Interaction between Dibucaine and Pig Erythrocyte Membranes as Studied by NOESY Experiments in ^{1}H-NMR Spectroscopy. Which Form of Dibucaine Interacts More Strongly, Cationic or Uncharged?

Yoshihiro KURODA,* Misako WAKITA, and Terumichi NAKAGAWA

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606–01, Japan.
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The interaction between amine local anesthetic dibucaine and pig erythrocyte membranes has been studied by ^{1}H-NMR spectroscopy. Two-dimensional NOESY spectra were observed to obtain the conformations of cationic and uncharged forms of dibucaine. The NMR spectra were measured at pH 7.4, and the temperature was raised (318—348 K) to increase the concentration of the uncharged form of dibucaine, taking the temperature dependence of the pK_a value of dibucaine into consideration. The dibucaine in a buffered solution showed the presence of two kinds of distinctly different species; one is assignable to the cationic form and the other to the uncharged form of dibucaine, suggesting that the protonation equilibrium between the two forms is slow in the presently employed experimental condition. The uncharged dibucaine showed well-defined NOE cross-peaks in the NOESY spectra of the solution containing no erythrocyte membranes, suggesting that its conformation is relatively fixed. Interestingly, however, it was only the cationic dibucaine that showed NOE cross-peaks when the solution contained the membranes, and experiments were performed at a much shorter mixing time for the buildup of NOEs, suggesting that it appeared only the cationic form of dibucaine is interacting with the membranes. It was concluded that the uncharged form of dibucaine, which was produced by raising the temperature, formed micelles in a buffered solution. Thus formed micelles didn't interact with membranes owing to the repulsive forces between the structured water surrounding the micelles and those at the surface of the membranes. This conclusion could be a promising reason why the cationic local anesthetics are much more active than their uncharged counterparts in blocking nerve conduction.

Keywords local anesthetic; dibucaine; erythrocyte membrane; ^{1}H-NMR

Since the publication of an excellent work by Narahashi *et al.*,[1] it has generally been believed that local anesthetics penetrate into the nerve sheath and nerve membrane with their uncharged forms and bind to the sodium channel at the axoplasmic side of nerve membranes with their cationic forms.[2] The binding site can be assumed to be amphiphilic in nature since, in addition to the fact that all the clinically important local anesthetics are amphiphilic, their anesthetic potencies correlate better with octanol than with hydrocarbon partition coefficients.[3] Recently, Greenberg and Tsong have isolated a protein which can function as a local anesthetic receptor site in axonal membranes.[4,5] If this is true, the molecular structure of a local anesthetic in membranes is expected to play an important role in causing anesthesia, as has been suggested by Sargent and Schwyzer for cases of membrane-catalyzed peptide-receptor interactions.[6] Previously, we investigated the conformation of the positively charged form of dibucaine (Fig. 1) in small unilamellar phosphatidylcholine vesicles by nuclear Overhauser effects (NOE) in ^{1}H-NMR spectroscopy.[7] The present work was initially undertaken with the intention of obtaining the conformations of both the cationic and uncharged forms of dibucaine which are interacting simultaneously with erythrocyte membranes. However, as will be described in the following, the uncharged form of dibucaine which was produced by raising the temperature of a sample solution showed no NOE cross-peak, the interpretation for which inevitably leads us to consider that the uncharged form of dibucaine cannot interact entirely with the membranes. This finding affords us a clear answer as to why the cationic local anesthetics are the active form for blocking nervous conduction,[1] on the one hand; however, it poses a serious question as to whether or not the uncharged form of local anesthetics really crosses the two barriers constructed by the nerve sheath and nerve membrane. In the present paper we report, in addition to the conformation of the cationic form of dibucaine, some interesting observations in ^{1}H-NMR spectra of dibucaine-ghost suspensions.

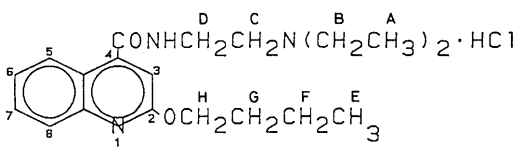

Fig. 1. Structure of Dibucaine Hydrochloride and Its Numbering Scheme

Experimental

Materials Dibucaine hydrochloride and egg yolk L-α-phosphatidylcholine (egg PC) were obtained from Sigma and used without further purification; the purity of the egg PC was checked by a thin layer chromatogram and found to be greater than 99%. Pig erythrocyte ghosts were prepared from fresh pig blood as described previously.[8] No treatment was made to reseal the hole in the ghost formed when the pig blood was hemolysed.

Determination of the pK_a Value of Dibucaine and Its Partition Coefficient K_p for the Pig Erythrocyte Ghost Membranes The pK_a value of dibucaine at its tertiary amine nitrogen was determined by titrating 0.5 mM dibucaine solution in D_2O with 0.01 M NaOD in a pH range of 2.94—8.53 (meter reading), and then simulating the resulting titration curve theoretically. The pK_a value at 25 °C was calculated to be 8.0; this value agrees well with that reported by Ohki (pK_a = 8.0),[9] but is a little smaller than that reported by Ritchie *et al.* (pK_a = 8.5).[10]

The partition coefficients of dibucaine for pig erythrocyte membranes suspended in a hypotonic (10 mOsm, 4.8 mM) phosphate buffer in D_2O at pH 5.6 (meter reading) were obtained by fluorometrically determining the concentrations of dibucaine not binding with the membranes. The solutions were obtained by ultrafiltration of the ghost suspension

solutions containing 0.5, 1.0, and 3 mM dibucaine. The ghost suspensions were allowed to stand for at least 12 h to equilibrate the binding of dibucaine. The excitation wavelength for the fluorescence measurements was 325 nm and the observation wavelength was 405 nm. The weights of the dry ghosts employed in the present measurements were 1.1—1.4 mg, and the final calculated K_p values for the ghost solutions containing 0.5, 1.0 and 3 mM dibucaine were, respectively, 134, 58 and 33.

Preparation of Sample Solutions for NMR Measurements Dibucaine in a buffer solution (3 mM) was prepared by using a hypotonic (10 mOsm, 4.8 mM) phosphate buffer in D_2O (pH 7.4, meter reading). The ghost suspension was prepared by using an isotonic (310 mOsm, 150 mM) phosphate buffer in D_2O (pH 7.4, meter reading); the concentration of the ghost was the same as that employed in our previous paper.[8] The proportion of a bound dibucaine molecule with the membranes estimated from the K_p value described above was 34% for 3mM dibucaine. Single bilayer vesicles of egg PC were prepared by ultrasonic irradiation of the dried egg PC suspended in an isotonic (310 mOsm) phosphate buffer of D_2O, for 20—30 min, followed by cooling in an ice/water bath and bubbling with nitrogen gas.

Measurements ^{1}H-NMR measurements were performed on a Bruker AM-600 spectrometer at a resonance frequency of 600 MHz. Phase-sensitive NOE and chemical exchange correlated spectra (NOESY) were measured by using a standard 90° three-pulse sequence and with a time-proportional phase increment (TPPI) phase-sensitive mode.[11] In the NOESY experiments, 1 kilo (real) × 1 kilo (real) data points were employed. Moreover, a squared shifted sinebell window was applied to the F1-dimension (vertical frequency axis) and a gaussian window to the F2-dimension (horizontal frequency axis). In order to obtain a solution containing both the cationic and uncharged forms of dibucaine at a physiological condition (*i.e.*, pH 7.4), NMR measurements were performed at an elevated tem perature (318—348 K). The chemical shifts were referenced to the residual HDO proton (4.8 ppm at 300 K); the chemical shifts for the solution at elevated temperatures were determined by cross-referencing the chemical shift of an impurity contained in the phosphate buffer at about 0.3 ppm at 300 K.

Molecular Dynamics (MD) Calculations MD and molecular mechanics (MM) calculations were performed as reported previously[7] with Molecular Simulations' NMRgraf software[12] running on a Silicon Graphics Iris 4D/25 computer.

Results

It is well known that an acid dissociation constant (pK_a) depends on temperature; the value decreases with increasing temperature.[13] According to the Perrin's equation, it is expected that if we raise the temperature by about 20—30 °C from room temperature, the pK_a value of dibucaine (8—8.5) would become nearly equal to the pH value of the bulk solution (pH 7.4). In the present work, we noticed this temperature dependence of the pK_a value in preparing the solution containing a millimolar order concentration of the uncharged form of dibucaine. This is because: (1) since the solubility of the uncharged molecular form of dibucaine was very low, we could not prepare the sample solution at an alkaline pH, which is near the pK_a value of dibucaine, and (2) the decomposition of the erythrocyte membrane in the alkaline media was expected to become a serious problem. At the beginning, in order to confirm the temperature dependence of the pK_a value, we observed the ^{1}H-NMR spectra of a dibucaine solution at pH 7.4 on raising the temperature, expecting temperature-dependent changes in the chemical shifts for each peak, especially for peaks of protons B, C, and D (see, Fig. 1) at the polar side chain of dibucaine. The results are shown in Fig. 2. Interestingly, however, the ^{1}H-NMR spectra of the solution at a temperature higher than 313 K showed new peaks which may originate from another species of dibucaine, the chemical shifts of

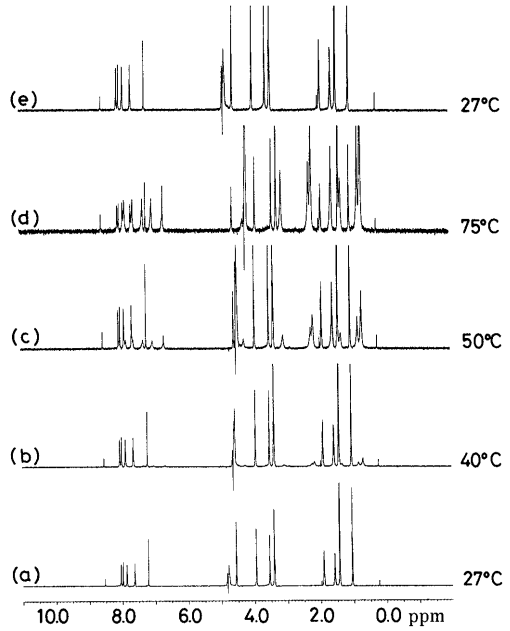

Fig. 2. 600 MHz ^{1}H-NMR Spectra of 3 mM Dibucaine Solution
(a) 300 K, (b) 313 K, (c) 323 K, (d) 348 K, (e) 300 K.

which appeared to be shifted to a higher field relative to their original positions. The most prominent changes in the chemical shifts were peaks due to the polar diethylamino group (0.7—1.3 ppm), followed by the other resonances from the aromatic (0.2—0.6 ppm) and the butoxy (0.2—0.3 ppm) groups. The peaks observed at room temperature (300 K) can be ascribed mainly to the cationic form of dibucaine, because the pH of the solution and the pK_a value (8.0—8.5) of dibucaine is well separated. On the other hand, the newly appearing peaks can be assigned to those due to the uncharged molecular form of dibucaine, because: (1) these peaks disappeared on lowering the temperature to 300 K (Fig. 2e), (2) a two-dimensional COSY observed at an elevated temperature approved the spin-coupling connectivities of a dibucaine molecule, and (3) the differences in chemical shifts between the original peaks and the newly appearing peaks are the largest for the protons around the diethylamino group. Reasonings (1) and (2) confirm that the dibucaine molecule was not decomposed and reasoning (3) suggests that deprotonation occurred at the tertiary amine nitrogen. Thus, it appears that the protonation equilibrium between the two forms is slow in the present case. The molecular form of dibucaine in the present solution may form micelles in some way[14,15] because: (1) the linewidths of the newly appearing peaks are somewhat broader as compared to the corresponding peaks of the cationic form; (2) since the solubility of the uncharged dibucaine should be very low, it is unlikely that it exists as a monomer in the solution of pH 7.4; and (3) all the protons including those due to butoxy and aromatic groups shifted to a higher field much larger than the magnitude expectable from deprotonation. A NOESY spectrum of the same solution at 333 K also supports the aggregation of the uncharged form of dibucaine. In Fig. 3, where we show the NOESY spectrum observed by using

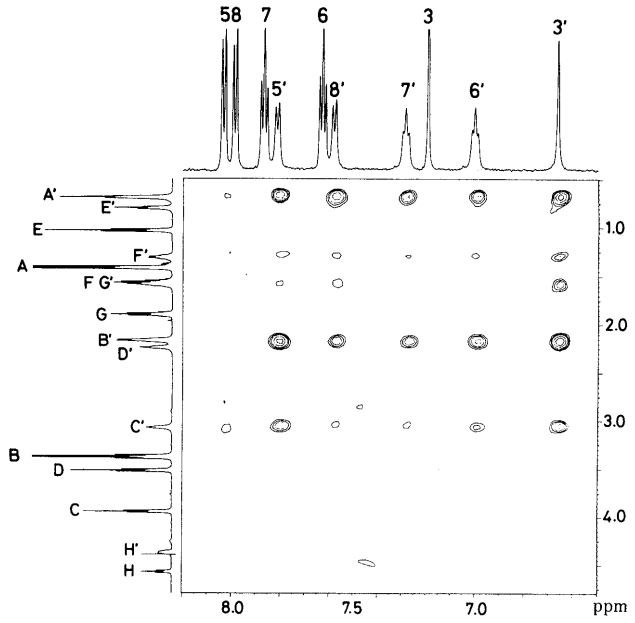

Fig. 3. 600 MHz NOESY Spectrum of Dibucaine (3 mM) in a Phosphate Buffer (pH 7.4) at 333 K; Mixing Time, 0.6 s

The peaks labelled with a prime denote newly appearing ones on raising the temperatue.

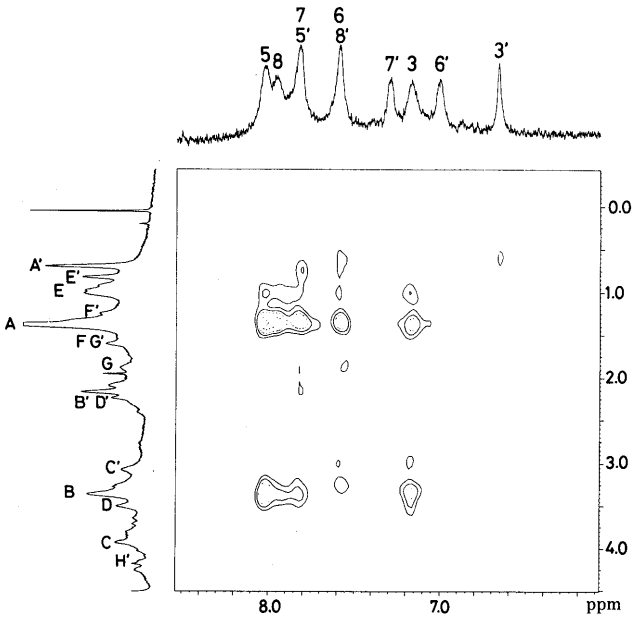

Fig. 4. 600 MHz NOESY Spectrum of Dibucaine (3 mM) in Pig Erythrocyte Ghosts Suspended in a Phosphate Buffer (pH 7.4) at 318 K; Mixing Time, 0.06 s

The peaks labelled with a prime indicate newly appearing ones on raising the temperature as in Fig. 3. NOE connectivities are summarized in Table I.

a mixing time of 0.6 s, only the newly appearing peaks, labeled together with a prime, showed NOE cross-peaks between the aromatic protons and the side chain protons of dibucaine. This finding indicates that the polar side chain of dibucaine is fairly fixed relative to the aromatic quinoline ring, owing to the aggregation of dibucaine molecules with one another.

Figure 4 shows a NOESY spectrum of dibucaine in pig erythrocyte ghosts suspended in a phosphate buffer at

Table I. Observed NOE Connectivities of Dibucaine-Pig Erythrocyte Ghost Suspension at 318 K

Ring proton[a]	Side-chain	Peak[a]
Ph-3, Ph-5, Ph-6, Ph-8	$NH^+(C\text{–}CH_3)_2$	A
	$NH^+(CH_2\text{–}C)_2$	B
	CH_3	E
Ph-7	$NH^+(C\text{–}CH_3)_2$	A
	$NH^+(CH_2\text{–}C)_2$	B
Ph-5′	$N(C\text{–}CH_3)_2$	A′

a) The peak/proton labelled with a prime denotes a newly appearing one on raising the temperature.

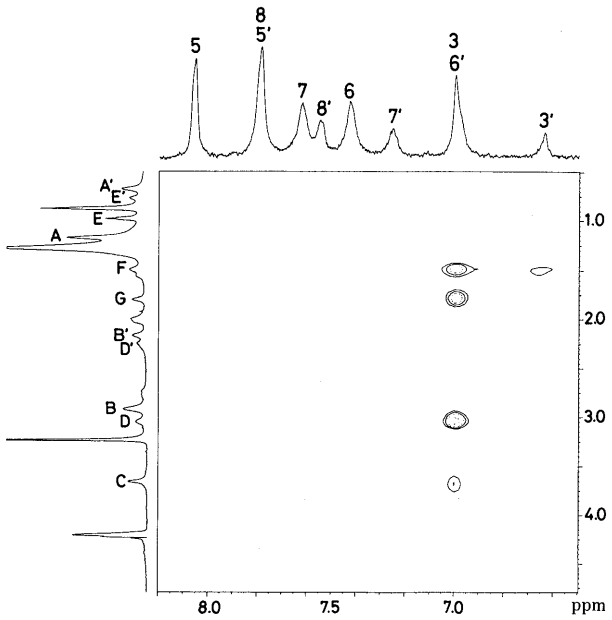

Fig. 5. 600 MHz NOESY Spectrum of Dibucaine (3 mM) in Egg PC Vesicles (3 mM) Solution in a Phosphate Buffer (pH 7.4) at 348 K; Mixing Time, 0.3 s

The peaks labelled with a prime indicate newly appearing ones on raising the temperature as in Fig. 3.

318 K. At this temperature of this solution, the ^{1}H-NMR spectrum also showed new peaks in addition to those arising from the cationic form of dibucaine, even though all the observed peaks were fairly broadened, implying that both cationic and uncharged forms of dibucaine are interacting with erythrocyte membranes, as expected. However it was only the cationic form of dibucaine that showed NOE cross-peaks between the aromatic ring protons and the polar sidechain protons of dibucaine. This result means that the uncharged form of dibucaine is not interacting with the erythrocyte ghosts, as opposed to the suggestion from the line-broadening data. The NOESY experiment has previously been performed at a mixing time of 0.06 s; when we performed the NOESY experiment at a longer mixing time than 0.1 s, NOE cross-peaks due to the uncharged form of dibucaine also appeared. However, these cross-peaks can be ascribed to the uncharged form of dibucaine in a buffer solution which correspond to those observed in Fig. 3. The NOE connectivities observed in Fig. 4 are summarized in Table I.

A similar phenomenon was also seen in the case of an egg PC vesicle solution at pH 7.4 which contains 3 mM

dibucaine. Figure 5 shows the NOESY spectrum observed at 348 K by using a mixing time of 0.3 s. Evidently, only the cationic form of dibucaine showed NOE cross-peaks between the aromatic proton at the 3-position and the peaks labelled F, G, D and C. Figure 6 shows a NOESY spectrum of an aromatic proton region of the same solution observed at a mixing time of 0.1 s. Interestingly, this NOESY spectrum showed clear exchange cross-peaks between the aromatic protons of the cationic and uncharged dibucaines, *i.e.*, between 3-3′, 5-5′, 6-6′, 7-7′, and 8-8′ protons. These observations lend further support to the view that the newly appeared peaks are due to the uncharged form of dibucaine. The linebroadening mentioned for Fig. 4 thus appears to be due to the exchange-induced linebroadening between the cationic and uncharged dibucaines.

Figure 7 shows ^{1}H-NMR spectra of dibucaine in the absence (a and c) and in the presence (b and d) of erythrocyte ghosts; spectra a and b were observed at 300 K and spectra c and d were observed at 348 K. Evidently, the peaks due to the cationic form of dibucaine shifted upfield as a result of interaction with the membranes; in contrast, no chemical shift changes were noted for the peaks due to the uncharged form of dibucaine, which

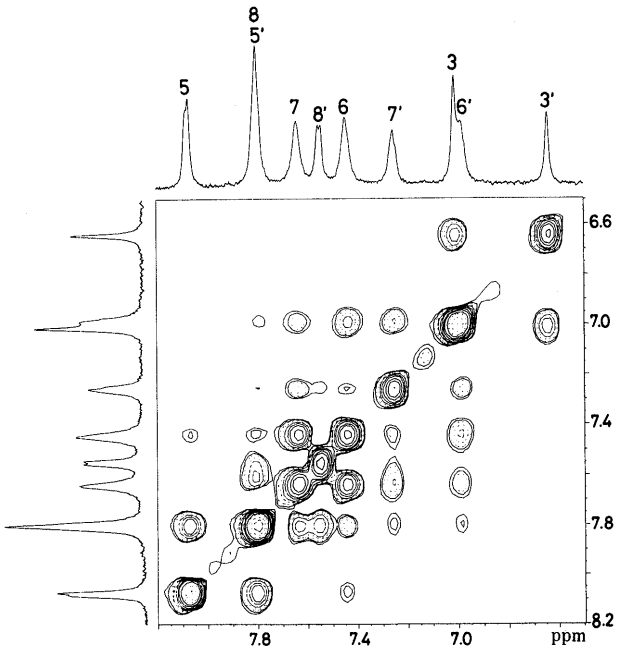

Fig. 6. 600 MHz NOESY Spectrum of Dibucaine (3 mM) in Egg PC Vesicles (3 mM) Solution in a Phosphate Buffer (pH 7.4) at 348 K; Mixing Time, 0.1 s

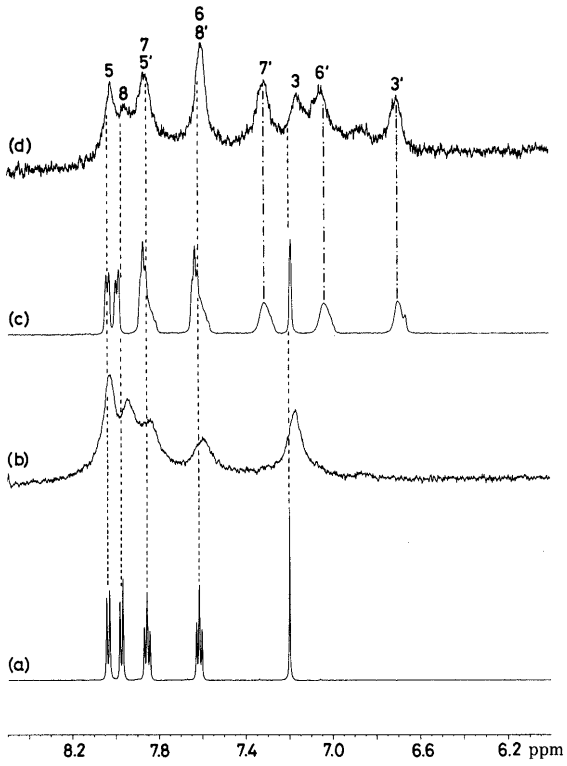

Fig. 7. (a) and (c): 600 MHz ^{1}H-NMR Spectra of Dibucaine (3 mM) in a Phosphate Buffer (pH 7.4) at 300 and 348 K, Respectively. b and d: 600 MHz ^{1}H-NMR Spectra of Dibucaine (3 mM) in Pig Erythrocyte Ghosts Suspended in a Phosphate Buffer (pH 7.4) at 300 and 348 K, Respectively

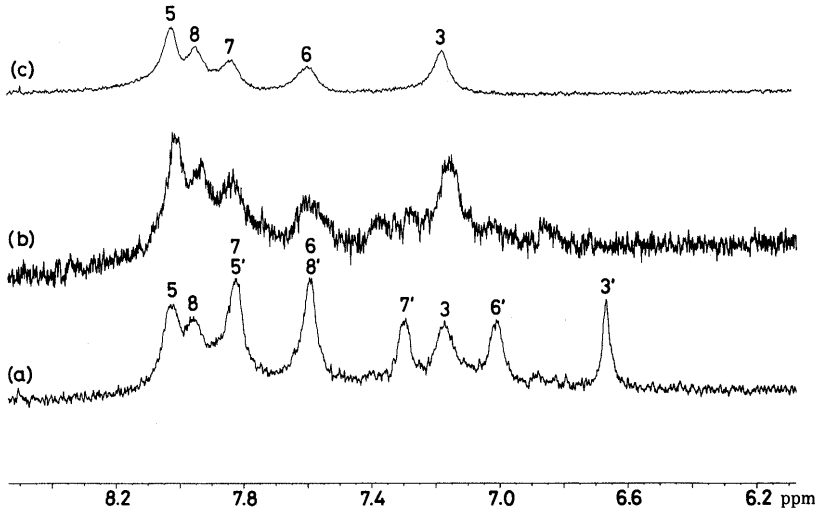

Fig. 8. (a) 600 MHz ^{1}H-NMR Spectrum of Dibucaine (3 mM) in Pig Erythrocyte Ghosts Suspended in a Phosphate Buffer (pH 7.4) at 318 K; (b) ^{1}H-NMR Spectrum of the Same Solution as in (a) at 318 K, but Observed 2 d Later; (c) ^{1}H-NMR Spectrum of the Same Solution as in (b), but Observed on Lowering the Temperature to 300 K

appeared on raising the temperature to 348 K. The same trend was also noted for the remaining resonances of dibucaine which appeared at a higher field. These results also mean that the uncharged form of dibucaine is not interacting with the membranes.

Figure 8 shows the time dependence of the ^{1}H-NMR spectra of a dibucaine-erythrocyte ghost solution at 318 K. Interestingly, the intensities of both the original and newly appeared peaks decreased greatly when the solution was allowed to stand for 2 d (Fig. 8b); particularly, the peaks due to the newly appeared uncharged form of dibucaine disappeared completely. This result does not indicate any decomposition of the dibucaine molecules, because the original peaks which were assigned to the cationic form of dibucaine reappeared on lowering the temperature of this solution to 300 K (Fig. 8c). The disappearance of the NMR peaks when the solution stood for 2 d at 318 K can be interpreted as being due to the accumulation of both forms of dibucaine into the erythrocyte membranes.

Inspection of the NOE distance constraints summarized in Table I informs us that a dibucaine molecule taking only one conformation cannot fully satisfy those constraints. We should consider more than two kinds of conformation. Moreover, we should consider the association of dibucaine molecules, as discussed in the case of dibucaine which is interacting with egg PC vesicles.[7] Because of the restraints required from the molecular structure of dibucaine, the following NOEs should be ascribed to intermolecular dipole-dipole interactions in origin: Ph-5–CH_3, Ph-8–$NH^+(C–CH_3)_2$, and Ph-8–$NH^+(CH_2–C)_2$. It is unlikely that spin diffusion[16,17] propagated intramolecularly caused these NOE cross-peaks, because no cross-peak was detected due to the intervening protons of these proton pairs. We considered the following three kinds (A—C) of conformation for a dibucaine molecule.[7] In conformation A, the diethylamino group is directed toward the Ph-3 proton, while the butoxy group is directed toward the Ph-8 proton; in conformation B, the diethylamino group is directed toward the Ph-5 proton, and the butoxy group toward the Ph-3 proton; finally, in conformation C, the diethylamino group is directed toward the Ph-5 proton, while the butoxy group is directed toward the Ph-8 proton. Calculations were made for both the trans and *cis* forms of the amide linkage of dibucaine. Moreover, we assumed that the associated dibucaines form a dimer. This assumption would be rationalized, because the stacking interaction between the two quinoline rings is expected to facilitate the formation of a dimer. By taking these points into consideration, we performed restrained molecular dynamics calculations; this method of calculation incorporates NOE distance constraints into the mechanical force fields of the dibucaine molecule, including bond stretch, angle bend, torsion, electrostatic, van der Waals and hydrogen bond energies. Thus calculated total mechanical strain energies of *trans*- and *cis*-amides of dibucaine and their dimers are summarized in Table II. The computer graphics pictured for energetically minimized dibucaine dimers are shown in Figs. 9 and 10, respectively, for the cases of the *trans*-amide of conformations A and B. As can be appreciated from the stabilization energy, ΔE, which is defined as the difference between twice the energy value of a monomer and the energy value of a dimer (Table II), the reduction of total energy as a result of the stacking interaction is evident for all the conformations considered here for a *trans*-form of amide linkage of dibucaine, but not always true for a *cis*-form of the amide linkage.

TABLE II. Calculated Meachanical Strain Energies of *trans*- and *cis*-Amides of Dibucaine and Their Dimers

Type of conformer	*trans*-Amide[a]			*cis*-Amide[a]		
	Monomer	Dimer	ΔE[b]	Monomer	Dimer	ΔE[b]
A	104.3	166.3	−42.3	112.7	175.1	−50.3
B	104.9	167.3	−42.5	103.5	220.5	+13.5
C	95.3	172.6	−77.3	97.6	240.6	+45.4

a) Energy (kcal/mol). *b*) ΔE = the stabilization energy, which is calculated by subtracting twice the energy value of a monomer from the energy value of a dimer.

Discussion

Since tertiary amine local anesthetics possess a hydrophobic aromatic ring, they are amphiphilic in nature. They can exist as both cationic and uncharged molecules, the proportion of which depends on the pK_a of the drug and the pH of the solution. To initiate the blockade of conduction in peripheral nerves using a local anesthetic, we must saddle the drug with a task to cross the two barriers constructed by the nerve sheath and nerve membrane which bears a protein known as a sodium channel. Since those barriers are hydrophobic in nature against polar amphiphilic chemicals, it seems reasonable to consider that it is the uncharged form of a local anesthetic that can penetrate into the membrane toward the inner monolayer. A drug which can arrive inside the nerve membrane can act in its cationic form as an anesthetic against the sodium channel.[1,2] The present NMR data show that if there exist both cationic and uncharged drugs in a solution, the cationic drug interacts much more strongly with erythrocyte membranes than does the uncharged counterpart. Ritchie *et al.*[18] and Narahashi *et al.*[1] have given clear evidence in their electrophysiological experiments that the active species of tertiary amine local anesthetics is the cationic forms.[2] The binding site has been shown to be an internal site within the ion conduction path for several reasons. For example, the values of anesthetic potency increase by a repetitive stimulation of nerves, *i.e.*, local anesthetics, show a so-called use-dependent block.[2,3] This implies that anesthetics occupy a site which can easily come and go between the binding site, which may be inside the pore of the sodium channels, and the intracellular fluid of nerve membranes. Our NMR data clearly explains why the active species is a cationic form of the local anesthetic, because no interaction with the erythrocyte (Figs. 4 and 7) and egg PC (Fig. 5) membranes was detected for the uncharged form of dibucaine. This extended application of the presently obtained results to the actual nerve membranes which contain sodium channels may be rationalized, because it has been suggested that the sodium channels are surrounded by phosphatidylserine (PS)[19,20]; on the other hand, in the erythrocyte membranes, it is known

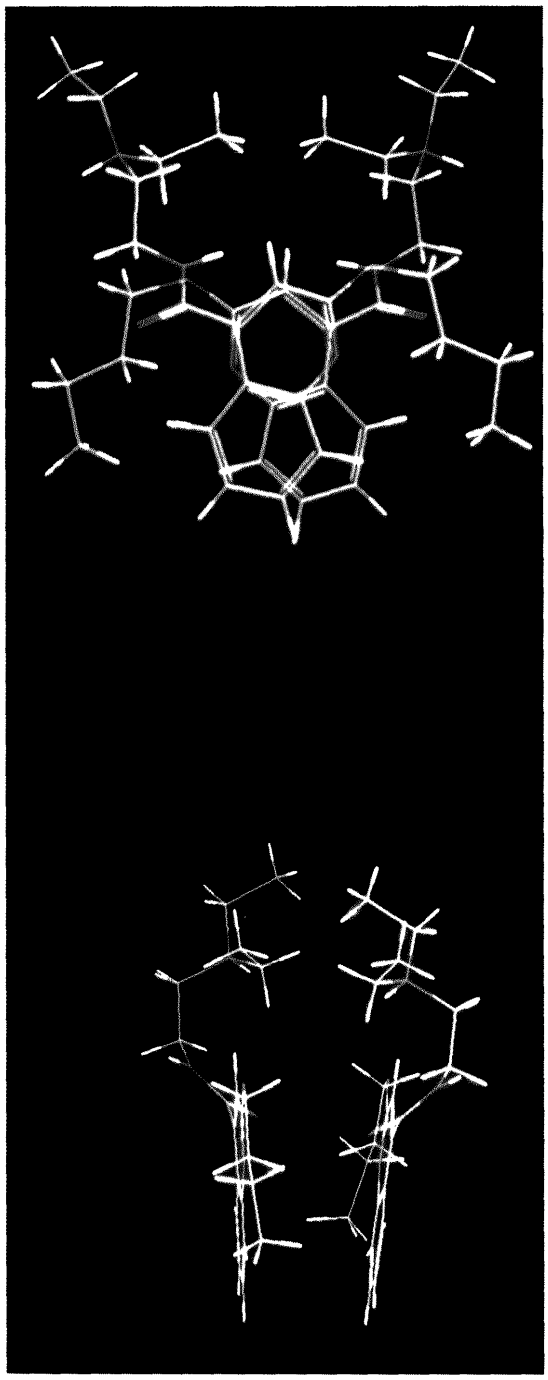

Fig. 9. Computer Graphics View of the Dibucaine Dimer Having Conformation A and the Trans Form of the Amide Linkage; the Lower Picture Shows a Stacking Interaction between the Two Quinoline Rings

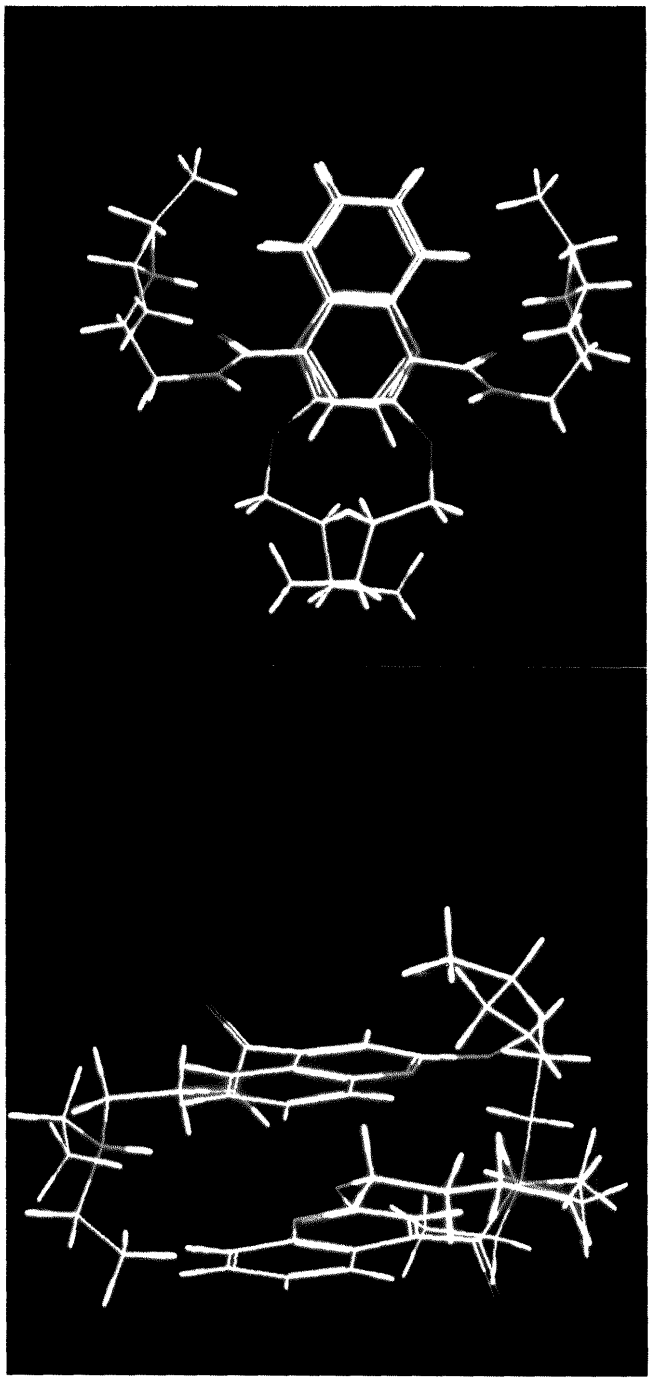

Fig. 10. As in Fig. 9, but with Conformation B

that the PS resides solely inside the bilayer membrane.[21] Since dibucaine is known to partition into the PS at least 20-fold more than into neutral phospholipids,[22] we may consider that the cationic dibucaine would interact mainly with the PS at the inside polar surface of the erythrocyte membranes, forming a dimer (or a larger aggregate) such as those shown in Figs. 9 and 10. The aggregation of cationic forms of local anesthetics was also noted in our previous work for both dibucaine and tetracaine interacting with egg PC vesicles,[7] and for 3 mM RAC-109[23] interacting with PS vesicles (unpublished data). Thus, the aggregate formation seems to be a universal property of tertiary amine local anesthetics for residing comfortably at an amphiphilic binding site; interestingly, our discussion agrees with that by Ueda *et al.*[24] Ueda *et al.* have discussed that the anesthetic molecules do not condense at the interface (between lipids and bulk water) until enough molecules at the interface are close enough together to attract each other.

The present NMR data, on the other hand, bring up a serious question as to whether or not the uncharged forms of local anesthetics really cross the nerve sheath and nerve membrane. We consider that if the uncharged drug

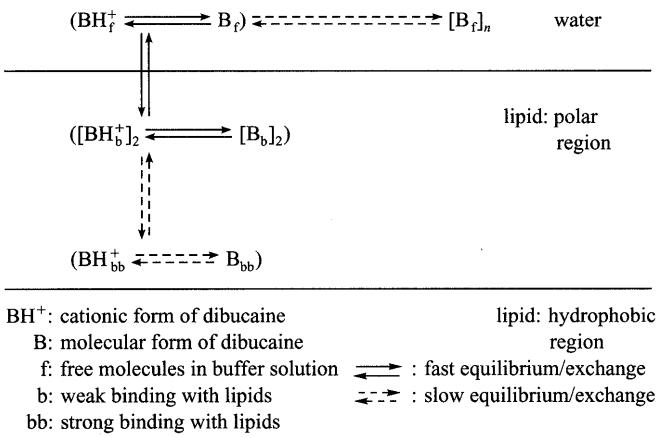

Fig. 11. Scheme of Possible Equilibria of Dibucaine between Water and Lipid Phases

moves across the membrane, it should also reside more or less at its polar surface, and can come and go between the membrane binding site and the bulk water. If this were really so, NOE cross-peaks should appear in Fig. 4, or the chemical shifts due to the uncharged dibucaine should change in Fig. 7. Mayer *et al.* have shown that if there exists an inside negative K^+ diffusion potential, dibucaine can traverse the hydrophobic barrier of the membrane lipids in its cationic form[25]; in this sense, however, our data bring no serious problem.

In order to resolve the conflicting results between the present ^{1}H-NMR data and the widely held view that cationic drugs traverse the bilayer in their uncharged deprotonated form, we propose, in Fig. 11, a scheme for the protonation equilibria and binding equilibria of dibucaine between water and lipid phases. This scheme is a modified version of a three-site exchange model proposed by Boulanger *et al.*[26] for the explanation of the ^{2}H-NMR spectra of a deuterated tetracaine which is interacting with egg PC membranes. In terms of the three-site exchange model, dibucaine is in a slow exchange between strongly and weakly bound sites, while there is a fast exchange between the weakly bound state and free dibucaine in solution. In Fig. 11, we denote the strongly binding species with a suffix "bb", weakly binding species "b", and free dibucaine "f", and the slow exchange by a dotted arrow, while the fast exchange is indicated by a solid arrow. The presently employed ^{1}H-NMR spectra can only show resonances for weakly binding species and free dibucaine in solution. According to Perrin,[13] the temperature coefficient, $-\mathrm{d}(\mathrm{p}K_a)/\mathrm{d}T$, for a $\mathrm{p}K_a$ value of dibucaine at room temperature (8.0—8.5) can be estimated to be 0.024—0.027. Accordingly, if we raised the temperature by, for example, 33 K from the room temperature, the $\mathrm{p}K_a$ of dibucaine is expected to decrease by 0.8—0.9 pK unit, which may result in an increased uncharged form by at least three times as compared to the concentration at the room temperature, as evidenced in Fig. 2. However, as mentioned above, no indications of interactions with the membranes were recognized for thus produced uncharged dibucaine molecules. Two reasonings are conceivable for explaining why the uncharged dibucaine did not interact with or penetrate into the membrane. First, there already existed a saturated amount of the uncharged dibucaine within the membrane, which is denoted as B_{bb} in Fig. 11, and the amount of B_b, if any, was very small. Second, it cannot really bind with the membrane because of some kind of repulsive force from the membrane surface. The first reasoning, however, can be denied from the observations in Fig. 8, where we noted the disappearance of both cationic and uncharged dibucaine when the solution stood at 318 K for 2 d; this can be interpreted as increased amount of B_{bb}. In the second reasoning, we noticed that the uncharged dibucaine which was produced by raising the temperature was forming micelles in some way, as mentioned for Figs. 2 and 3. The formation of micelles seems reasonable and can be explained as follows: on raising the temperature, an excess amount of uncharged dibucaine, B_f, which cannot partition instantaneously into the membrane, was created because of a very slow exchange rate between the weakly binding and strongly binding sites (Fig. 11). The majority of these neutral dibucaine molecules cannot help forming thermodynamically metastable micelles, $(B_f)_n$, in a buffer solution, leaving only a small amount of B_f as a monomer, which may be in rapid equilibrium with the cationic counterpart, BH_f^+ (Fig. 11). The micelles thus created can be considered to be surrounded by a so-called structured water. According to Israelachvili,[27] repulsive forces are expected to work between the structured water around the uncharged dibucaine and that surrounding the erythrocyte membranes or egg PC vesicles. Thus, the NMR peaks arising from $(B_f)_n$ showed no indication of the interaction with the membranes. The reason the amount of B_b was very small comes from the very low solubility of B_f; this fact, in turn, resulted in a very slow exchange rate of dibucaine between the weakly and strongly binding sites. For a more thorough discussion regarding the scheme drawn in Fig. 11, however, we should confirm whether or not a tightly binding species is present within the membranes which could not be detected on account of the linebroadening of the peaks in the ^{1}H-NMR spectra. The observations in Fig. 8 imply that both cationic and uncharged dibucaines partition entirely into the erythrocyte membranes, although the partition coefficient (K_p) of dibucaine measured at pH 5.6 and at a room temperature was not as large ($K_p = 33$ for a 3 mM dibucaine, see Experimental). In order to solve these unanswered problems, we observed the ^{2}H-NMR spectra of the deuterated dibucaine at the 3-position in its quinoline ring and have confirmed the existence of the strongly binding species of both cationic and uncharged dibucaines in the erythrocyte membranes; detailed data will be presented in a future article.

Since the solubility of the uncharged forms of local anesthetics is lower than that of their charged counterparts, it can generally be expected that the uncharged molecular forms of local anesthetics constitute micelles in a bulk solution just as in the present case for dibucaine. Micelles thus formed cannot interract with membranes because of the repulsive forces between them. This finding affords a clear reasoning in response to the question of why the cationic local anesthetics, and not the uncharged ones, are the active form for producing a block to nerve conduction.

Finally, it should be stressed that the importance of the

temperature dependence of the pK_a values of tertiary amines seems to not have been fully recognized. Since the pK_a values of the tertiary amines lie in the range 7.5—9, any experiment concerning local anesthetics under a physiological condition, including electrophysiological, biochemical, physicochemical, and spectroscopic experiments, must be performed bearing in mind the changes in the pK_a value with temperature, because the concentration of the cationic or the uncharged species varies logarithmically. This caution also applies to the time when we are ready to use the local anesthetics clinically.

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