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Interaction of Antidepressant Drugs with Lipid Bilayers Studied by High-Resolution Carbon-13 (^{13}C-) and High-Power Deuterium Nuclear Magnetic Resonance (^{2}H-NMR) Spectroscopy: The Manner of Binding as Deduced from the Differential Line-Broadening of ^{13}C-NMR Signals

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The manner of binding of imipramine (IMIP) and amitriptyline (AM) as well as chlorpromazine (CPZ) to single- or multi-bilayers of egg phosphatidylcholine (PC) was analyzed by carbon-13 (^{13}C-) and deuterium nuclear magnetic resonance (^{2}H-NMR) methods. For this purpose, we first tried to assign or re-assign the aromatic ^{13}C chemical shifts by the selective proton-decoupling method. We observed that the ^{13}C-NMR signals from a specific portion of the aromatic moiety of IMIP and AM are differently broadened as a result of binding to single bilayers of egg PC. In contrast, the signals of all carbons adjacent to protons in the aromatic moiety of CPZ were completely suppressed in the presence of egg PC. The observation of such differential line-broadening for IMIP and AM was interpreted in terms of the presence of anisotropic diffusion at the tricyclic moiety incorporated into the bilayers. However, drugs tightly bound to lipids such as CPZ cannot afford signals showing such differential line-broadening because of the slowed molecular motion in the bilayers. The extent of binding to lipids as viewed from the characteristic change of the ^{13}C-NMR signals is in parallel with that determined from binding experiments by centrifugation and equilibrium dialysis. The reason why the ^{13}C-NMR signals of IMIP and AM are visible even in the presence of lipid bilayers is considered to be the larger proportion of these drugs involved in fast exchange between lipids and the aqueous phase, on the basis of the observation of a major central peak in the ^{2}H-NMR spectra of [2,4,6,8-$^{2}H_4$]IMIP in the presence of multibilayers of egg PC. Nevertheless, there appears to be some portion of IMIP which penetrates deeply into the hydrophobic portion of egg PC and has a slow exchange rate compared with the inverse of the quadrupole splitting (16 kHz).

Keywords——antidepressant drug; lipid bilayer; ^{13}C-NMR; ^{2}H-NMR; differential line-broadening

Tricyclic antidepressants (TCA's) are clinically used for the treatment of depressive psychosis.[2)] The exact mechanism whereby TCA's produce their antidepressant effects in human beings is not known. There is considerable evidence that a functional abnormality of one or more of the biogenic amine systems of the brain is involved in the etiology of depressive psychoses or affective disorders.[3)] Amines which have been investigated most thoroughly in this respect are norepinephrine (NE) and 5-hydroxytryptamine (5HT).[4,5)] The most striking neurochemical effect of TCA's is their potency in inhibiting the transport processes involved in the reuptake of these neurotransmitters into presynaptic nerve endings.[6)] Binding sites for imipramine (IMIP), one of the widely used TCA's, have been suggested to be identical with or at least closely related to the serotonin reuptake site in both brain and platelet membranes.[7,8)] However, very little is known about the structure of the transport site or the mode of interaction of IMIP and other TCA's.

Nevertheless, membrane lipids have been shown to be essential components of binding

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sites to drugs such as IMIP and chlorpromazine (CPZ).[9-11] Binding values similar to those of biological membranes were obtained for protein-free liposomes of lipids extracted from microsomes, mitochondrial, erythrocytes and of egg phosphatidylcholine (PC).[11] It appears that binding to lipid is prerequisite for the drugs to reach the active site which is supposed to be in membranes.

It has been found that nuclear magnetic resonance (NMR) is one of the most promising techniques for analyzing the interaction of drugs or xenobiotics with model or biological membranes on the basis of its ability to elucidate the extent or the manner of binding to lipids, the phase behavior, the dynamic features of bound drugs and the perturbation of the organization of membrane lipids.[12-29] We aimed in this work to delineate the manner of binding of IMIP and amitriptyline (AM) as compared with that of CPZ to vesicles of egg PC by means of carbon-13 (^{13}C)- and deuterium (^{2}H)-NMR spectroscopy. ^{2}H-NMR analysis employing ^{2}H-labelled drugs is more suitable than proton (^{1}H)- and ^{13}C-NMR because drugs incorporated into bilayers in many instances give indeterminate ^{1}H- and ^{13}C-NMR signals. In such cases, the manner of binding as well as the dynamic features of such immobilized drugs can be conveniently obtained by analysis of the ^{2}H-NMR spectra. Nevertheless, ^{13}C-NMR is a still very convenient tool to obtain similar information when drugs are rather loosely bound to lipids.

We found, in this work, that the manner of binding of IMIP and AM to egg PC vesicles can be clearly analyzed in terms of differential line-broadening in aromatic ^{13}C signals of these drugs caused by anisotropic diffusion in the bilayers. The same conclusion was obtained from analysis of the ^{2}H-NMR spectra of [2,4,6,8-^{2}H$_4$] IMIP bound to multibilayers of egg PC. In contrast, the ^{13}C signals of aromatic carbons attached to protons of CPZ are completely suppressed, because this molecule is tightly bound to the interior of egg PC bilayers.[11,27]

Experimental

Materials——Imipramine hydrochloride was a generous gift from Ciba-Geigy (Japan) Ltd., Takarazuka, Japan (Lot. No. 000383). Amitriptyline hydrochloride was a generous gift from Nippon Merck-Banyu Co., Ltd., Tokyo, Japan (Lot. No. A38603). Chlorpromazine hydrochloride was purchased from Sigma Chemical Company, MO, U.S.A. The chemical structure and the numbering system used in this paper are summarized in Fig. 1. [1-^{2}H] Acetic acid and D_2SO_4 used for selective deuteration of IMIP by ^{1}H–^{2}H exchange reaction were purchased from Aldrich Chemical Co., MI, U.S.A. and Merck Co., Germany, respectively. Deuterium-depleted water was purchased from Aldrich Chemical Co., MI, U.S.A. Egg phosphatidylcholine was isolated from fresh yolk and purified by the procedure of Bligh and Dyer.[30]

Preparation of [2,4,6,8-^{2}H$_4$]IMIP by ^{1}H–^{2}H Exchange——Imipramine hydrochloride (800 mg) was dissolved in 5% D_2SO_4–CH_3COOD (40 ml), transferred into an ampoule and sealed. The ampoule was placed in a water bath for 1.5 h (100 °C), then reaction mixture was extracted with chloroform and the products were separated on a thin-layer plate. The deuterated imipramine hydrochloride (550 mg) was obtained by recrystallization from ethyl ether–acetone solution after formation of the hydrochloride. The purity was checked by thin-layer chromatography and confirmation of the complete absence of two kinds of signals (H-2(8) and H-4(6)) by ^{1}H-NMR spectroscopy. The positions of deuteration were supported by the following two points. First, it has been established that the *ortho* and

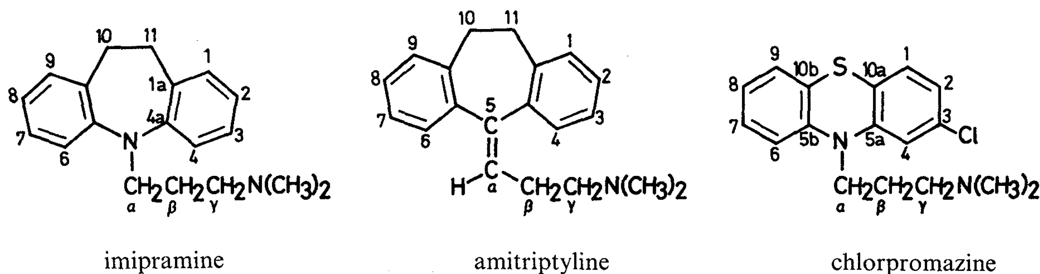

Fig. 1. Numbering of the Drugs Used

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para positions with respect to a basic nitrogen atom, namely H-2(8) and H-4(6), are susceptible to electrophilic deuterium exchange.[31] Second, the H-4(6) ^{1}H-NMR signal can be identified by the significant peak displacements with varying pH.

Preparation of Vesicles or Multibilayers of Egg PC——Chloroform from stock PC solution was evaporated under a nitrogen stream and completely removed by placing the sample in a high vacuum. Lipid films thus obtained were hydrated with ^2H_2O (for ^{13}C-NMR measurements). Freezing and thawing were repeated several times to achieve homogeneous multibilayers. Single-bilayer vesicles were prepared by sonicating the multibilayers while cooling them with ice-water for 15 min. An appropriate amount of drug was added to PC either in chloroform solution before hydration or in sonicated vesicles. Exactly the same results were obtained for both kinds of preparations.

Measurements of the Amount of Drugs in the Lipid Phase——Lamellar multibilayers or sonicated vesicles containing drugs (drug/lipid 2:5) were centrifuged at 40000 rpm (100000 × g) for 20 h at 24 °C. The volume of supernatant thus obtained was measured then it was diluted several times with distilled water for ultraviolet (UV) measurements (λ_{max}, 254 nm (IMIP); 242 nm (AM) and 308 nm (CPZ)). The amount of drug in the aqueous phase was determined from the UV absorption spectra and the amount bound to lipids was calculated from the above value and the total amount of drug.[15] The apparent K_p value was determined as the ratio of the concentration of drug in the lipid phase to that in the aqueous phase (5% accuracy).[15]

^{13}C-NMR Spectroscopy——^{13}C-NMR spectra were recorded on a Bruker CXP-300 spectrometer at 75.46 MHz. Spectra were usually obtained with a 50 ° pulse (15 μs), repetition time of 0.8 s, spectral width of 15 kHz and 16 K data points. In many instances, transients were accumulated over 8000 times. In order to achieve reliable assignment of ^{13}C signals of protonated aromatic carbons of drugs, we performed selective proton decoupling by successive irradiation of assigned ^{1}H-NMR signals.[32] Spin-lattice relaxation times (T_1's) were measured by the 180 °–t–90 ° pulse sequence[33] with repetition times between 8 and 12 s to achieve a duration longer than $5 \times T_1$. Transients were usually accumulated 300—1000 times. Nuclear Overhauser enhancements (NOE's) were obtained by comparing the peak intensities of signals of proton noise-decoupled spectra with those of spectra in which the proton noise-decoupler was gated off to remove the NOE's.[34] ^{13}C chemical shifts were referred to external tetramethylsilane (TMS) sealed in a capillary.

^{2}H-NMR Spectroscopy——^{2}H-NMR spectra were recorded on a Bruker CXP-300 spectrometer at 46.06 MHz. Multibilayer samples instead of sonicated vesicles were used in this experiment. A quadrupole echo pulse sequence,[35] $90°_{\pm x} - t_1 - 90°_y - t_2 - T$, was used to preserve the inhomogeneously broadened portion of the ^{2}H-NMR signal, which is normally lost through spectrometer dead time, where T is the repetition time. The phase of the first 90 ° pulse was shifted by 180 ° on alternate scans to reduce artefacts due to coil ringing and imperfect 90 ° pulses. The 90 ° pulse was 7 μs long, and the repetition time was 0.5 s, with $t_1 = t_2 = 50$ μs. Spectra were accumulated over 100000 times.

Results

Assignment of ^{13}C-NMR Peaks of Drugs by Selective Proton Decoupling——We attempted to assign or re-assign the ^{13}C-NMR signals of the aromatic moieties of IMIP, AM and CPZ by the selective proton decoupling method. This assignment would be straightforward for carbons attached to protons if well-resolved ^{1}H-NMR signals could be assigned correctly, as in the case of IMIP and CPZ. It was found that the spectral features of the ^{1}H-NMR spectra of these drugs are highly concentration-dependent and maximal separation of peaks was achieved only at high concentration (>30 mg/ml), probably because of the onset of micelle formation.

IMIP——As summarized in Fig. 2, the ^{13}C-NMR signals of the aromatic moiety of IMIP were assigned on the basis of the 300 MHz ^{1}H-NMR spectrum (Fig. 2A). The H-4(6) signal can be assigned to the low-field doublet by taking into account that this proton is located near the nitrogen atom. The other peaks were straightforwardly assigned to the H-2, H-3 and H-4 protons by successive ^{1}H-decoupling experiments. The assignment was confirmed by the fact that the H-4(6) and H-2(8) signals completely disappeared for [2,4,6,8-$^2H_4$]IMIP prepared by ^{1}H–^{2}H exchange. Next, the C-1, C-2, C-3 and C-4 signals were identified by searching for singlet peaks that sharpened on irradiation of the H-1, H-2, H-3 and H-4 signals, respectively (Figs. 2B—D). Distinction between the C-1a and C-4a signals was based on the fact that the C-4a signal is broadened to some extent due to residual ^{1}H–^{13}C long-range couplings with the H-10 and -11 methylene protons in spite of irradiation at the aromatic proton signals. The present assignment, summarized in the bottom trace of Fig. 2, is

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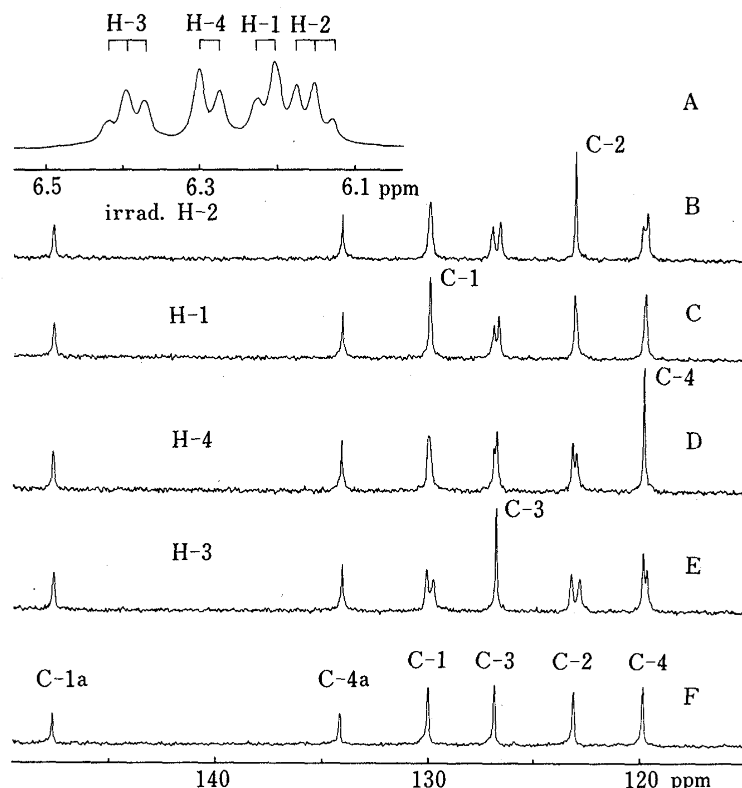

Fig. 2. ^{1}H- and ^{13}C-NMR Spectra of the Aromatic Region of IMIP in Aqueous Solution (40 mg/ml)

A, 300 MHz ^{1}H-NMR spectrum. B—E, ^{13}C-NMR spectra with selective ^{1}H irradiation. F, ^{13}C-NMR spectrum obtained by proton noise-decoupling.

almost the same as that of Abraham *et al.*[36] except that the C-2 and C-4 assignments are reversed. The assignment of the side-chain moiety was performed in a similar manner and is the same as that given in a previous paper.[36]

CPZ——We also assigned the ^{13}C signals of carbons attached to protons of the aromatic moieties of CPZ on the basis of the ^{1}H-NMR spectrum shown in the top trace of Fig. 3. The H-1, H-2 and H-4 ^{1}H signals were easily distinguished by examining the profile of spin multiplets and by means of proton-decoupling experiments. In particular, the H-4 and H-2 signals are broadened to some extent because of the presence of chlorine, which causes line-broadening by scalar relaxation of the second kind.[37] Further, another two doublet peaks are ascribable to the H-6 and H-9 protons. The low-field doublet was assigned to H-6 by taking into account the nearby nitrogen atom, as in the case of IMIP. Connectivity of the H-6–H-9 signals was confirmed by successive proton decoupling as shown in Fig. 3. The assignment of the ^{13}C signals of carbons attached to protons was straightforward by selective proton decoupling on the basis of the above proton assignments. (Figs. 3A—G). To assign the four ^{13}C signals of carbons not attached to protons, we used the empirical rule of substituent effect,[38] although ambiguity remains in this portion. This assignment differs from the previous data[27,39] in many respects; we consider that the present assignment is more reliable.

AM——The ^{1}H-NMR spectrum of AM is not well resolved as compared with those of IMIP and CPZ, as shown in Fig. 4. Nevertheless, we achieved some correlation between ^{1}H- and ^{13}C-NMR signals. First, we labelled ^{1}H-NMR peaks of aromatic and olefinic protons as indicated by ①—⑦ for selective proton irradiation. Clearly, the ^{13}C signals sharpened (asterisked) by the selective ^{1}H irradiation are well related to the ^{1}H-NMR peaks (Fig. 4), although there remains ambiguity in peak ② (superposition of the doublet and triplet signals)

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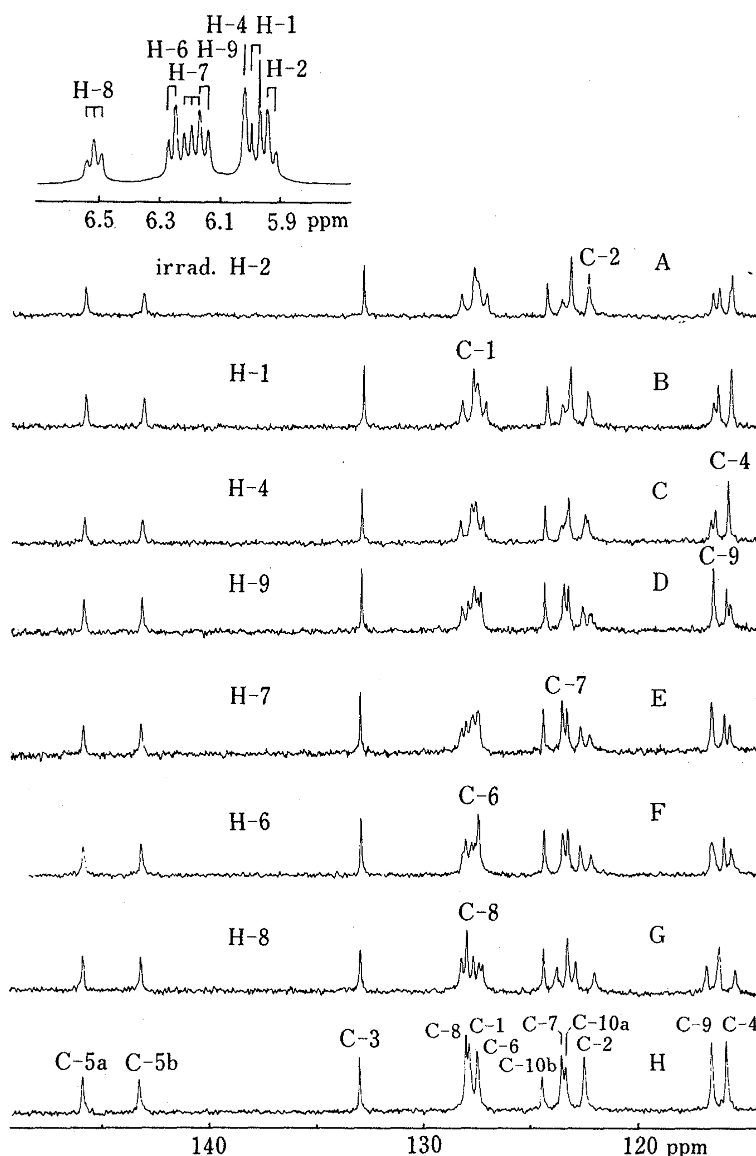

Fig. 3. ^{1}H- and ^{13}C-NMR Spectra of the Aromatic Region of CPZ in Aqueous Solution (33.3 mg/ml)

Top trace, 300 MHz ^{1}H-NMR spectrum. A—G, ^{13}C-NMR spectra with selective ^{1}H irradiation. H, ^{13}C-NMR spectrum obtained by proton noise-decoupling.

and peak ③ (two triplet signals superimposed). Thus, the ^{13}C peaks 6, 7 and 9 can be unequivocally assigned to three of C-1, C-4, C-6 and C-9, because these signals are sharpened by irradiation of the doublet ⑥, ① and ④ ^{1}H peaks, respectively. In addition, either peak 8 or one peaks 11 and 12 (separable at lower concentration) could be ascribed to the other carbon mentioned above. The peak marked C_α is straightforwardly assigned to the olefinic C_α carbon (C-5). The assignment of the side-chain moiety was also performed by this method.

The Amount of Drugs in the Lipid Phase——The amount of drugs in the lipid phase was determined at the concentration used for the present NMR experiments, although the values are known to vary considerably with the concentration of samples and pH.[15,40] The pH values used in this study were usually 4.0—4.2, unless otherwise specified. These data as well as the apparent K_p values are summarized in Table I. It appears that the amounts of IMIP and AM in the lipid phase are considerably smaller than the amount of CPZ, although there remain several difficulties in assessing the absolute values.[40]

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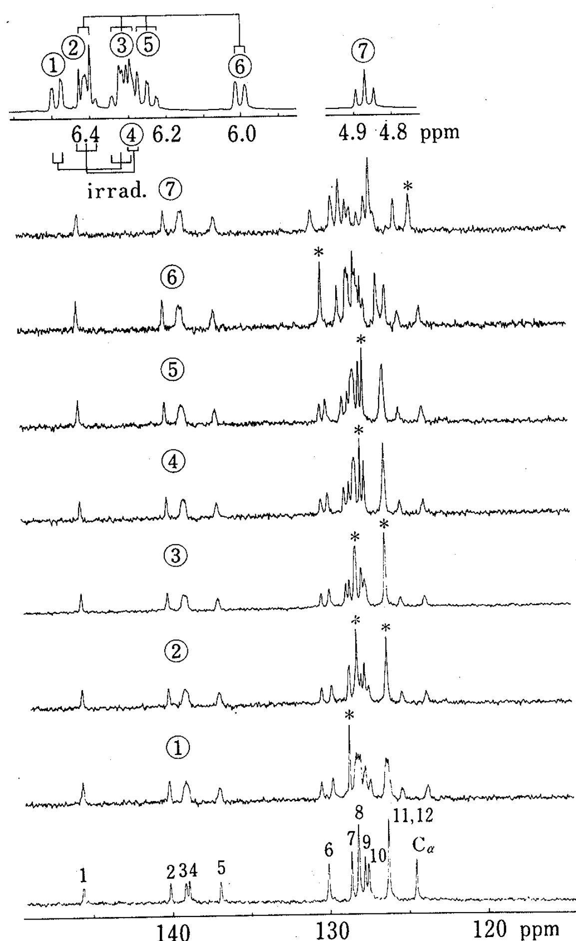

Fig. 4. ^{1}H- and ^{13}C-NMR Spectra with Selective Region of AM in Aqueous Solution (33.3 mg/ml)

Top trace, 300 MHz ^{1}H-NMR spectrum. Bottom trace, ^{13}C-NMR spectrum obtained by proton noise-decoupling.

Table I. The Amounts of IMIP, AM and CPZ Bound to the Lipid Phase[a]

Lipid bilayer		Lipid phase (%)	Apparent partition coefficient $(K_p)^{b)}$
IMIP	Multibilayer	90	10.6
	Vesicle	69	4.3
AM	Multibilayer	84	4.0
	Vesicle	50	3.2
CPZ	Multibilayer	75	3.6
	Vesicle	49	3.0

a) Drugs/PC 2:5; pH 4.0. IMIP, 0.0473 mmol; PC, 0.119 mmol. AM, 0.0478 mmol; PC, 0.120 mmol. CPZ, 0.0563 mmol; PC, 0.141 mmol. *b*) 5% accuracy, calculated based on ref. 15.

Study of Drug–Lipid Interaction by ^{13}C-NMR Spectroscopy

In the present ^{13}C-NMR approach, we used sonicated single-walled vesicles. For this purpose, sonication was continued until milky multibilayer samples changed to translucent single bilayer vesicles (*ca.* 15 min).

IMIP——Figure 5 shows the ^{13}C-NMR spectra of IMIP bound to egg PC vesicles together with those of IMIP and egg PC alone. The spectral features clearly vary with the ratio of IMIP to egg PC, but no displacement of the ^{13}C chemical shifts of IMIP was noted. The spectrum of IMIP/PC 4:1 shows well-resolved ^{13}C signals of both IMIP and egg PC, probably because mixed micelles (clear solution) are formed (Fig. 5D). This view is justified by

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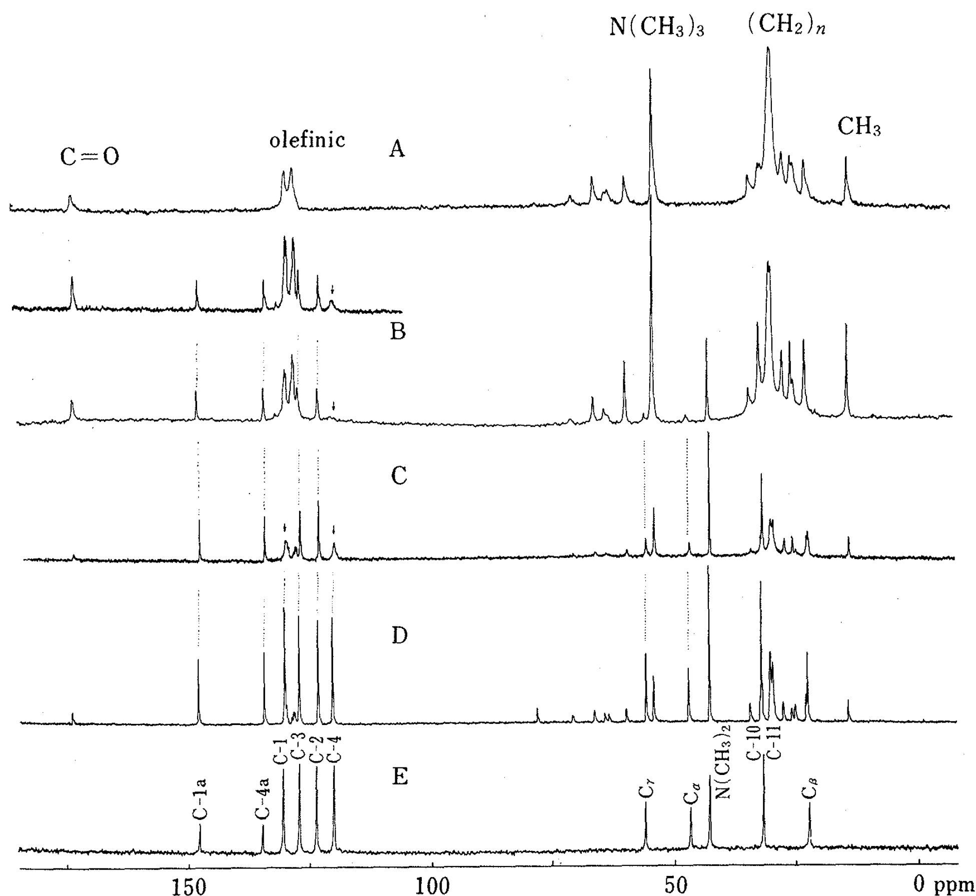

Fig. 5. The 75.46 MHz ^{13}C-NMR Spectra of IMIP in the Presence of Vesicles of Egg PC

A, egg PC vesicle alone. B, IMIP/PC 2:5 (IMIP, 0.0473 mmol; PC, 0.119 mmol). Inset: sonicated for 30 min. C, IMIP/PC 2:1 (IMIP, 0.0473 mmol; PC, 0.0237 mmol). D, IMIP/PC 4:1 (IMIP 0.0967 mmol; PC, 0.0237 mmol). E, IMIP, 0.0967 mmol.

Table II. ^{13}C Spin-Lattice Relaxation Times (T_1's; s), Nuclear Overhauser Enhancements (NOE's), and Linewidths ($\Delta\nu$; Hz) of IMIP in Micelles or Bilayers of Egg PC

		Aromatic						C-10, 11	Side-chain			
		C-1	C-2	C-3	C-4	C-1a	C-4a		C_α	C_β	C_γ	$N(CH_3)_2$
Aqueous soln.												
16.7 mg/ml	T_1	0.65	0.66	0.48	0.69	2.24	1.67	0.69	0.45	0.48	0.71	1.12
	NOE	2.7	3.1	2.8	2.6	1.9	1.6	3.0	2.8	2.4	2.8	3.0
33.3 mg/ml	T_1	0.33	0.25	0.29	0.32	1.79	1.34	0.38	0.13	0.28	0.40	0.82
IMIP/PC 4:1	T_1	0.24	0.22	0.23	0.25	1.37	1.29	—	0.17	0.26	0.28	0.77
	NOE	2.0	1.7	2.0	1.7	1.2	1.1	—	1.8	2.3	1.9	2.3
	$\Delta\nu$	13	13	13	13	7.4	9.2	—	17	—	7.4	7.4
2:1	T_1	0.36[a]	0.30	0.34	0.31	1.12	1.05	—	0.18	—	0.34	0.69
	NOE	—	1.8	2.4	1.7	1.2	1.1	—	1.6	—	2.0	2.1
	$\Delta\nu$	48	11	16	44	5.6	6.5	—	26	—	29	7.4
2:5	T_1	—	0.17	0.32[a]	0.16	1.41	0.97	—	0.18	—	0.36	0.61
	NOE	—	1.8	1.6	1.5	1.3	1.4	—	1.6	—	—	2.0
	$\Delta\nu$	—	17	28	75	18	26	—	50	—	32	13
	$\Delta\nu$[b]	—	17	36	200	11	14	—	50	—	22	11

a) Partially overlapped with signals of egg PC. *b*) Sample after prolonged sonication.

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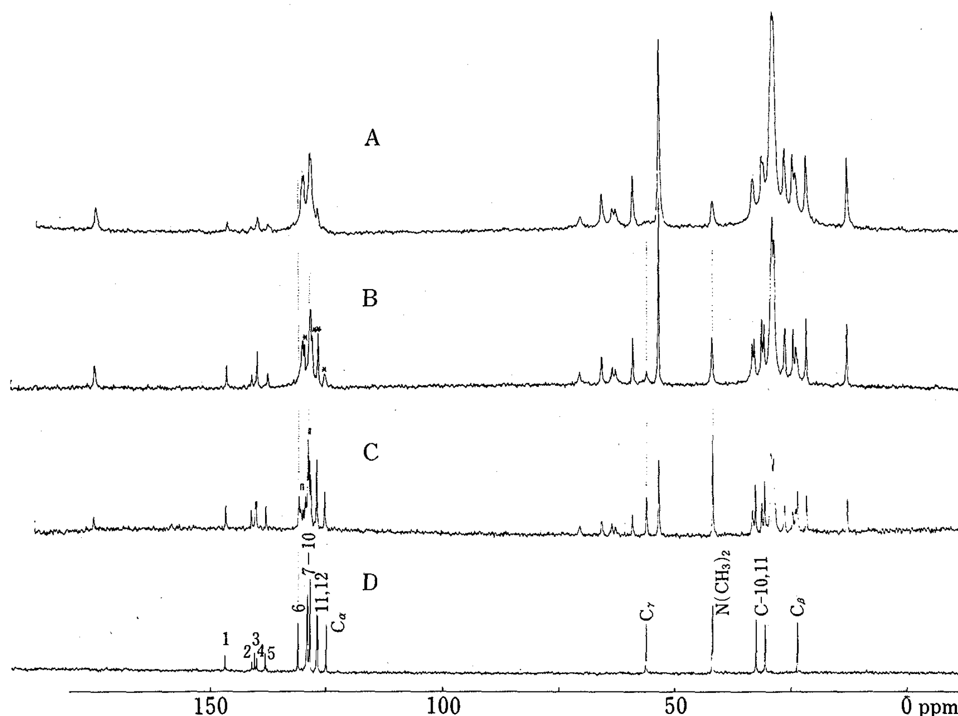

Fig. 6. The 75.46 MHz ^{13}C-NMR Spectra of AM in the Presence of Vesicles of Egg PC

A, AM/PC 2 : 5 (AM, 0.0478 mmol; PC, 0.120 mmol). B, AM/PC 4 : 5 (AM, 0.0553 mmol; PC, 0.0692 mmol). C, AM/PC 2 : 1 (AM, 0.0478 mmol; PC, 0.0239 mmol). D, AM, 0.0478 mmol.

the comparison of the spin-lattice relaxation times (T_1's) with those of IMIP in aqueous solution containing a similar amount of IMIP (Table II). Interestingly, we found that the C-1 and C-4 signals of the aromatic moiety of IMIP are considerably broadened (three- to four-fold broadening as compared with the C-2 and C-3 signals, see Table II) in the case of IMIP/PC 2 : 1 (Fig. 5C). This situation becomes more striking for IMIP/PC 2 : 5 (Fig. 5B): the C-1 and C-4 signals are further broadened to 200 Hz. The inset of Fig. 5B shows the result obtained with another preparation (sonicated for 30 min); the remaining spectral features were the same as in Fig. 5B. It is also noteworthy that the C_α and C_γ signals of Fig. 5B and 5C are broadened as compared with the $N(CH_3)_2$ signal, although the C_β and C-10, 11 signals are superimposed on peaks arising from egg PC. In particular, the extent of the line-broadening of the C_α and C_γ signals is significant for samples of higher PC content, although the $N(CH_3)_2$ signal is not influenced by lipid composition. Closer examination of the linewidths (summarized in Table II) shows that there appears to be a gradient in the linewidths of the side-chain: $C_\alpha > C_\gamma > N(CH_3)_2$. In spite of such drastic changes of the linewidths of aromatic carbons, no significant differences in the T_1 and NOE values were noted among the C-1, C-2, C-3 and C-4 signals.

AM——Figure 6 summarizes the ^{13}C-NMR spectra of AM in the presence of egg PC, in order to clarify whether or not similar findings are observed for another clinically used TCA, although the complete assignment of the ^{13}C chemical shifts is not established as yet. In contrast to the case of IMIP, the aromatic ^{13}C chemical shifts of AM exhibit slight but distinct displacements of chemical shifts on changing the ratio of AM to PC. In fact, peaks 2, 3 and 5 of the aromatic carbons not attached to protons in AM/PC 2 : 5 are shifted by -0.5, $+0.5$ and $+0.4$ ppm, respectively, as compared with those of the aqueous solution (16.7 mg/ml) as shown in the bottom trace. These displacements are large compared with the usual

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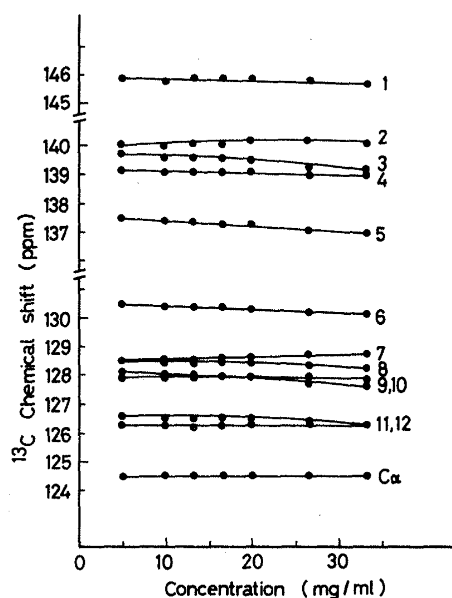

Fig. 7. A Plot of ^{13}C Chemical Shifts of AM against AM Concentration in Aqueous Solution

TABLE III. Variation of the ^{13}C-NMR Linewidths (Hz)[a] of AM and CPZ in the Presence of Egg PC

		Quaternary carbon of aromatic moiety	Side-chain			
			C_α	C_β	C_γ	$N(CH_3)_2$
AM/PC	2:1	7[b]	12	—	15	12
	4:5	7	52	—	34	26
	2:5	13	130	—	100	54
CPZ/PC	5:7	35[c]	96	50	14	9
	4:5	40	100	—	28	13
	3:4	37	120	—	32	15
	2:5	36	—	—	30	20

a) Estimated error ±15%. *b*) Peak 1. *c*) Peak C-10b.

displacement of ^{13}C chemical shifts. Such displacement of the ^{13}C chemical shifts could be explained by assuming that the environment of aromatic moieties of AM varies from the aqueous phase to the hydrophobic lipid phase within the lipid bilayers, on increasing the proportion of egg PC. This view is substantiated by the finding that the trend of displacement of the chemical shifts is well reproduced by changing the concentration of AM in aqueous solution, as illustrated in Fig. 7. More hydrophobic environment could be produced by increasing the concentration of AM, provided that the critical micelle concentration lies in this concentration range.

In the case of AM/PC 2:1, the ^{13}C-NMR signals of both the drug and PC are well-resolved because of micelle formation (clear solution) as in the case of IMIP/PC 4:1 (Fig. 5D). The peak intensities of several aromatic carbons attached to protons are diminished or suppressed when the relative proportion of PC is raised (Fig. 6A and 6B). In particular, at least four asterisked peaks (8, 10, 11/12, and C_α) are visible at AM/PC 4:5 and only peak 11/12 can be seen at AM/PC 2:5, peaks in other regions are considerably broadened. On the basis of the selective proton decouplings summarized in Fig. 4, the visible three peaks (8, 10 and 11/12) can be ascribed to the C-2, C-3, C-7 and C-8 and invisible peaks (at least 6) to the group of C-1, C-4, C-6 and C-9, consistent with the observations in the IMIP/PC system

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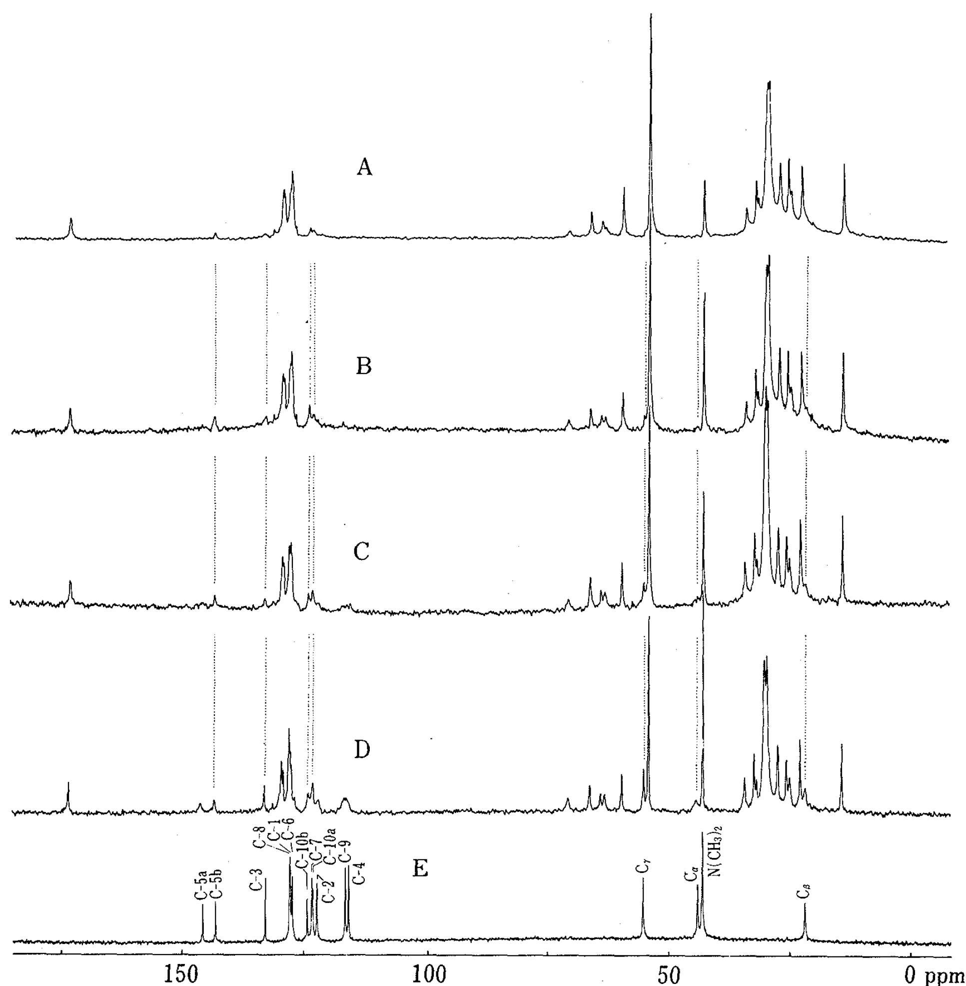

Fig. 8. The 75.46 MHz ^{13}C-NMR Spectra of CPZ in the Presence of Vesicles of Egg PC

A, CPZ/PC 2:5 (CPZ, 0.0563 mmol; PC, 0.141 mmol). B, CPZ/PC 3:4 (CPZ, 0.0422 mmol; PC, 0.0563 mmol). C, CPZ/PC 4:5 (CPZ, 0.0563 mmol; PC 0.0703 mmol). D, CPZ/PC 5:7 (CPZ, 0.0985 mmol; PC, 0.138 mmol). E, CPZ, 0.0985 mmol.

described above. It is also interesting to note that the linewidths of the side-chain carbons are considerably broadened (up to *ca.* 100 Hz) for the sample of AM/PC 2:5 (Table III).

CPZ——In the same manner as for IMIP and AM, Fig. 8 shows that the linewidths of aromatic carbons attached to protons of CPZ are significantly broadened as the proportion of PC is increased, in accordance with the previous data by Kitamura *et al.*[27] The notable feature of this system, however, is that no differential line-broadening is observed.

^{2}H-NMR Spectra of [2,4,6,8-$^2H_4$]IMIP Bound to Multibilayers of Egg PC——Figure 9 shows the ^{2}H-NMR spectra of [2,4,6,8-$^2H_4$]IMIP bound to multibilayers of egg PC at pH 4.0 and 9.5. The observed spectra consist of the two kinds of peaks, the intense central signal (*ca.* 80%, linewidths 2.1 and 1.6 kHz for the spectra at pH 4.0 and 9.5, respectively) and the quadrupole doublet (*ca.* 16 kHz). The central isotropic signal is usually ascribed to the presence of the free drug in aqueous solution or micelles. However, it is unlikely that this central peak arises solely from the drug in the free state in view of the considerably broadened linewidths (over 1500 Hz). The presence of such a considerably broadened central peak can be interpreted in terms of fast chemical exchange between IMIP loosely bound to lipids and IMIP in the aqueous phase with a life time (t) shorter than the inverse of the quadrupole splitting (D_q),[41]

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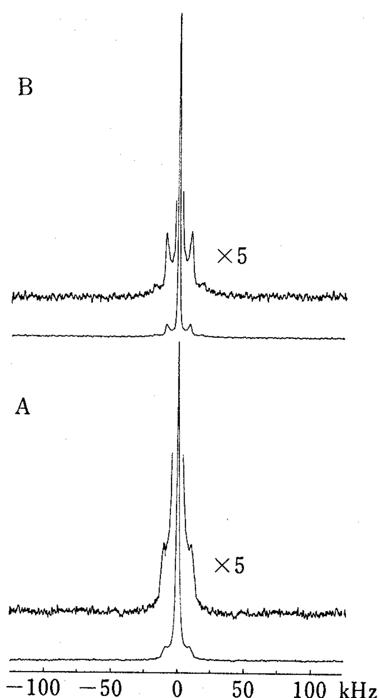

Fig. 9. The 46.06 MHz ^{2}H-NMR Spectra of [2,4,6,8-$^2H_4$]IMIP in the Presence of Multibilayers of Egg PC

A, pH 4.0. B, pH 9.5. Line-broadening of 300 Hz was used.

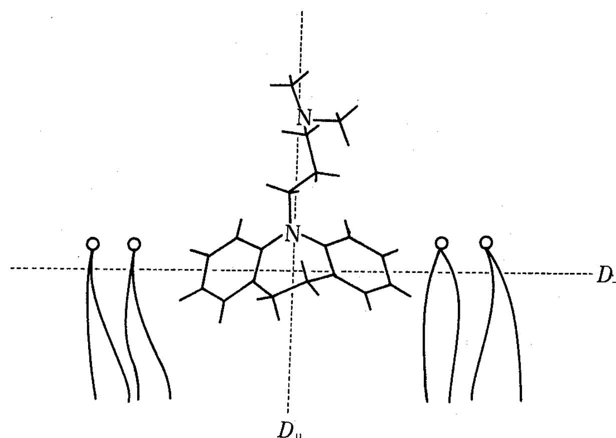

Fig. 10. Incorporation of IMIP into Bilayers of Egg PC on the Basis of the ^{2}H- and ^{13}C-NMR Data

$$t \ll 1/D_q \tag{1}$$

In this case, the ^{2}H-NMR signal is expressed by

$$\nu_q = bD_q + (1-b)\nu_{1/2} \tag{2}$$

where b stands for the fraction giving the quadrupole splitting and $\nu_{1/2}$ is the linewidth of [2,4,6,8-$^2H_4$]IMIP in the aqueous (or micelle) phase undergoing isotropic reorientation. The quadrupole splitting, however, should be caused by the presence of IMIP which is in slower exchange with the species described above as compared with the inverse of the quadrupole splitting. It appears that the intensity of the doublet peak is not strongly influenced by pH, in contrast to the cases of local anesthetics,[15] although we observed sharpening of the central and doublet peaks owing to the slowed exchanged rate.

Theoretically, the ^{2}H quadrupole splitting D_q is related to the angle of the principal axis of the electric field gradient tensor (C–^{2}H vector) with respect to the applied magnetic field.[42,43]

$$D_q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)(3\cos^2\theta - 1) \tag{3}$$

where e^2qQ/h is the quadrupole coupling constant (180 kHz for sp^2-hybridized C–^{2}H[43]). For rigid polycrystalline solids, all values of θ are possible and we obtain a so-called "powder pattern" having a singularity separation (135 kHz for $\theta = 90°$) and an edge separation (270 kHz for $\theta = 0°$). Clearly, the quadrupole splitting in Fig. 9 is too small to correspond to static orientation of IMIP in the multibilayers, suggesting the presence of fast rotational diffusion ($\gg 10^5\,s^{-1}$) about the axis connecting the midpoint of the two methylene carbons of the seven-membered ring and the nitrogen atom. Other possible motions such as a 2-fold flip

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can be ruled out because of the appearance of the quadrupole splitting with symmetric electric field tensor.[42,44] In the presence of fast rotational diffusion, the quadrupole splitting is given by:

$$D_q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)\left(\frac{3\cos^2\alpha - 1}{2}\right)\left(\frac{3\cos^2\beta - 1}{2}\right) \tag{4}$$

where α and β stand for the angle of the C–^{2}H vector under consideration with respect to the director (the axis of fast rotational diffusion) and the angle between the director and the normal of the bilayers, respectively. There exist two distinct conformers (A and B) of IMIP hydrochloride in the crystalline state:[45] the dihedral angle between the benzene planes is 130 and 123° for molecules A and B, respectively, while the angles between the benzene rings and the N-C(methylene)-C(methylene) plane of the seven-membered ring are 18 and 116° (for A) and 125° (for B); in the latter case, the ring nitrogen atom is pyramidal, lying 0.19 Å (for A) or 0.29 Å (for B) from the plane formed by the three carbon atoms to which it is bonded. It can be roughly estimated from the data described above that C–^{2}H (H-2 and H-8) and C–^{2}H (H-4 and H-6) make angles of 60° (*ca.* ± 10°) and 0° (*ca.* ± 10°) with the director, which is assumed to be the line connecting the midpoint of the two methylene carbons of the seven-membered ring and the nitrogen atom. Thus, the expected quadrupole splitting is estimated as 17 and 135 kHz from Eq. 2 for H-2(8) and H-4(6) deuteriums, respectively, if the director is in parallel with the normal of the bilayer ($\beta \approx 0$). The observation of the quadrupole splitting of 16 kHz is consistent with this expectation, although the peak-height of the wider doublet (135 kHz) becomes very low as compared with that of the narrow doublet (16 kHz) and complete excitation of this region is rather difficult because of the rather long 90° pulse (7 μs) used.

Nevertheless, this experiment shows that IMIP exists at least in the following three different environments: (1) deeply buried within the bilayer, (2) bound to lipids but in fast exchange with the drug in the aqueous (or micelle) phase, and (3) in the aqueous (or micelle) phase. However, it should be emphasized that IMIP in site (2) should still be in the bilayer in such a manner that anisotropic fast rotational diffusion can occur to afford differential line-broadening of the ^{13}C-NMR signals (Fig. 10).

Discussion

The most noticeable change of the ^{13}C-NMR signals of the drugs studied in this paper is that the ^{13}C signals of aromatic carbons attached to protons as well as carbons at the side-chain moiety are broadened significantly when the drugs are bound to vesicles. A gradual change of the linewidths from the C_α to C_γ in the side-chain appears, reflecting a gradient in mobility. Naturally, the $N(CH_3)_2$ group gives rise to a sharp signal because of the motional freedom about the C_γ–$N(CH_3)_2$ bond. Rotation about N–$C_\alpha H_2$ of CPZ is strongly restricted because the C_α linewidth is much more broadened than that of AM ($C{=}C_\alpha H$), in which no rotation is allowed. This result can be explained by considering that the aromatic as well as N–$C_\alpha H_2$ moiety of CPZ is incorporated within the bilayers. Further, only a small amount of the drug remains in the aqueous phase, as can be seen from the measurement of the amount of CPZ in the lipid phase (Table I). As judged from the extent of the line-broadening of both the aromatic moiety and side-chain moiety, CPZ is most strongly bound to lipids, while IMIP is most loosely bound. In this connection, Romer and Bickel previously showed on the basis of equilibrium dialysis and fluorometry studies that CPZ binds to the surface of the membrane and also penetrates into the inner hydrocarbon phase of the bilayers, whereas IMIP only binds near the surface region of the liposomes.[11] In fact, the ^{13}C signals of carbons attached to protons of CPZ are completely suppressed at the CPZ/PC ratio of 4 : 5, although the ^{13}C signals of carbons not attached to protons are visible except for that of C-10a. This situation

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could be simply visualized as follows. The correlation time of molecular reorientation of CPZ immobilized in the bilayers could be of the order of 10^{-7}—10^{-6} s either from the tumbling rate of vesicles as a whole[46] or on the basis of the linewidths (100—1000 Hz) from the isotropic tumbling model.[47] The effect of the dipolar field on the linewidths from neighboring protons for the carbons not attached to protons is reduced to at least 1/40 with respect to that of carbons attached to protons, taking into account the differences in the C–H distances.[48] Thus, the linewidth of the carbons not attached to protons is about 13 Hz, even if the carbons attached to protons have linewidths as large as 500 Hz.

In the cases of IMIP and AM, however, we noticed that the ^{13}C signals of these drugs are still visible under the same conditions as used for CPZ but some signals from specific positions are significantly broadened. Those are the C-1, C-4, C-6 and C-9 positions adjacent to the seven-membered ring. No such differential broadening, however, occurs for the TCA's incorporated into micelles of higher TCA/PC ratio, because the tumbling rate of micelles as a whole is much less than that of vesicles owing to the smaller "size" in the former. It should be pointed out that similar differential line-broadening could be noted, even in isotropic media, for a number of ^{2}H-NMR linewidths of specifically deuterated aromatic molecules, if molecular motions are anisotropic to some extent in solution.[43] A similar situation was also suggested from observations of differential T_1 values.[49] However, no such significant change was observed in the T_1 and NOE values among carbons whose linewidths were significantly changed (see Table II). To resolve this apparent contradiction, it should be taken into account that the T_1 and NOE values are mainly influenced by fast motion while the T_2 values are rather sensitive to slow motion, if several kinds of motions are present for the molecules within bilayers, as suggested by the results that $T_1 \gg T_2$. In fact, the dynamic process of lipid bilayers alone is very complicated. Recently, Brown showed on the basis of the ^{2}H T_1 data for dipalmitoylphosphatidylcholine (DPPC) bilayers that the relaxation rate reflects fast local segmental motions (0.5—3×10^{-10} s) and collective slow motions which are characterized by a distribution of correlation times.[50] To make the situation more complicated, the observed T_1 and T_2 ($1/\pi$(linewidth)) of the drugs should be considered as averages between two rapidly exchanging systems, lipid and aqueous (micelle) phases,

$$\frac{1}{T_{1,2}} = b\left(\frac{1}{T_{1,2}}\right)_b + (1-b)\left(\frac{1}{T_{1,2}}\right)_f \tag{4}$$

where $T_{1,2}$ denotes either T_1 or T_2 and b is the fraction of the drug bound to lipids. Subscripts b and f denote the bound and free state, respectively. The presence of fast exchange in this case is inferred from the absence of two components in the ^{13}C-NMR signals due to drug molecules bound to or free from lipids. This view is also confirmed by the observation of a larger proportion of the central peak in the ^{2}H-NMR spectra of [2,4,6,8-$^{2}H_4$]IMIP in the presence of lipids (Fig. 10). Undoubtedly, the b value varies with changes of the drug-to-lipid ratio. Nevertheless, there appears to be very little change in the T_1 values of IMIP when the ratio of IMIP to lipid is varied (Table II), although the T_1 values in these cases are significantly shortened as compared with those in aqueous solution (16.7 mg/ml). This situation probably arises because of a significant contribution from micelles to make the $(T_1)_f$ shorter than that of isotropic solution, as seen from the shortened T_1 values of micelles with IMIP/PC 4:1.

Clearly, the differential line-broadening arises from the contribution of only the first term in Eq. 4, because the second term is related to the isotropic tumbling for aqueous or micelle solution. Thus, the differential line-broadening of the drugs bound to lipids can be treated in terms of the anisotropic rotational diffusion model as depicted in Fig. 10. The T_2 relaxation rate ($1/T_2$) is given by[51]

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$$\frac{1}{T_2}=h^2\gamma_H^2\gamma_C^2\sum r_{ij}^{-6}\chi\tau_{eff}$$

$$\chi=\frac{1}{4}(3\cos^2\theta-1)+18(5+\rho)^{-1}\sin^2\theta\cos^2\theta+\frac{9}{4}(1+2\rho)^{-1}\sin^4\theta \quad (5)$$

$$\rho=D_{\parallel}/D_{\perp}$$

where θ is the angle between the C–H vector and the axis of major diffusion (director vector), r_{ij} is the distance between carbon i and proton j, γ_H and γ_C is the gyromagnetic ratio for ^{1}H and ^{13}C, respectively, and τ_{eff} is the effective correlation time. $D_{\parallel}$ ($=(6\tau_{eff})^{-1}$) and $D_{\perp}$ are diffusion constants parallel and perpendicular to the major diffusion axis, respectively. It appears that the presence of fast motion prevents the observation of the differential T_1 values, although a similar formula can be applied to the T_1 relaxation rate. The manner of incorporation of IMIP, as well as AM, into lipid bilayers may be depicted as shown in Fig. 10, on the basis of the ^{13}C- and ^{2}H-NMR data. In such a case, the rotational diffusion coefficient about the axis of the bilayer normal ($D_{\parallel}$; $\sim 10^{10}\,s^{-1}$ as estimated from the fast local segmental motion of lipids[50]) becomes much larger than that perpendicular to the normal ($D_{\perp}$; $\sim 10^6\,s^{-1}$ as estimated from the overall tumbling rate of vesicles[46]). In the limit of $D_{\parallel}>D_{\perp}$, so that $\rho \gg 1$,[51]

$$\chi=\left(\frac{3}{2}\cos^2\theta-\frac{1}{2}\right)^2$$

It is clear from the argument in the previous section that this angular dependence makes the linewidths of the C-1, C-4, C-6 and C-9 signals eight-fold larger than those of the others, which is consistent with the experimental data. It has also been shown that the T_1 and T_2 relaxation rates are strongly dependent on the order parameters,[46,52] but it seems difficult to explain the present experimental data in terms of this approach, because the angular dependence seems to be less significant.

In conclusion, we emphasize that the present ^{13}C-NMR approach as well as the ^{2}H-NMR method affords detailed information as to the binding of IMIP and AM to bilayers of egg PC.

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