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Locations and dynamical perturbations for lipids of cationic forms of procaine, tetracaine, and dibucaine in small unilamellar phosphatidylcholine vesicles as studied by nuclear Overhauser effects in ^{1}H nuclear magnetic resonance spectroscopy

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Locations and dynamical perturbations for lipids of local anesthetics (procaine · HCl, tetracaine · HCl, and dibucaine · HCl) in sonicated egg yolk phosphatidylcholine (PC) vesicles have been studied by ^{1}H–^{1}H nuclear Overhauser effect (NOE) measurements. It was found that tetracaine and dibucaine bind much strongly to the neutral lipids than does procaine and that their mobilities are lowered to such an extent that spin diffusion is transmitted (i.e., $\omega^2\tau_c^2 \gg 1$). The intermolecular NOEs between drugs and PC were more effective in the case of dibucaine than with tetracaine, indicating that dibucaine binds to the lipids more strongly than tetracaine; this order agrees well with that of anesthetic potency. However, it was only tetracaine that gave any appreciable dynamical perturbation to the PC vesicles when they were monitored by the extent of transfer of the negative NOE from α-methylene protons to choline methyls, olefinic methines, acyl methylenes and terminal methyl protons. This finding was interpreted as being due to the differences in the locations of these drugs in small unilamellar vesicles: (1) procaine interacts with lipids very weakly at the outer surface of the vesicles; (2) tetracaine binds to the lipids both at the outer and inner halves of the bilayer, inserting its rod-like molecule in a forest of acyl chains of PC; (3) dibucaine binds tightly to the polar head-group of PC, which resides only at the outer half of the bilayer vesicles. It was concluded that the relative order of anesthetic potency within these drugs can be correlated not with the ability to affect membrane fluidity but with the ability to bind to lipids at the polar head-group of the bilayer vesicles.

Introduction

Although there have been some controversial proposals for the mechanism of action of local anesthetics, interactions of the anesthetics with membrane lipids appear to be a most important step in bringing about anesthesia [1–3]. Thus, studies on the interactions have been made extensively from spectroscopic, physico- and biochemical points of view [3–7]. Nuclear magnetic resonance spectroscopy is one of the most powerful methods in the field of the spectroscopic approach in studying this interaction. For example, recent investigations by ^{2}H- [8–12], ^{31}P- [9,11,13], ^{13}C- [13] and ^{14}N- [14] NMR and previous pioneering works by ^{1}H-NMR [15–17] on the interactions of local anesthetics with lipid multilamellar dispersions or with sonicated small bilayer vesicles have

Abbreviations: NOE, nuclear Overhauser effect; PC, phosphatidylcholine.

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revealed the importance of both electrostatic and hydrophobic interactions to explain the mechanism of action of local anesthetics and the relative order of anesthetic efficiency.

^1H–^1H nuclear Overhauser effect measurements in ^1H-NMR spectroscopy are known to be a powerful technique to obtain information on the distance between the protons which can interact by dipole–dipole mechanisms [18]. In our recent studies on the interactions of chlorpromazine with PC vesicles, we showed that this NOE appears as a negative NOE between protons which belong within chlorpromazine and PC (i.e., intramolecular) or between protons of chlorpromazine and PC (i.e., intermolecular), indicating that chlorpromazine incorporated into the bilayer of PC vesicles moves with the same time-scale as that of the vesicles [19]. More interestingly, this negative NOE was observed not only between the nearby protons but also between remote protons; that is, there exists spin diffusion in the vesicles of the drug-lipid system. Since the efficiency of this spin diffusion depends not only on the interproton distances but also on the mobilities of the protons through which the spin energy is transmitted [20,21], measurements of NOEs for vesicles of a drug-lipid system can be a good probe to obtain information on the site of molecular contact of the drug with lipids or on the effect of the interactions upon the dynamics of lipid molecules. The present work has been undertaken to inquire into the location of a drug and a possible perturbation for lipids induced as a result of the interaction between the drug and PC molecules in bilayer vesicles. Local anesthetics chosen in the present study were hydrochlorides of procaine, tetracaine and dibucaine, since the anesthetic potency of these drugs is known to increase in this order [3,5].

Materials and Methods

Materials

Egg yolk L-α-phosphatidylcholine (egg PC) and all the anesthetics, i.e., procaine hydrochloride, tetracaine hydrochloride, and dibucaine hydrochloride (Fig. 1) were purchased from Sigma and used without further purification.

Preparation of sample solution

Single bilayer vesicles were prepared by ultrasonic irradiation of a ^2H_2O suspension of dried egg PC for 20–30 min, cooling in an ice/water bath and bubbling with nitrogen gas. A weighed amount of a drug, dissolved or not dissolved in ^2H_2O, was added to the solution of pre-formed vesicles (40–50 mM). No buffer was used, to avoid interactions between the buffer and drug molecules and also to avoid competitive interactions with lipids. The resulting actual pH values (meter reading) were lower than 5.5 for those which include the drugs and about 6.0 for the PC vesicles solution which contains no drug. No de-

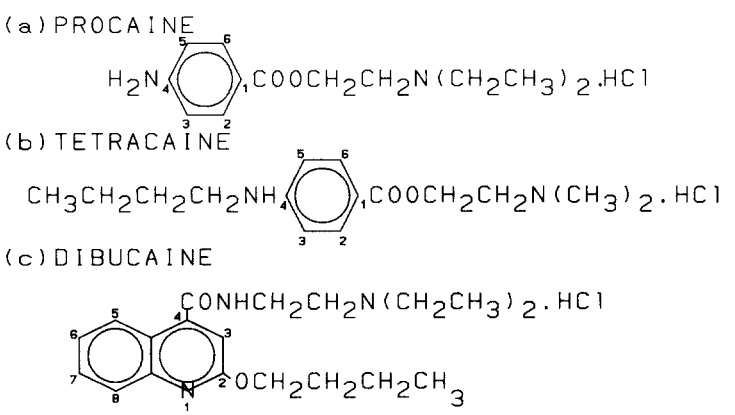

Fig. 1. Structures of the hydrochlorides of (a) procaine, (b) tetracaine, and (c) dibucaine and numbering schemes in their aromatic rings.

oxygenation was made for these sample solutions before NOE measurements. The pH value of 5.5 is low enough to secure the cationic forms of the drugs employed even for their membrane-associated forms, for which the pK values of the tertiary amino group of the drugs (i.e., procaine, 9; tetracaine, 8.5, dibucaine, 8.5) [22] are shown to decrease by approx. 1 pK unit [23] from the corresponding values in the aqueous phase, i.e., 7.36 in procaine [10], 7.23 [10] or 7.05 [24] in tetracaine, and 7.5 in dibucaine [25]. Partition coefficients (K_p) reported so far for the drug–egg PC systems in their acidic states are 2 for procaine (pH 5.5) [8], 22–25.7 for tetracaine (pH 5.5) [8,24] and 29 for dibucaine (pH 6.0, for a charged form of dibucaine at the lipid concentration of 40 mM) [26]. Thus, if we assume that a K_p value in multilamellar dispersions of egg PC is the same as that in small unilamellar vesicles, the amounts of the drugs bound relative to those added to the 40 mM vesicles solution can be estimated to be about 6% in procaine, 40–44% in tetracaine, and 47% in dibucaine. Each of these values can be considered to be an upper limit for the percentage of the bound drugs, because the K_p value for a charged anesthetic is known to decrease with an increase in the concentration at which it is determined [27–29]; the drug concentrations employed in the present experiments are, however, higher than the corresponding ones employed to determine each K_p value.

Measurements

The ^{1}H-NMR experiments were carried out on a JEOL FX-200 (200 MHz), Varian XL-300 (300 MHz) or Bruker AM-400 (400 MHz) spectrometer. NOE difference spectra were obtained as described previously [19,30] by employing preirradiation times of 2.0 and 0.2 s; the former irradiation time gives steady-state NOE values, whereas the latter gives a measure of the build-up rate of an NOE value by comparing the corresponding steady-state NOE value. The pre-irradiation power ($\gamma H_2/2\pi$) was 8–9 Hz. Chemical shifts were referenced to the signal from the terminal methyl protons of acyl chains of egg PC. ^{31}P-NMR spectra were recorded on a Varian XL-300 (121.4 MHz) spectrometer. Chemical shifts were referenced to external 85% H_3PO_4 which contained in a coaxial capillary tube. All the NMR experiments were performed at room temperature (21–23°C).

Results

Normal ^{1}H-NMR spectra and peak assignments

Fig. 2 shows the ^{1}H-NMR spectra of (a) dibucaine in ^2H_2O (16 mM), (b) PC vesicles in ^2H_2O (47 mM), and (c) dibucaine-PC vesicles (1 : 3) in ^2H_2O. Observed chemical shifts for the peaks labeled A–N (Fig. 2c) in the drug-PC (1 : 3) system are summarized in Table I, together with the chemical shifts of the ^{1}H-NMR resonances labeled in a similar manner in the PC, procaine-PC and tetracaine-PC systems. The assignments of the PC protons followed mainly those of Hauser et al. [31]; those of drugs were determined from the expected chemical shifts and/or spin decoupling experiments for drugs in ^2H_2O. As shown in Fig. 2, no appreciable changes in linewidth were seen for signals due to PC on addition of a 1/3 molar ratio of the drug to the PC vesicle solution, implying that changes in the mobility of a PC molecule, if any, are small. However, the linewidths of signals due to the drug were changed, which indicates that the mobility of the drug was lowered as a result of the interactions with PC vesicles. The chemical shifts of ^{1}H-NMR signals in tetracaine-PC and dibucaine-PC systems were also changed from those of the corresponding resonances of PC and drugs, except for the signals due to acyl methylenes and terminal methyl protons of PC; however, no chemical shift changes were noted for signals from PC in the procaine-PC system (Table I). The most prominent changes in the chemical shifts were peak E (α-CH_2), followed by the other resonances from the polar head group (peaks H, I, and J), indicating that these drugs locate at these regions.

NOE difference spectra at 200 MHz

Fig. 3 shows NOE difference spectra for a dibucaine-PC (1 : 3) system, where resonances M (spectra a and a′) and E (spectra b and b′) were irradiated for 2.0 and 0.2 s. As shown in these spectra, irradiation on the resonances due to PC caused not only intramolecular NOEs for all the other protons of PC, but also intermolecular NOEs

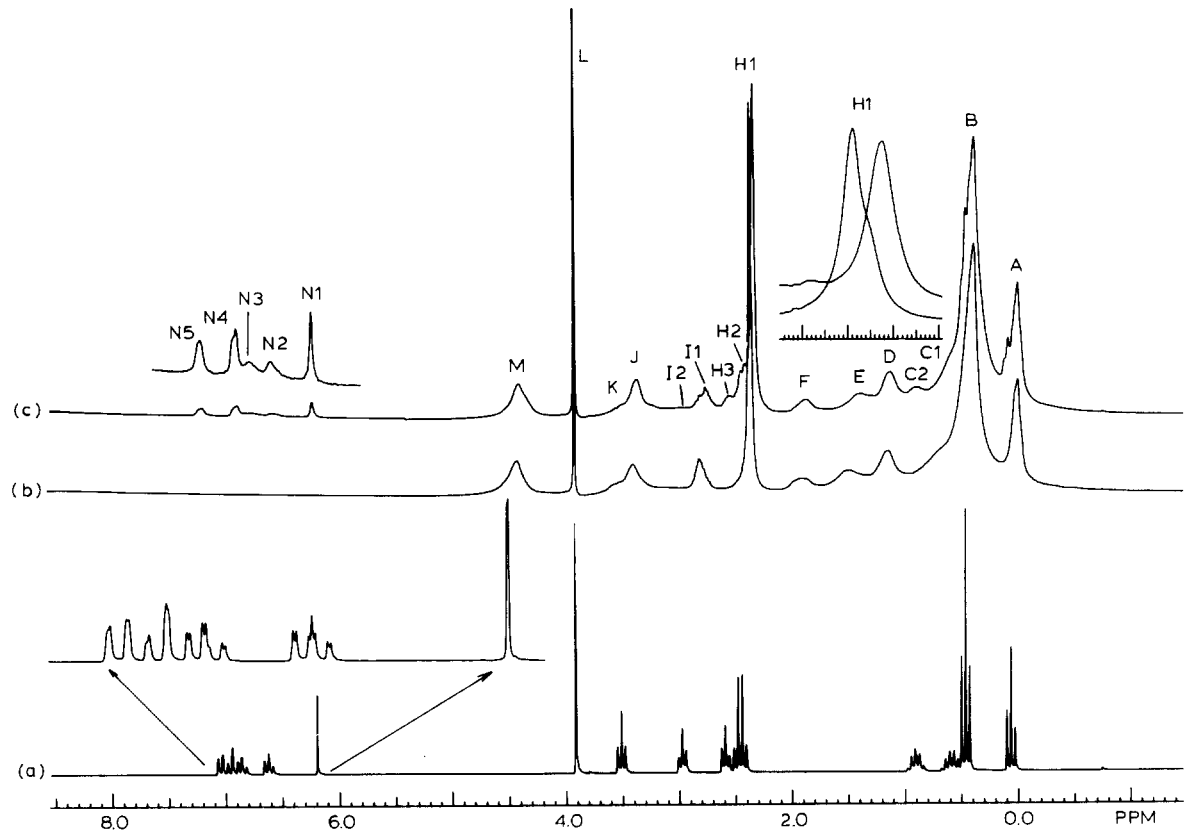

Fig. 2. (a) ^{1}H-NMR spectrum (200 MHz) of dibucaine hydrochloride in ^2H_2O (16 mM); (b) ^{1}H-NMR spectrum (200 MHz) of PC vesicles in ^2H_2O (47 mM); (c) ^{1}H-NMR spectrum (200 MHz) of dibucaine hydrochloride (16 mM)-PC vesicle (47 mM) solution. Insets for H1 (spectra b (lower) and c (upper)) are the 8-fold expanded spectra; one division is equal to 1 Hz.

for the protons arising from dibucaine (see peaks N1–N5), as in the case of a chlorpromazine-PC system [19]. These spin diffusion phenomena clearly indicate that dibucaine was incorporated into the bilayer of PC vesicles and has molecular contact with PC. The mobility of the dibucaine molecules was slowed down to an extent that satisfies the condition $\omega^2\tau_c^2 \gg 1$ (i.e., $\tau_c \gg 10^{-9}$ s at 200 MHz), where ω is the Larmor frequency of the proton and τ_c the molecular rotational correlation time [20,21]. A similar observation was noted for a tetracaine-PC system, whereas, in the case of a procaine-PC system, the intermolecular NOEs were much smaller. Observed intermolecular NOE values between protons of PC (peaks M and E) and aromatic ring protons of dibucaine, tetracaine and procaine (peaks N1–N5) are listed in Table II, where we show only steady-state NOE values. Table II informs us that the degree of interaction of the drugs with PC vesicles decreases in the order dibucaine > tetracaine ≫ procaine, since the intermolecular NOE values decreased in this order. The differences in the extent of broadening of the signals from the aromatic ring protons supported this result. The decreasing order agrees with that of the amounts of membrane-bound drugs which were described in Materials and Methods and also with the order of anesthetic potency.

In order to obtain information on the locations and conformations of these drugs, we further measured the NOEs on irradiating resonances due to aromatic ring protons. Their numberings are shown in Fig. 1. Fig. 4 shows the results for a tetracaine-PC system, where resonances N2 (spectra a and a′) and N1 (spectra b and b′) were irradiated for 2.0 and 0.2 s. Comparisons of the

TABLE I

CHEMICAL SHIFTS AND ASSIGNMENTS OF PEAKS A–N (FIG. 2) OF DRUG (12 mM) -PC (35 mM) SYSTEMS

PR, procaine; TE, tetracaine; DI, dibucaine.

Peak	Chemical shifts [a]				Assignment [b]			
	PC [c]	PR-PC [d]	TE-PC [e]	DI-PC [f]	PC	PR	TE	DI
A	0.00	0.00	0.00	0.00	CH_3		CH_3	CH_3
B	0.39	0.39	0.38	0.38	$(CH_2)_n$	$NH^+(C\text{-}CH_3)_2$	$CH_3CH_2^*$	$NH^+(C\text{-}CH_3)_2$
C(C1)	0.70	0.70	0.70	0.62	$\beta\text{-}CH_2$		$CH_2^*CH_2NH$	$CH_2^*CH_3$
C2				0.91				$OCH_2CH_2^*$
D	1.15	1.15	1.15	1.13	$CH_2C=C$			
E	1.51	1.52	1.48	1.43	$\alpha\text{-}CH_2$			
F	1.93	1.93	1.92	1.90	$=C\text{-}CH_2\text{-}C=$			
G			2.11				$NH^+(CH_3)_2$	
H(H1)	2.37	2.37	2.35	2.34	$N(CH_3)_3^+$		CH_2NH	
H2		2.46		2.45		$NH^+(CH_2\text{-}C)_2$		$NH^+(CH_2\text{-}C)_2$
H3				2.58				$CONHCH_2^*$
I(I1)	2.81	2.72	2.78	2.77	CH_2N^+	CH_2NH^+	CH_2NH^+	
I2		2.79		2.97				CH_2NH^+
J	3.41	3.41	3.39	3.38	O_3POCH_2			
K		3.74	3.72	3.53	CH_2OCO	OCH_2	OCH_2	OCH_2
L	3.91	3.91	3.90	3.90	H^2HO			
M	4.43	4.43	4.43	4.42	CH = CH,CHOCO			
N1		5.93	5.82	6.24		ϕ3,5	ϕ3,5	ϕ3
N2		6.97	6.99	6.61		ϕ2,6	ϕ2,6	ϕ6
N3				6.79				ϕ7
N4				6.92				ϕ8
N5				7.21				ϕ5

[a] In ppm, ±0.02 ppm.
[b] Ring protons are denoted as ϕi, j, where numberings i and j are shown in Fig. 1.
[c] pH 5.9, 40 mM.
[d] pH 5.2.
[e] pH 5.2.
[f] pH 4.8.

peak intensities in these NOE spectra show that (1) ϕ2,6 (peak N2) is nearer to OCH_2 and $NH^+(CH_3)_2$, whereas ϕ3,5 (peak N1) is nearer to CH_2NH (the high-field root of peak H) of tetracaine than is the corresponding counterpart, and that (2) ϕ3,5 is nearer to $N(CH_3)_3^+$ (peak H), O_3POCH_2 (peak J), and the $CH_2C=C$ (peak D) of PC than is ϕ2,6. Irradiation on the $N(CH_3)_3^+$ resonance also confirmed that the $N(CH_3)_3^+$ is closer to the ϕ3,5 than is to the ϕ2,6.

Moreover, since the intensity of peak B in spectrum b is fairly strong as compared with that of peak C ($CH_2^*CH_2NH$) in the same spectrum, the peak B can be considered to be due mainly to the $(CH_2)_n$ of PC. The peak A is also considered to be due mainly to the CH_3 of PC by the same reasoning. Thus, it is evident that there exists intermolecular spin diffusion from ϕ3,5 to acyl methylenes of PC, presumably through more effective intramolecular spin diffusion within the methylene protons of the butylamino group of tetracaine.

In order for these observations to be satisfied consistently, we had to consider two kinds of molecular structure of the tetracaine in lipid bilayers, as shown in Fig. 5. One is a somewhat bent structure (Fig. 5a) whose polar part lies parallel to the bilayer surface. This structure satisfies the requirement that ϕ3,5 be close to the $N(CH_3)_3^+$ and O_3POCH_2. The other is a rod-like structure lying parallel to the bilayer normal (Fig. 5b) and satisfies the requirement that the ϕ3,5 be also

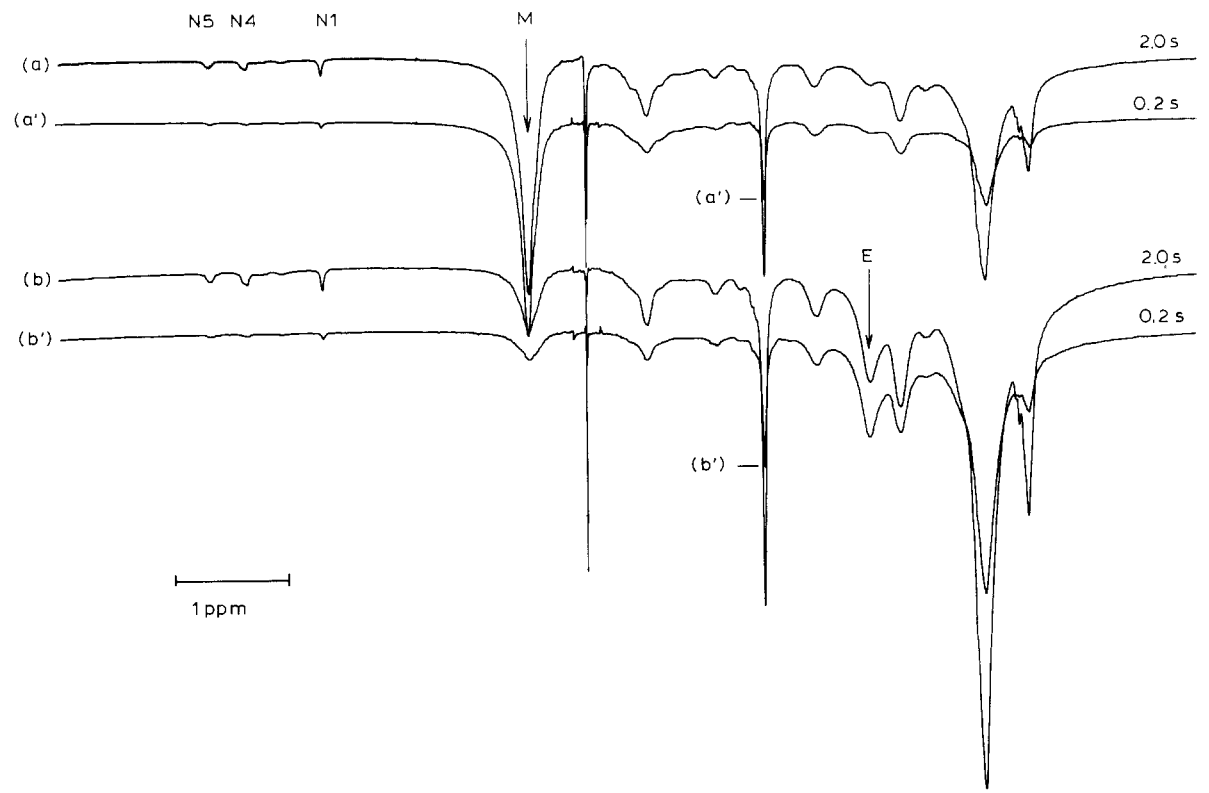

Fig. 3. NOE difference spectra (200 MHz) of dibucaine hydrochloride (16 mM)-PC vesicle (47 mM) solution: (a) irradiation at the resonance frequency of peak M for 2.0 s; (a′) as in (a), but irradiated for 0.2 s; (b) irradiation at the resonance frequency of peak E for 2.0 s; (b′) as in (b), but irradiated for 0.2 s.

close to the $CH_2C=C$. The intermolecular spin diffusion from the ϕ3,5 to the acyl chains of PC is also to be expected for this rod-like structure which may penetrate deeper in the bilayer. In the bent structure, we can consider two electrostatic interactions, between the cationic head of tetracaine and PO_4^- of PC and between the C = O of tetracaine and $N(CH_3)_3^+$ of PC. On the other hand, in the latter rod-like structure, only the interaction between the cationic head of tetracaine and PO_4^- of PC is possible. Thus, the intercalation of tetracaine to a lipid bilayer may not inhibit the motion of the lipid but may conversely increase it to some extent, since the strong electrostatic interactions between the head groups of PC are violated by the tetracaine molecules. The two kinds of structure of tetracaine shown in Fig. 5a and b, respectively, may correspond to the tetracaine of weakly and strongly bound species which were revealed by ^{2}H-NMR studies on the interaction of tetracaine with multilamellar dispersions of egg PC [8,12].

TABLE II

STEADY-STATE NOE VALUES (%) BETWEEN PC (PEAKS M AND E) AND AROMATIC RING PROTONS (PEAKS N1–N5) OF DIBUCAINE, TETRACAINE, AND PROCAINE

NOE values for a pre-irradiation time of 2.0 s are shown here. Drug (16 mM):PC (47 mM) = 1:3. Peak assignments are shown in Table I.

Peak irrad. (d)	NOE obsd.						
	DI-PC			TE-PC		PR-PC	
	N1	N3	N5	N1	N2	N1	N2
M	−14	−11	−12	−6	−6	−2	−2
E	−19	−17	−16	−14	−12	−2	−2

Fig. 6 shows the NOE spectra for a dibucaine-

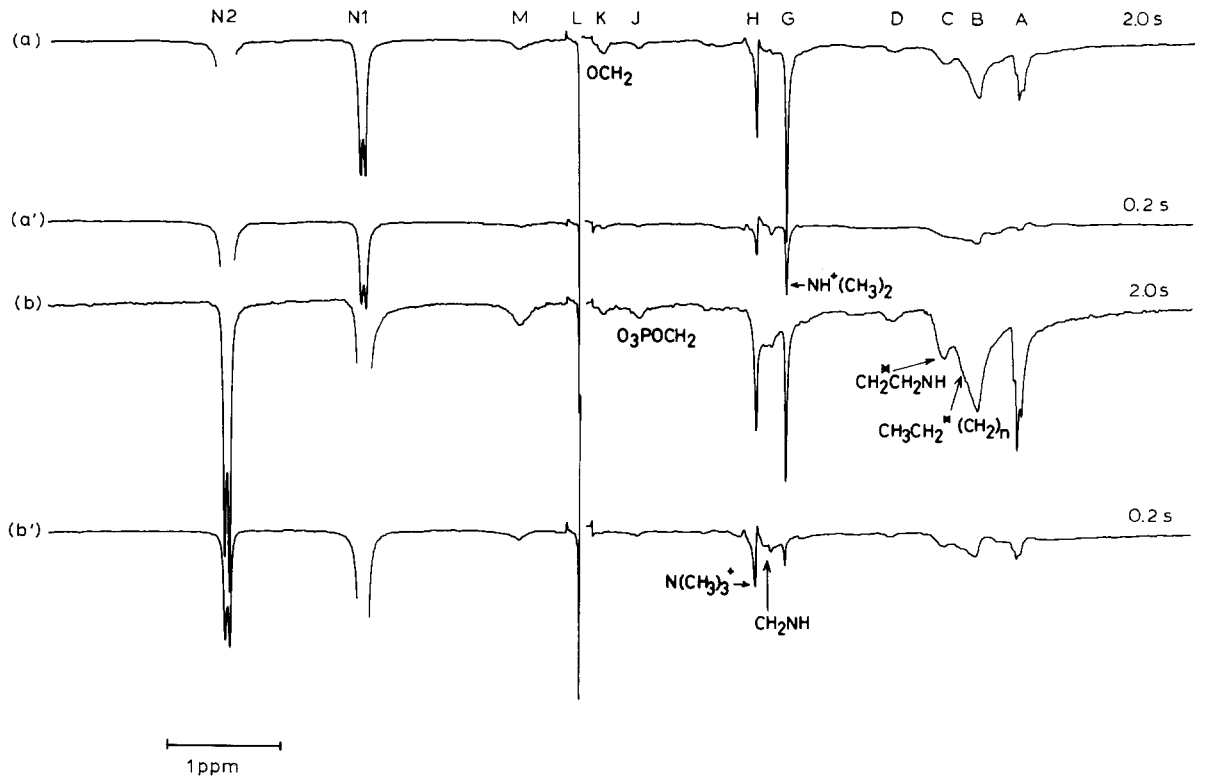

Fig. 4. NOE difference spectra (200 MHz) of tetracaine hydrochloride (16 mM)-PC vesicle (47 mM) solution: (a) irradiation at the resonance frequency of peak N2 for 2.0 s; (a′) as in (a), but irradiated for 0.2 s; (b) irradiation at the resonance frequency of peak N1 for 2.0 s; (b′) as in (b), but irradiated for 0.2 s.

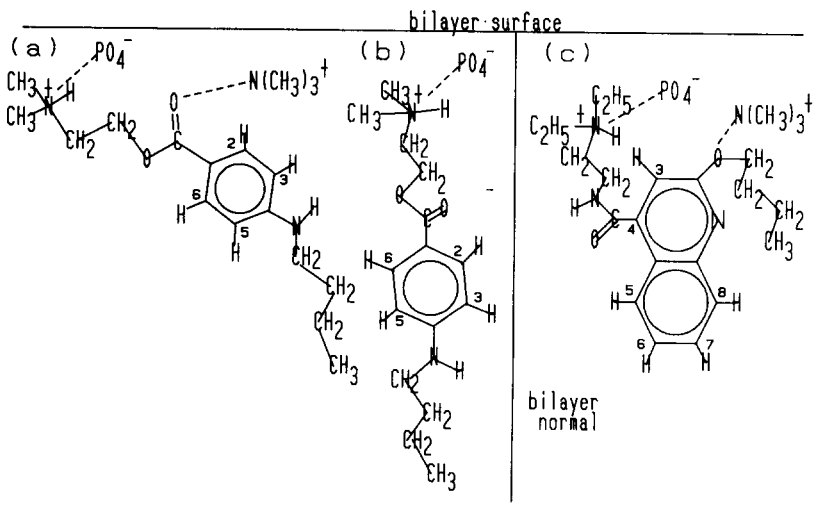

Fig. 5. Schematic drawings of the structures and positions of tetracaine (a and b) and dibucaine (c) in lipid bilayer vesicles. Bond lengths and the depths of the molecules along the bilayer normal are not always drawn to scale. The Dreiding model (Büchi) indicates that when the protonated ammonium ion of a drug is positioned at the level of PO_4^- of PC, the terminal methyl of the butylamino group of tetracaine reaches the levels of C-3–C-4 and C-7–C-8 methylenes of the acyl chains of PC in (a) and (b), respectively, whereas in (c) ϕ6 and ϕ7 protons of dibucaine reach the level of C-2 or C-3 methylenes. Electrostatic interactions between drugs and PC are indicated by dashed lines.

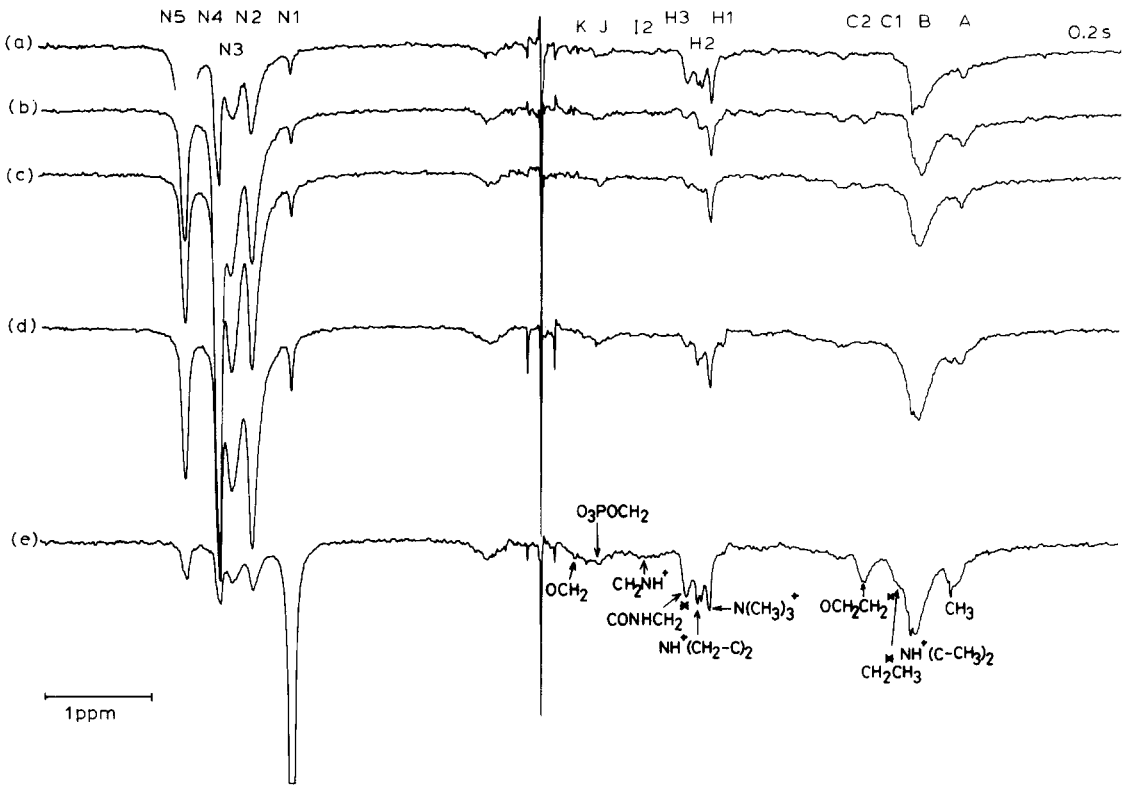

Fig. 6. NOE difference spectra (200 MHz) of dibucaine hydrochloride (16 mM)-PC vesicle (47 mM) solution: (a) irradiation on the resonance frequency of peak N5 for 0.2 s; (b)–(e) as in (a), but irradiated on the resonance frequencies of peaks N4–N1, respectively.

PC system, where resonances N1–N5 were each irradiated for 0.2 s; the results from the pre-irradiation time of 2.0 s are omitted here. The spectra a and e suggest that both ϕ5 (peak N5) and ϕ3 (peak N1) can locate in the vicinity of CON-HCH_2^* (peak H3) and $NH^+(CH_2-C)_2$ (peak H2) of dibucaine. This result means that the polar substituent at C4 is extending perpendicular to the quinoline ring. This situation resembles with the conformation of lidocaine in CCl_4 solution which was determined by a lanthanide probe method [32]. However, in this molecular structure, the distance between ϕ3 (or ϕ5) and NH^+ $(CH_2-C)_2$ becomes quite large; thus, the NOEs seen in peak H2 may be a result of an intramolecular spin diffusion. This effective spin diffusion can originate from the tight binding of the polar substituent of dibucaine to PC vesicles, probably from the binding of its NH^+ to their negatively charged phosphate groups. ^{31}P-NMR experiments which are described later gave evidence for this binding. The tight binding can also be realized from the very broad resonance of CH_2NH^+ (peak I2 in Fig. 2). The differential line broadening (Fig. 2) seen in ϕ7 (peak N3) and ϕ6 (peak N2), compared with the other aromatic protons (ϕ3, ϕ8 and ϕ5), suggests that the quinoline ring is penetrating into the rigid part of the glycerol moiety of PC vesicles at the portion of its ϕ7 and ϕ6 protons; thus the butoxy group cannot penetrate beyond the glycerol region on account of the restraints imposed by its molecular structure. However, the spectrum e in Fig. 6 shows that the spin diffusion transmits from ϕ3 (peak N1) to the butoxy group (peaks K, C2, C1, and A). This means that the mobility of the butoxy group is also quite decreased. In contrast to the case of tetracaine, the peak B in spectrum e is considered to be due mainly to the $NH^+(C-CH_3)_2$ of dibucaine, as will be realized from comparison of the intensity of peaks H2 and C2 with

that of peak B. Thus, the spin diffusion from $\phi 3$ to the $(CH_2)_n$ of PC is considered to be small. This may be due to the location and the structure of dibucaine as mentioned above. Although Fig. 6a–e show nearly an identical magnitude of NOEs for the $N(CH_3)_3^+$ (peak H1) resonance with one another, build-up of the NOE at peak H1 was fastest when the irradiation was done on $\phi 3$ (peak N1) and slowest on $\phi 8$ (peak N4); this result indicates that the $\phi 3$ proton locates near $N(CH_3)_3^+$ and the $\phi 8$ at the most apart from the $N(CH_3)_3^+$. Fig. 6e also shows that the $\phi 3$ locates near the CH_2NH^+ (peak I2) of dibucaine and O_3POCH_2 (peak J) of PC. A possible molecular structure of dibucaine which satisfies these observations is illustrated in Fig. 5c, where we showed a *cis* conformation for the amide linkage (CONH) of dibucaine. The *cis* conformation was suggested by the appearance of the NOE peak between the $\phi 3$ or $\phi 5$ and the $CONHCH_2$ protons (peak 3 in Fig. 6e or a). The *cis* conformation facilitates the electrostatic interaction between the protonated ammonium ion of dibucaine and PO_4^- of PC, while allowing the lone-pair electrons of the butoxy group of the same dibucaine molecule to interact electrostatically with the $N(CH_3)_3^+$ of PC.

In contrast to the above two systems, the NOE experiments for a procaine-PC (1 : 3) system gave no information on the location and/or the molecular conformation, since the NOE values in this system were very small. However, irradiation on the resonance due to H^2HO was found to give larger NOEs for $\phi 3$, 5 (peak N1) and $\phi 2,6$ (peak N2) protons than on a resonance due to PC or procaine. This finding means that the interactions of procaine with the structured water at the surface of vesicles is even stronger than with PC molecules. A similar observation was noted for nicotinamide-PC vesicle solution in our previous work [19].

Observations of ^{31}P-NMR spectra

In order to investigate whether the cationic forms of procaine, tetracaine and dibucaine bind to a negatively charged phosphate group, ^{31}P-NMR spectra were measured varying the drug/PC ratios from 1 : 3 to 5 : 3. Fig. 7 shows the effect of the increased drug/PC ratios on the ^{31}P chemical shifts of procaine-PC, tetracaine-PC and dibucaine-PC systems. Evidently, dibucaine shifted the ^{31}P resonance position of PC to the high field much more than tetracaine and procaine. Since the amount of the drug which is bound to the lipids is considered to differ very little between the tetracaine-PC and dibucaine-PC systems, this observation can be interpreted as being due to the strong interaction of the charged diethylammonium moiety of dibucaine with the phosphate group of PC and/or to the location of the quinoline ring of dibucaine near the phosphate group (Fig. 5c).

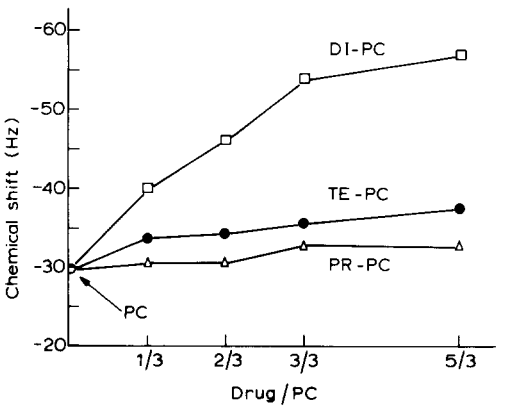

Fig. 7. Changes in ^{31}P-NMR chemical shifts of PC upon the addition of the drugs to PC vesicle solution in ^2H_2O. The chemical shifts are expressed in Hz (at 121.4 MHz) from external 85% H_3PO_4. The negative value of the chemical shifts means a high-field shift. DI, TE, PR, dibucaine, tetracaine, procaine, respectively.

Effect of the interactions of drugs with PC vesicles on the dynamics of PC molecules

Alteration of the fluidity of membranes caused by anesthetics is a possible reason for anesthesia [1–7]. Thus, we examined the effect of interactions of drugs with PC vesicles upon the dynamics of PC molecules by the present NOE method. Table III shows the results of spin diffusion (NOE) measurements within PC protons on irradiating the resonance due to α-CH_2 (peak E) for 2.0 s. This resonance is quite suitable for irradiation, because (1) it does not overlap with the resonances from drugs, and (2) the α-CH_2 locates at a boundary region between the polar head-group and hydrophobic acyl chains, and thus can monitor the changes in the mobility of PC molecules in

the two regions separately. Interestingly, as shown in Table III, it was only the tetracaine-PC system that showed any significant deviation in NOE values from those measured as a control with a PC vesicle solution. These NOE values are mean values of the two trials, except those of the tetracaine-PC system, for which four trials were performed, since the deviations in each monitored resonance were small. Measurements were performed at the same experimental settings and within 2 days, for the sample systems prepared from the same batches of preparations of the sonicated vesicles. If the experiments were performed for another preparation of the sonicated vesicles, each NOE value took a different value, indicating that the efficiency of spin diffusion is sensitive to the size of the vesicles in solution; however, the relative magnitude in these sample systems showed the same trend as in Table III. The NOE values in the tetracaine-PC system were smaller in absolute magnitude and in all the peaks monitored than those of the corresponding values in PC, procaine-PC, and dibucaine-PC systems. This indicates that the mobilities of PC molecules at both the polar and hydrophobic regions were increased as a consequence of the interactions

TABLE III

STEADY-STATE NOE VALUES (%) BETWEEN α-CH_2 OF ACYL CHAINS (PEAK E) AND $N(CH_3)_3^+$, $(CH_2)_n$, CH = CH(CHOCO), AND CH_3 OF PC, PROCAINE-PC (1:3), TETRACAINE-PC (1:3) AND DIBUCAINE-PC (1:3) SYSTEMS

PC (40 mM), drug (12 mM):PC (35 mM) = 1:3. Data represent mean ± mean deviation for two experiments in the PC, procaine-PC, and dibucaine-PC systems and four experiments in the tetracaine-PC system. These measurements were performed at the same experimental settings and within 2 days for the sample systems prepared from the same batches of preparations of the sonicated vesicles. Peak assignments are given in Table I.

Peak obsd.	System			
	PC	PR-PC	TE-PC	DI-PC
H (H1)	−8.5 ± 0.3	−8.1 ± 1.0	−7.0 ± 0.4	−9.9 ± 1.0
B	−16.8 ± 0.8	−16.1 ± 0.2	−13.4 ± 1.2	−19.0 ± 0.7
M	−19.2 ± 0.9	−19.0 ± 0.4	−15.4 ± 1.2	−20.7 ± 0.3
A	−14.2 ± 0.5	−13.9 ± 0.3	−11.3 ± 0.8	−15.3 ± 0.6

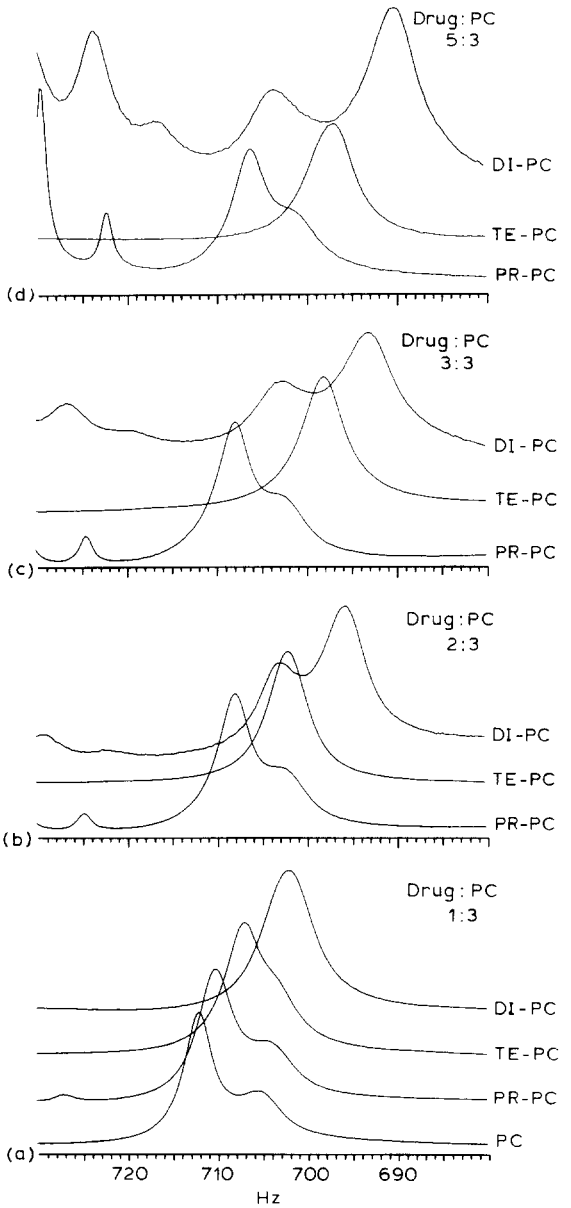

Fig. 8. ^{1}H-NMR spectra (300 MHz) of the $N(CH_3)_3^+$ resonances from the inner and outer halves of bilayer vesicles of PC and drug-PC systems. Drug/PC ratios were: (a) drug (12 mM)/PC (35 mM) = 1:3; (b) drug (20 mM)/PC (31 mM) = 2:3; (c) drug (27 mM)/PC (27 mM) = 3:3; (d) drug (38 mM)/PC (23 mM) = 5:3. The concentration for the PC vesicle solution which contains no drug was 40 mM. The chemical shifts are expressed in Hz from the terminal methyl protons of acyl chains of egg PC. Peaks which appear at more than 720 Hz are due to the drugs.

with tetracaine. In contrast, in the dibucaine-PC system, the NOE values increased slightly. This means that the mobility of a PC molecule was decreased on addition of dibucaine, agreeing well with the finding that the polar substituent of dibucaine is binding tightly to PC.

In order to examine the sensitivity of the spin diffusion with respect to the changes in mobility of the PC molecule, the NOE experiments were performed by raising the temperature by 15 Cdeg, that is, at 38°C. This trial was made on PC vesicles which contain no drugs and it was found that the NOE values in peaks H, B and M decrease by 20% and those in peak A by 30% as a rate. These rates of decrease in NOE values are roughly comparable to those caused on addition of tetracaine to a solution of PC vesicles (Table III). Thus, although the effect of local anesthetics may not always correspond to temperature changes which cause the overall fluidization of the membrane [33], it can be concluded that the addition of a 1/3 molar ratio of tetracaine increases the mobility of PC molecules to an extent comparable to raising the temperature by 15 Cdeg.

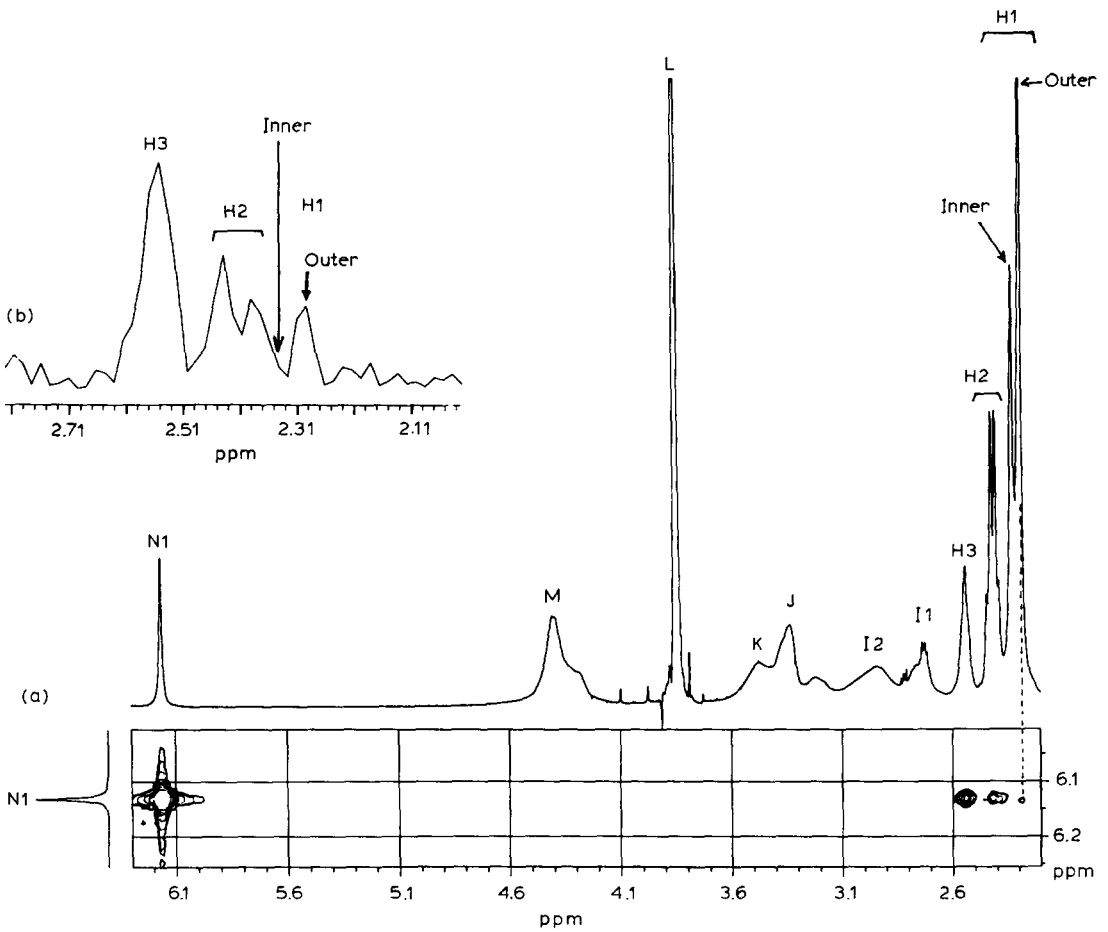

Fig. 9. (a) Two-dimensional NOE experiments for dibucaine hydrochloride (45 mM)-PC vesicle (45 mM) solution showing a diagonal peak of N1 and cross-peaks between the N1 and H3, H2, and only the outer component of H1. 'Inner' and 'outer' for the H1 specify, respectively, $N(CH_3)_3^+$ resonances from the inner and outer halves of the bilayer vesicles. Contour plots of the absolute value spectra obtained after symmetrization are shown. (b) The cross-section parallel to the ω_2 axis. Observed conditions are: digital resolution, 6.4 Hz per point; mixing time, 150 ms with ±11% random variation; homo-gated irradiation on the ^{2}HHO (peak L) resonance; Fourier transform in both dimensions with sine-bell shifted 5°.

Effects of the binding of drugs to PC on the chemical shifts of the choline methyl protons

In order to find out whether the cationic forms of local anesthetics move across the membrane to the inner monolayer, changes in chemical shifts of choline methyl protons ($N(CH_3)_3^+$) by the addition of the various amounts of local anesthetics have been investigated. Fig. 8 shows the 300 MHz ^{1}H-NMR spectra of the $N(CH_3)_3^+$ resonances of PC, procaine-PC, tetracaine-PC and dibucaine-PC systems at various drug/PC ratios. As shown in Fig. 8a, the resonance from $N(CH_3)_3^+$ of PC splits into two peaks; the lower-field peak has been assigned to the $N(CH_3)_3^+$ of the outer half of bilayer vesicles and the higher-field one to that of the inner half of the vesicles [34]. Thus, if a drug was added to the solution of pre-formed PC vesicles, and also if the cationic form of a drug does not move across the membrane, only the lower-field component would show a high-field shift on account of the ring current effect due to the aromatic ring moiety of the drug. Fig. 8 shows that this expectation holds for procaine and dibucaine, but not always for tetracaine, although it appears that the higher-field components of procaine and dibucaine also shifted slightly (1–2 Hz) to the high field. In the cases of procaine and dibucaine, if the drug/PC ratio was increased from 1 : 3 to 3 : 3 or 5 : 3 (Fig. 8a–d), the $N(CH_3)_3^+$ resonance of the lower-field component (i.e., external $N(CH_3)_3^+$) shifted to the high field. The magnitude is much larger in dibucaine than in procaine; hence, the lower- and higher-field components were interchanged in the dibucaine-PC system (Fig. 8b–d). These observations clearly indicate that both procaine and dibucaine locate predominantly at the outer half of the bilayer. In contrast, in the tetracaine-PC system both the lower- and the higher-field components were shifted to the high field. This indicates that the tetracaine molecules are also located at the inner half of the bilayer. Although this situation is not so clear in Fig. 8a, it is likely that even at the 1 : 3 tetracaine/PC ratio, the tetracaine molecules have also penetrated into the inner half of the bilayers. Since the observable NOEs (spin diffusion) within PC molecules are those of the weighted averages from the PC molecules which reside in both outer and inner halves of the bilayers, the perturbations due to the interactions of tetracaine with PC can be more effectively reflected in the NOE values shown in Table III than those in the procaine-PC and dibucaine-PC systems.

Fig. 9 clearly demonstrates that dibucaine is located only at the outer half of the bilayer. It would be expected that if the dibucaine molecule does not reside in the inner half of the bilayer, NOE experiments for the sample system of a dibucaine/PC ratio of, for example, 3 : 3 (see, Fig. 8c) would reveal that there exists an NOE between dibucaine and only the outer component of $N(CH_3)_3^+$. Fig. 9 gives clear evidence that this is indeed so, where we show the results of two-dimensional NOE experiments [35] performed at 400 MHz ^{1}H resonance frequency to spread the peak separation (15.2 Hz at the 400 MHz) between the two components from $N(CH_3)_3^+$. As shown in the contour plot (Fig. 9a) or in its cross-section spectrum (Fig. 9b), the NOE cross-peak between peaks N1 (ϕ3) and H1 ($N(CH_3)_3^+$) was clearly observed only for the outer component of the $N(CH_3)_3^+$.

Discussion

Recent studies of the effects of tetracaine and dibucaine on the changes in fluidity in multilamellar dispersions of synthetic lipids [9], rat total brain lipid liposomes [33], and in multibilayers of ox brain white matter lipids [36,37] by ^{2}H-NMR [9] and electron spin resonance (ESR) [33,36,37] spectroscopies have demonstrated that these drugs disorder the lipids and increase the membrane fluidity at their somewhat high concentrations. Colorimetric and fluorescence polarization measurements also indicated that dibucaine fluidizes the PC membranes at relatively higher concentrations [38]. It is recognized that in the cases of the ^{2}H-NMR and ESR studies, the changes in the fluidity as manifested by the changes in an order parameter do not necessarily imply an increase in the mobility of the lipid, because the order parameter does not give information on the rate of a segmental motion but rather on the time-averaged orientation of the isotope- or spin-labelled segment [39–41]. Nevertheless, the magnitude of the order parameter has been shown to correlate to some extent with the mobility of a lipid molecule,

i.e., increasing mobility with decreasing order parameter [39,42]. Thus, it might be expected that at a drug concentration of 12 mM, which was our experimental condition to test the present approach for obtaining information on the changes in lipid mobility, both tetracaine and dibucaine would also increase the mobility of the sonicated egg PC molecules. However, Table III shows that this is indeed true for tetracaine but not for dibucaine.

In the tetracaine-PC system, relatively large decreases in NOE values, as compared with those in PC alone, could be seen about the protons due to the acyl chains of PC, whereas a small one was observed about the choline methyl protons, both showing the increased mobility by tetracaine. Boulanger et al. [9] have shown that the charged form of tetracaine increases the magnitude of the quadrupole splitting of deuterons in ^{2}H-NMR spectrum of PC-d_9 where all choline methyl protons are deuterated, while it decreases the quadrupole splittings from acyl chain-labelled molecules. They ascribed the former observation to a conformational change at the PC head-group and the latter one to the increased disordering of the acyl chains. Our present work confirms that the disordering effect by tetracaine certainly accompanies the increase in mobility of PC molecules. The changes in the mobility were reflected as the overall alterations in the correlation times of successive proton pairs through which spin energy transmits. Chan et al. [43–45] roughly classified the motion of the acyl chains of a lipid into two groups, i.e., reorientation and rotational isomerization; the correlation times for the former mode lie in a range 10^{-8}–10^{-9} s and those for the latter 10^{-10}–10^{-11} s in the case of sonicated single bilayers. We can consider that the present NOE (spin diffusion) method sensed the changes in the mobility of the reorientational motion of acyl chains, because the correlation times followed by the spin diffusion phenomena should be longer than about 10^{-9} s at our ^{1}H-NMR frequency of 200 MHz; hence, the present NOE method has this characteristic, although it must be applied to sonicated unilamellar vesicles in order to follow the changes in signal intensity quantitatively.

Evidently, the changes in mobility of the egg PC molecules by the addition of tetracaine are not due to the formation of a tetracaine–egg PC mixed micelle [46,47], because our experimental concentration (12 mM) is low enough to prevent the mixed micelle formation, for which a critical micelle concentration (cmc) of about 50 mM is reported by Fernández for a solution of pH 6.5 [47]. It is also reported by Frezzatti et al. that the mixed micelle formation occurs abruptly at concentrations near the cmc and is reversible on dilution to concentrations below the cmc [48]. As evidenced in Fig. 8 the tetracaine easily intercalate into the inner half of bilayer vesicles. This intercalation into the inner half of the bilayer can enhance the observed effect on the mobility of a PC molecule caused by the tetracaine as described in the Results section. However, the intercalation phenomena contrast markedly with the finding by Yeagle et al. [13]. Based on ^{31}P-NMR spectra for sonicated PC vesicles with Pr^{3+} trapped only on the inside aqueous phase, they showed that tetracaine does not penetrate into the inner half of the bilayer over a period of 4 h [13]; however, our NMR data are reproducible for a tetracaine-PC solution prepared within a much shorter time. It is probable that the tetracaine which resides in the inner monolayer is partly present as an uncharged form which may not necessarily displace the Pr^{3+} from the phosphates of the interior lipids, since both our experiments and those of Yeagle et al. were performed in an unbuffered medium. Further study is required to determine whether the discrepancy in the transbilayer movement of tetracaine results from the presence of Pr^{3+} at the interior lipids, as in the case of in the experimental conditions of Yeagle et al. Nevertheless, in our judgment, it is just this ability to tetracaine to permeate across the bilayer that can disrupt the unilamellar egg PC vesicles and also can lead to the formation of mixed micelles.

On the other hand, in the dibucaine-PC system, Table III showed that the mobility of the PC molecule was slightly decreased by the addition of dibucaine in spite of the higher concentration of dibucaine in the lipid phase than that of tetracaine. A possible explanation for this discrepancy between dibucaine and tetracaine seems to come from the differences in their molecular shapes and the resulting differences in the locations in single bilayer vesicles (Fig. 5). The cylindrical tetracaine

penetrates more deeply into the lipid bilayer than does dibucaine (Fig. 5b); consequently, it acts as a wedge that separates the PC molecules with each other. This weakens both the electrostatic binding at the polar head groups and the hydrophobic interaction at the acyl chains among PC molecules and consequently increases their mobilities. In contrast, more bulky dibucaine binds shallowly with the lipids at the (outer) surface of the bilayer, developing a positive surface charge which effectively stabilizes the bilayer vesicles (Fig. 5c); consequently, it acts as a clamp that grasps the PC molecules and reduces their mobility. Previously found fluidizing effects of dibucaine on rat total brain lipid liposomes [33] and on multilamellar vesicles of PC [38] at pH 7.2–7.4 may be due to deeper penetration of its neutral form.

The decrease in mobility of the PC molecules by the 'clamp' effect of dibucaine can easily be conceived if we take into account the packing

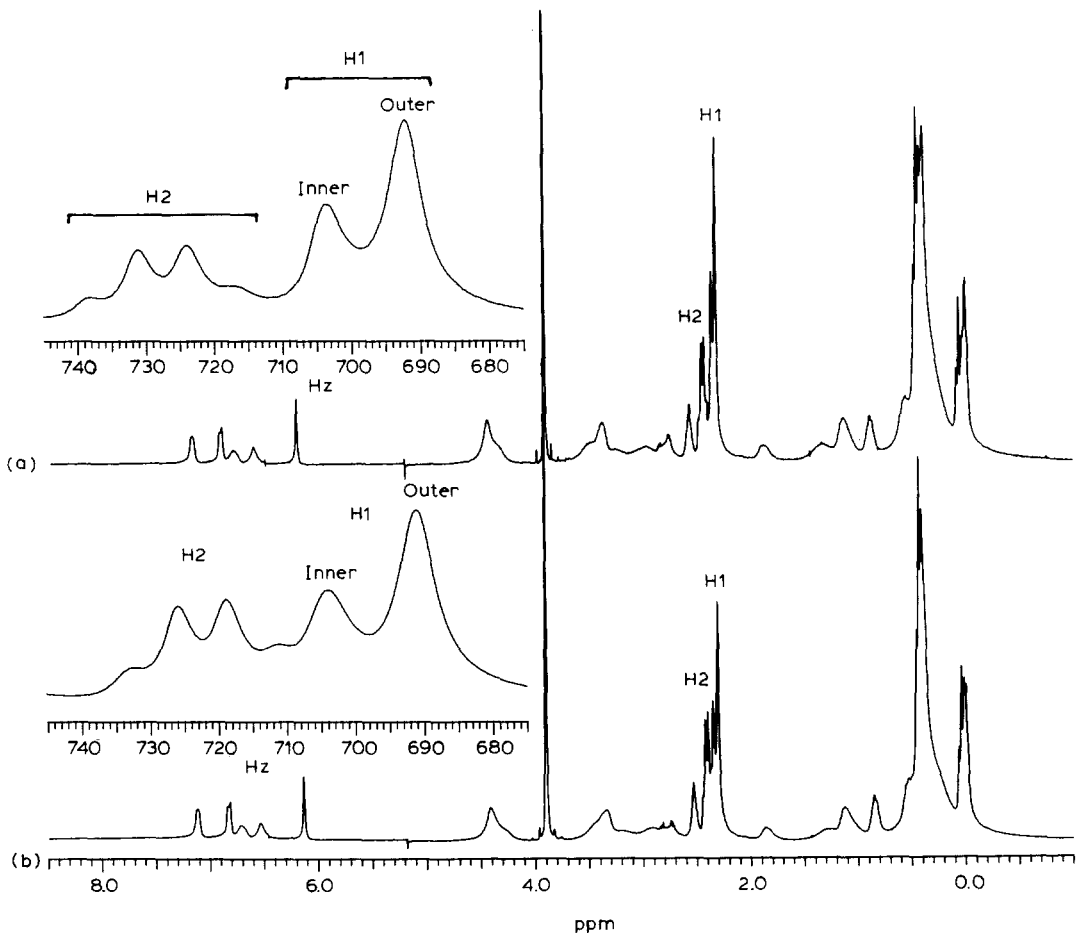

Fig. 10. (a) ^{1}H-NMR spectrum (300 MHz) of dibucaine hydrochloride (50 mM)-PC vesicle (50 mM) solution (pH 5.3). A weighed amount of the drug was added to the pre-formed PC vesicle solution, as in all the other experiments in this paper. (b) ^{1}H-NMR spectrum (300 MHz) of dibucaine hydrochloride (48 mM)-PC vesicle (43 mM) solution (pH 5.4). A weighed amount of the drug was added to $^{2}H_2O$ before the sonication. In both (a) and (b), insets are the expanded spectra at a region between 2.25 and 2.48 ppm and the chemical shifts are expressed in Hz from the acyl chain terminal methyl proton resonance. 'Inner' and 'outer' for peak H1 specify, respectively, $N(CH_3)_3^+$ resonances from the inner and outer halves of the bilayer vesicles; H2 is due to the drug. Note the close resemblance between the two spectra; also, it should be noted that in (b), only the outer component of $N(CH_3)_3^+$ is shifted to higher field.

asymmetry for lipids in sonicated small vesicles [34,49–52]. The packing asymmetry due to the small radius of curvature of the vesicle bilayer results in a looser packing of PC head-groups in the outer half of the bilayer and the converse is true for the inner counterpart. The dibucaine clamps just the loosely packed head-groups of the PC molecules at the outer monolayer and decreases their mobility. The lipid packing constraints imposed by the small radius of curvature at the inner monolayer might have shut out the dibucaine which wished, if possible, to locate at the inside surface. In fact, a similar differential partitioning between the two monolayers of small lipid vesicles has already been reported for cholesterol [53,54] and dansylglycine [55]. Since the tertiary amines such as tetracaine and dibucaine may easily pass through the bilayer via their uncharged forms [56,57], it is probable that dibucaine also exists in the inside aqueous phase. Thus, in order to examine whether or not the dibucaine which truly exists in the inside aqueous phase can reside at the inside surface, we have further observed ^{1}H-NMR spectrum for a dibucaine-PC (1 : 1) system as prepared by dissolving the dibucaine in ^2H_2O before the sonication. The results are shown in Fig. 10 together with the ^{1}H-NMR spectrum for a dibucaine-PC system prepared by adding dibucaine to the pre-formed vesicle solution (Fig. 10a). Interestingly, as a whole, the two spectra closely resembled each other, including their chemical shifts and the extents of a line broadening; more importantly, it should be noted that in spectrum b only the outer component of choline methyl protons shifted to higher field, leaving the inner component as in spectrum a. These observations strongly support the view that dibucaine cannot reside on the highly curved inner surface.

In conclusion, the local anesthetics chosen differ in their strength of binding to the neutral egg PC and also in their ability to perturb the mobility of the lipids. Dibucaine binds to the PC most strongly whereas tetracaine perturbs the organization and mobility of the lipid bilayers most effectively. Procaine binds very weakly to the PC and therefore perturbs the lipid bilayer least of all. These differences arise from those in the molecular structures and the resulting differences in the locations of the drugs in a bilayer. It can thus be concluded that, although perturbations of fluidity of lipid bilayers by the anesthetic molecules are a plausible mechanism of anesthetic action, the relative order of the anesthetic potency within these 'caine'-type local anesthetics can be correlated not with their ability to affect the membrane fluidity but with the tightness of their binding to the lipids.

Acknowledgments

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