action of CPZ at this position, *i.e.*, the order parameter, *S*, was gradually increased as the CPZ/lipid ratio was increased, and became approximately equal to the control value at CPZ/lipid = 1 (Fig. 5A, ▲). The observed decrease in the value of the order parameter for the 12-DS probe in the liposomes by the addition of CPZ may be explained as a consequence of the expansion of the spaces between fatty acyl chains at the middle portion, caused by the penetration of CPZ into the polar-hydrocarbon interfaces of the lipid bilayers. With respect to the disappearance of the disordering effect of CPZ at a higher concentration, as described above, the present study did not provide the experimental data necessary to explain this complicated phenomenon. However, the following assumption about this mechanism may be possible. First, CPZ molecules which entered into the polar-hydrocarbon interfaces may have been transferred to the inner compartments as a result of the molecular rearrangement of the lipids occurring during the consecutive contact of CPZ with lipids, and the compartments between the fatty acyl chains at the middle portion were presumably filled up by CPZ. This phenomenon would presumably cause disappearance of the expected membrane perturbing action of CPZ at the CPZ/lipid = 1. Secondly, the possible formation of CPZ micelles in the solution surrounding the liposomes would appear to reduce the total amount of CPZ which could contribute to the entrance of CPZ molecules into the liposomes, and therefore the membrane could not have been sufficiently disordered. To verify this assumption, the cmc value for CPZ in saline solution was measured by fluorometry as described in the experimental section. As shown in Fig. 6A, the excitation maximum λ_{max} for the spectrum of CPZ at its concentration of 0.083 mM was observed at 307.2 nm, and was red-shifted by increasing the concentration, *e.g.*, 352.6 nm at 3.3 mM. The wavelength, λ_{max}, was plotted as a function of CPZ concentration, and the result was shown in Fig. 6B. Up to CPZ concentrations of 0.3 mM, the λ_{max} value was almost constant at approximately 310 nm. A further increase in CPZ concentration caused a significant shift in λ_{max} toward a higher wavelength. From these data, it was assumed that the CPZ molecules formed aggregates, at least at the concentration of 0.3 mM, which could be referred to as the cmc for CPZ in the saline solution. This obtained value was in good agreement with the cmc in the same solution measured by the surface tension meter.[11] On the basis of the cmc value for CPZ, the second assumption described above was found to be unreasonable, *i.e.*, CPZ micelles could exist at extremely lower concentrations than 6.67 mM, corresponding to the concentration of CPZ/lipid ratio = 0.4, at which the disordering action of CPZ started to diminish in relation to an increase in the ratio of CPZ/lipid.

The values of a hyperfine splitting tensor, $T_{//}$, were plotted as a function of CPZ/lipid ratio as shown in Fig. 5B. As observed, the changes in the $T_{//}$ values with CPZ/lipid ratio for 5-DS and 12-DS (○, ▲) were in fair agreement with those in the order parameters (●, ▲ in Fig. 5A), suggesting that the $T_{//}$ value was also capable of

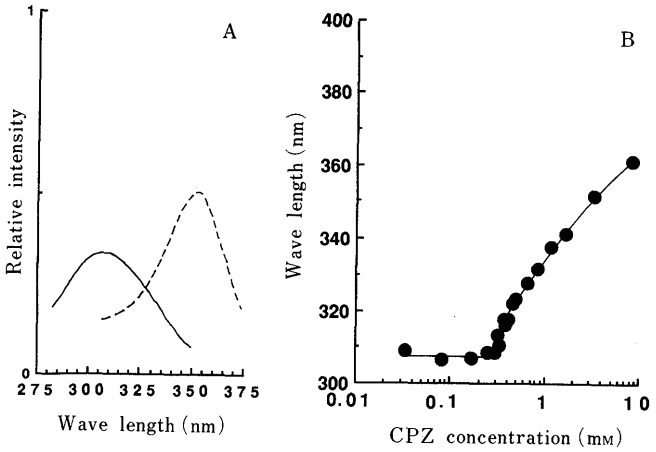

Fig. 6. A, Excitation Spectra of Chlorpromazine at 0.083 mM (—) and 3.33 mM (– – –); B, Excitation Maximum of Spectra Shown in A as a Function of CPZ Concentration

The fluorescence was measured at 450 nm.

reporting the molecular mobility of hydrocarbons at the site where the nitroxide moiety of the probe existed. As shown in Fig. 5B, □, the hydrophobic region labeled with 16-DS probe, was remarkably perturbed. Liposomes used in this experiment consisted of PC, CHL and DCP (SA), as described in the experimental section. The PC molecules contained 30% of a palmitoyl group (C16 : 0), 30% of an oleoyl group (C18 : 1), and other groups. The length of CHL was a little shorter than these hydrophobic chains. On the basis of these considerations, it was understood that the 16-DS probe could report on the environment near the center of the bilayers. The strong influence of CPZ on the 16-DS probe reporting portion discribed above can probably be explained as a result of the entry of the undissociated CPZ molecules into the center of the bilayers, which led to a reduction in hydrophobic interactions between the facing terminal methyl groups of the bilayers.

As observed in Fig. 4a, the spectrum of 16-DS in the liposomes had two components. An explanation for this appearance could be as follows. When the fatty acid spin probe was partially ionized, two forms of this probe, which would be anchored at different positions in the polar interface, might have exhibited two spectral components.[12] As the pH of the saline (5.7) which was used to hydrate the lipids in this experiment, this spin probe was expected to exist as undissociated form in large part.[12a] Thus, the appearance of the two spectral components could not be explained by the partial ionization of the probe. An alternative possibility is that the undissociated probe might have a great amplitude of flexing motion about the perpendicular to the bilayer, allowing the nitroxide group to be located at the other hydrocarbon phase over the center of the bilayers.[12b] Under this consideration, the two components observed in the spectrum (Fig. 4a) could likely be attributed to the different location of spin probe in the bilayers, one with the label group at the center of the bilayers, and the other with that at the hydrocarbon phase where the label must be more restricted in motion than at the center of the bilayers. As shown in Figs. 4b and c, the spectra for the liposomes treated with CPZ had

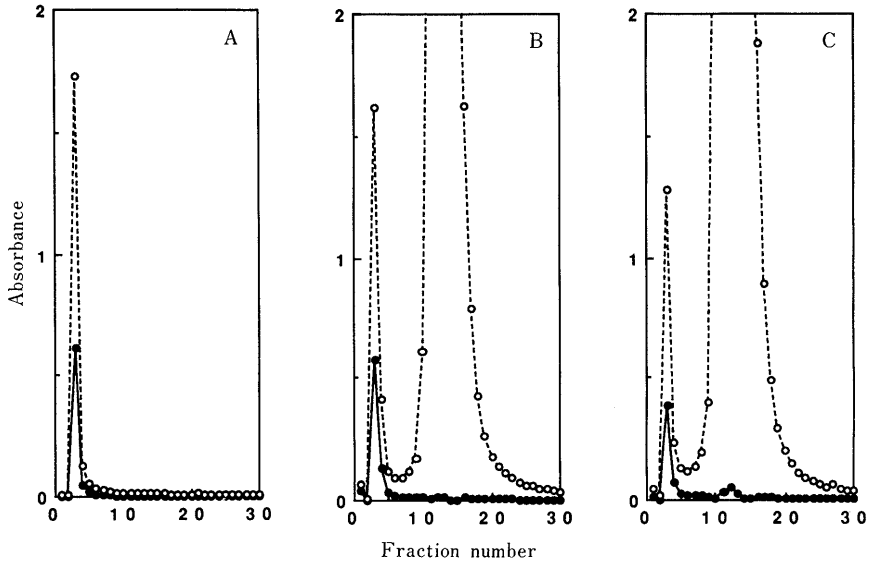

Fig. 7. Elution Profiles of PC/CHL/DCP (1 : 0.75 : 0.1)-Liposomes

A, alone; B, C with CPZ at CPZ/lipid = 1, or 2, respectively, from columns of Sephadex G-50. ●—●, absorbance at 450 nm (turbidity); ○—○, at 253 nm.

only one component with small $T_{//}$ values, in comparison with the control liposomes (Fig. 4a). Such a phenomenon suggests that the nitroxide group of the 16-DS probe in the liposomes treated with CPZ is not located at the hydrocarbon phase. Even in the presence of CPZ at the CPZ/lipid ratio of 0.2 to 4 and at a pH range of 5.6 to 4.9, respectively, the almost undissociated probe would be flexing perpendicularly to the bilayers. Thus, in this case, the center of the bilayers was presumably widened by the entrance of the undissociated CPZ molecules. Lieber *et al.*[2] reported that the CPZ-laden erythrocyte membranes were increased in thickness more than two- fold in comparison to the control, and possibly formed a CPZ-rich zone at the center of the bilayers. This finding was highly consistent with our proposed mechanism of CPZ action at the site where 16-DS was reporting.

CPZ Action on the Negatively Charged Liposomes at a Ratio of CPZ/Lipid >1 By the addition of CPZ to the negatively charged liposomes labeled with 12-DS or 16-DS, at a ratio of CPZ/lipid >1, no changes were observed with the parameters, S and $T_{//}$, and the values were almost equal to that in case of the CPZ/lipid =1 (Fig. 5, ▲, △ and □). On the other hand, the parameter for the 5-DS probe in the liposomes was increasingly decreased as the CPZ/lipid ratio was increased (Fig. 5, ○, ●). The 5-DS probe reported the mobility of the fatty acyl chain near the surface, and it appeared that such mobilization was a possible result of the lysis of liposomes. When CPZ was added to the liposome suspension at a ratio of CPZ/lipid $\geqq 2$, an optically cleared solution was obtained. This phenomenon implied the occurrence of the lysis of liposomes at a much higher concentration of CPZ to lipids. To ascertain the liposome lysis, another experiment was introduced using a gel filtration technique. Figure 7A shows the elution data of the liposome sample not treated with CPZ. It was demonstrated that the intact liposomes were eluted at the third fraction (fraction No. 3), as determined by the turbidity at 450 nm (●). The peak of absorbance at 253 nm (○) observed at the same fraction was considered to be due to the lipid molecules composing the liposomes. Figure 7B and C, respectively show the elution profile of liposomes treated with CPZ at a CPZ/lipid ratio = 1 or 2. The liposomes were eluted at fraction No. 3, and the CPZ molecules which were not incorporated into the liposomes were delayed in elution and were detected at fractions 9 to 20, as determined by the absorbance at 253 nm. The intensity of the turbidity peak for the liposome fraction obtained after treatment with CPZ at a CPZ/lipid ratio = 1 (No. 3 in Fig. 7B) was almost equal to that for the liposome fraction obtained with the control (No. 3 in Fig. 7A), suggesting that the addition of equimolar CPZ to lipid did not cause any notable lysis of the liposomes. When CPZ/lipid =2, the intensity of the turbidity peak for the liposome fraction (No. 3 in Fig. 7C) decreased remarkably as compared with the control (No. 3 in Fig. 7A), and at the same time a small but apparent turbidity peak appeared at the 12th fraction, which corresponded well with the fraction for CPZ. The occurrence of such a turbidity peak, as well as the decrease in peak intensity at the liposome fraction, might indicate that CPZ molecules induced the lysis, and this collapse presumably formed small vesicles consisting of lipids and CPZ.

It was reported that the lysis of liposomes was induced by the addition of CPZ at concentrations higher than the cmc.[10] In our experiment, however, the lysis of the liposomes was not observed at a CPZ concentration of 16.6 mM, which corresponded to CPZ/lipid =1, despite this CPZ concentration being much higher than the cmc (0.3 mM). Such a difference might be explained by the fact that the lipid concentration we used was about ten times as high as that used in the Luxnat *et al.* experiment. The lysis of liposomes possibly occurred when CPZ molecules entered into the lipid membranes, sufficiently exceeding the limited proportion of CPZ to the lipids. The ratio of the absorbance at 253 nm to that at 450 nm, A_{253}/A_{450}, for the liposome fraction obtained after the

treatment with CPZ at CPZ/lipid =2 (No. 3 in Fig. 7C) was calculated to be 3.3, and this value was very large compared to the value calculated for the untreated liposomes or those treated with CPZ at CPZ/lipid =1 (Fig. 7A, B), 2.8. Such an increase in the A_{253}/A_{450} value suggested the excessive entrance of CPZ into the liposomes, and this entrance possibly caused the lysis of the liposomes.

With respect to the membrane fluidity of 5-DS labeled liposomes in the presence of CPZ above the cmc, Luxnat and Gella[10] showed that CPZ, even at their lytic concentrations, did not change the order of lipids in the liquid crystalline phase. This result was obtained with the liposome sample after removing the lysate formed in the presence of CPZ by centrifugation. In this case, the spin probe located in the lysate did not report on the environment where it was. When we measured the CPZ-induced membrane disordering action using the whole liposome suspension without removing the lysate, however, the 5-DS labeled liposomes was extremely disordered in the presence of CPZ at CPZ/lipid >1, as shown in Fig. 5. Thus, our obtained disordering action of CPZ was found to be due to the formation of a lysate (small vesicles) in which the nitroxide group of the 5-DS probe was loosely packed.

Membrane Perturbing Action of CPZ on Positively Charged Liposomes When positively charged liposomes consisting of PC, CHL and SA were treated with CPZ, the value of the order parameter, S, for the 5-DS probe in the liposomes was not altered at a CPZ/lipid ratio $\leqq 1$, but decreased remarkably at a CPZ/lipid ratio >1 as shown in Table I. Such a decrease in S value suggested that the positively charged liposomes were disrupted by the action of CPZ. This obtained result was consistent with the case of the negatively charged liposomes as discribed above. From this finding, it was understood that the disruption of the liposomes induced by CPZ was not presumably related to the result of interaction based on the electrical charges of the liposomes. As for the middle portion of the lipidic fatty acyl chains where the 12-DS was reporting, the packing order at this portion of the positively charged liposomes was estimated to be unchangeable, even if CPZ was added to the liposome suspension at a molar ratio of four times CPZ to lipids (Table II). Meanwhile, with the negatively charged liposomes, the mobility of the fatty acyl chain at the middle portion reported by 12-DS was enhanced by the addition of CPZ at a ratio of CPZ/lipid <1 (Fig. 5A, △). A comparison of these two results provided by the 12-DS spin probe pointed out that the 12-DS reporting area of the negatively charged liposomes was possibly disordered by the action of the cationized CPZ, which could enter into the hydrocarbon-polar interface, and probably expanded the space between the fatty acyl chains at the middle portion. In case of the positively charged liposomes, the electric repulsion between the positively charged CPZ and the positively charged liposomes was considered to reduce the disordering action of CPZ by preventing CPZ from entrance into the hydrocarbon-polar interface. As indicated in Table III, the center of the bilayer of the positively charged liposomes was fully disordered when CPZ/lipid =1, as in the case of negatively charged one. From these results, it was presumed that undissociated CPZ, which exsists at a volume of only 0.01% in the outside of the liposomes, entered into the center of the bilayers and perturbed the fatty acyl chains at the innermost portion, as reported by the 16-DS spin probe.

Table I. Effect of CPZ on the Order Parameter for 5-DS in Positively Charged Liposomes Consisting of PC/CHL/SA (1:0.75:0.1)

CPZ/lipid ratio	Order parameter
0	0.714±0.006
0.2	0.704±0.002
0.4	0.705±0.007
0.6	0.705±0.001
1.0	0.702±0.002[a]
2.0	0.696±0.006[b]
3.0	0.684±0.006[c]
4.0	0.689±0.004[c]

Each value of the order parameter represents the mean±S.D. of three values. Significantly different from the control (CPZ/lipid=0): *a*) $p<0.05$, *b*) $p<0.005$, *c*) $p<0.001$, respectively.

Table II. Effect of CPZ on the Order Parameter for 12-DS in Positively Charged Liposomes Consisting of PC/CHL/SA (1:0.75:0.1)

CPZ/lipid ratio	Order parameter
0	0.591±0.005
0.2	0.592±0.001
0.4	0.585±0.002
0.6	0.583±0.002
1.0	0.581±0.001
2.0	0.582±0.005
4.0	0.585±0.006

Each value of the order parameter represents the mean±S.D. of three values. Significant difference from the control (CPZ/lipid=0) was not detected.

Table III. Effect of CPZ on the Hyperfine Splitting for 16-DS in Positively Charged Liposomes Consisting of PC/CHL/SA (1:0.75:0.1)

CPZ/lipid ratio	Hyperfine splitting
0	18.52±0.13
0.2	18.09±0.20[a]
0.4	17.92±0.32[a]
0.6	17.88±0.18[b]
1.0	17.91±0.29[a]
2.0	17.52±0.12[c]
4.0	17.56±0.57[a]

Each value of the hyperfine splitting represents the mean±S.D. of three values. Significantly different from the control (CPZ/lipid=0): *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$, respectively.

Conclusion

The mechanism of interaction between CPZ and artificial membranes, positively and negatively charged liposomes was studied using an ESR technique. Analysis by a 5-DS spin probe indicated that regardless of electrical charges of liposomes, CPZ hardly changed the order of the hydrophobic region near the surface when CPZ/lipid $\leqq 1$, and remarkably disturbed at CPZ/lipid >1, suggesting that such observed action was not related to the result of an interaction based on the electrical charges

of liposomes. The middle portion of the fatty acyl chain was disordered with negatively charged liposomes but not with positively charged ones at ratios of CPZ/lipid = 0.4, 0.6. In comparing these two opposite results, it is conceivable that the disordering at this site could result from the entrance of cationized CPZ into the hydrocarbon-polar interface. This entrance would expand the space between the hydrocarbon chains at the middle portion. The perturbation observed at the innermost portions of the bilayers, with both negatively and positively charged liposomes, was presumed to be the result of the entry of undissociated CPZ into the center of the bilayers, which could lead to a reduction in the hydrophobic interactions of the lipid molecules. When the concentration of CPZ was higher than the lipid concentration (CPZ/lipid >1), the lysis of liposomes was observed, and it was suggested to be due to the excessive entrance of CPZ molecules into the inner portion of liposomes.

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