Characterization of the Binding of the Local Anesthetics Procaine and Tetracaine to Model Membranes of Phosphatidylethanolamine: A Deuterium Nuclear Magnetic Resonance Study†

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ABSTRACT: The interaction of the local anesthetics tetracaine and procaine with multilamellar dispersions of phosphatidylethanolamine has been investigated by using ^2H NMR of specifically deuterated anesthetics. Tetracaine was found to partition more strongly than procaine into the lipid. The ^2H NMR spectra showed a quadrupole doublet and a narrow line, with the former corresponding to membrane-bound anesthetic and the latter to anesthetic free in solution. The integrated areas of the narrow line and of the doublet correspond to the concentrations of free and bound anesthetic predicted from the K_p values. There is no strong pH dependence for the quadrupole splittings of tetracaine, suggesting a similar depth of penetration into the lipid bilayer over the entire pH range. The data are consistent with a model in which tetracaine acts as a wedge to stabilize the phosphatidylethanolamine bilayer against transition to a hexagonal structure. Procaine is proposed to sit higher in the phosphatidylethanolamine bilayer than does tetracaine. The T_1 values were generally shorter in the membrane than in solution, suggesting slower motions, particularly for the aromatic ring of tetracaine.

The molecular mechanism of local anesthetic action on nerve membranes is not well understood (Trudell, 1980). Several theories have been proposed, involving both specific and nonspecific sites of action (Trudell, 1980; Lee, 1976; Richards et al., 1980), but the wide range of chemical structures exhibiting anesthetic potency suggests a nonspecific site. The observed correlation between anesthetic potency and hydrocarbon solubility suggests a lipid environment as this site of action (Meyer, 1899; Overton, 1901), while a recent model suggests a heterogeneous site of anesthesia which could include both lipid binding and protein binding environments (Trudell, 1980).

The interaction of local anesthetics with model and biological membranes has been studied by a number of techniques, including ESR[1] (Butler et al., 1973; Giotta et al., 1974; Neal et al., 1975; Eriksson & Westman, 1981), high-resolution NMR (Cerbon, 1972), neutron diffraction (Coster et al., 1981), and a novel use of polarized light-absorption spectroscopy (Johannsson & Lindblom, 1981). All these studies suggest that the anesthetic intercalates partially into the lipid bilayer.

A recent ^2H NMR study of the interaction of deuterated tetracaine and procaine with phosphatidylcholine indicated that the local anesthetics exist in both a strongly bound and a weakly bound environment, as well as free in solution (Boulanger et al., 1980). The strongly bound site was characterized by quadrupole splittings arising from deuterium incorporated at several positions on the tetracaine molecule. Tetracaine was found to be intercalated into the bilayer, with a deeper penetration observed at pH 9.5 where the anesthetic is primarily uncharged (Boulanger et al., 1980; Boulanger, 1981). Later work on the effect of attaining a true equilibrium on the ^2H NMR line shape showed that repeated freeze–thaw cycles eliminated the quadrupole patterns observed for PRC-d_4 and TTC-d_6 at most pH values (Westman et al., 1982). (See Figure 1 for the structures of PRC-d_2, PRC-d_4, TTC-d_2, TTC-d_3, TTC-d_6, and TTC-d_9.) Only at pH 5.5 did TTC-d_6 maintain its quadrupole pattern. Temperature studies on this latter sample showed that the quadrupole pattern collapsed to a broad line on heating to 60 °C but reappeared when the temperature was restored to 30 °C (E. C. Kelusky and I. C. P. Smith, unpublished results). For TTC-d_2 in egg PC, at both pH 5.5 and pH 9.5, the quadrupole pattern is reduced in intensity after repeated freeze–thawing; however, it is never totally eliminated, and the narrow line is too wide and too intense to be due to TTC free in solution. The TTC-d_2 spectra also show a temperature dependence, similar to that of TTC-d_6. All these studies tend to suggest that when equilibrium is attained, a three-site exchange is likely but complicated by exchange rates which are of the order of magnitude of the quadrupole splitting.

In order to further our studies of anesthetic–lipid interactions and to seek a simpler, more favorable exchange environment, we have investigated the interaction of specifically deuterated tetracaine and procaine with phosphatidylethanolamine (PE) by ^2H NMR. With PE, the anesthetic spectra are significantly different than those observed in egg PC. There are only two sites for the anesthetic, strongly bound and free in solution, and the exchange rate is slow on the NMR time scale ($\leq 1 \times 10^3$ s^{-1}).

Materials and Methods

Materials. Procaine hydrochloride and tetracaine hydrochloride were purchased from Sigma Chemical Co. PRC-d_2, PRC-d_4, TTC-d_3, and TTC-d_6 were the generous gift of Yvan Boulanger (Boulanger & Leitch, 1981). Deuterium-depleted water, used for all samples, was obtained from Aldrich Chemical Co. Bromobutane-d_9 was purchased from Merck, Sharp & Dohme, Canada; all other materials were analytical grade.

Synthesis. TTC-d_2 was prepared by refluxing a solution of tetracaine in D_2O with DCl as a catalyst (Boulanger & Leitch, 1981). TTC-d_9 was prepared by a method adapted from Boulanger & Leitch (1981) by using bromobutane-d_9.

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[1] Abbreviations: ESR, electron spin resonance; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PC, phosphatidylcholine; BPC, borate–phosphate–citrate buffer; TTC, tetracaine; PRC, procaine; PS, phosphatidylserine.

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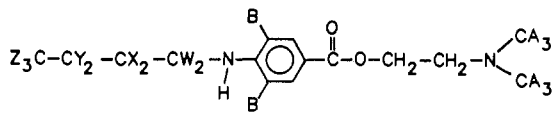
TETRACAINE



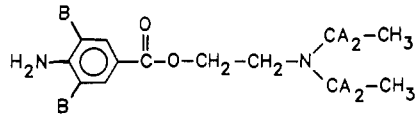
TTC-d_6 A = ^2H ; B,W,X,Y,Z = H

TTC-d_2 B = ^2H ; A,W,X,Y,Z = H

TTC-d_3 Z = ^2H ; A,B,W,X,Y = H

TTC-d_9 W,X,Y,Z = ^2H ; A,B = H

PROCAINE



PRC-d_2 B = ^2H ; A = H

PRC-d_4 A = ^2H ; B = H

FIGURE 1: Structures of the specifically deuterated tetracaines and procaines.

The deuterated anesthetics were recrystallized twice from hot absolute ethanol. The structures of the labeled anesthetics are shown in Figure 1.

Phosphatidylethanolamine was prepared from egg phosphatidylcholine by phospholipase D mediated head-group exchange (Cullis & DeKruiff, 1976). The egg PC was isolated from fresh eggs (Singleton et al., 1965), and partially purified phospholipase D was isolated from fresh Savoy cabbages (Davidson & Long, 1958). The PE was purified by column chromatography on a silicic acid column employing a chloroform/methanol gradient. The fractions containing PE were collected, dried, and reprecipitated from hexane/cold acetone, with special care being taken to avoid exposure to air. Purity was checked by thin-layer chromatography (using a PE standard), and the PE was visualized with both phosphate and ninhydrin sprays. This semisynthetic PE, hereafter called egg PE, has the same fatty acid distribution as the egg PC and is much less susceptible to oxidation than the actual egg PE (which has approximately 15% of the 22:6 fatty acid) (Mantsch et al., 1981).

Sample Preparation. Egg PE, in a chloroform/methanol solution, was dried under a stream of nitrogen to a volume of 0.5 mL. This solution and the deuterated anesthetic were placed in a 10-mm NMR tube with a 5-mm capillary at the open end. The remaining solvent was blown off under a stream of nitrogen, and the sample was dried on a vacuum line for 12 h. Buffer was added, and the NMR tube was sealed under vacuum. The sample was then vortexed extensively and heated above the bilayer–hexagonal transition temperature of 65 °C (Mantsch et al., 1981) to ensure hydration of the PE (Mantsch et al., 1983). In order to attain complete equilibration of the anesthetic, each sample was then subjected to five freeze–thaw–vortex cycles (Westman et al., 1982). Samples prepared in this manner gave very reproducible results and did not change even on standing at 15 °C for several months.

The buffer was a borate–phosphate–citrate buffer (BPC) made up in deuterium-depleted water in order to minimize the HDO signal. The buffer consisted of citric acid (3.8 mM), boric acid (2.9 mM), sodium hydroxide (17.1 mM), and 85% H_3PO_4 (2.4 mM) in ^1H_2O. The pH was adjusted to 5.5, 7.0, and 9.5 with concentrated HCl. The ^2H NMR spectrum of the neat BPC buffer was run under the same conditions as the anesthetic/egg PE samples. The resulting spectrum indicated that HDO contributed 1% to the intensity of the central narrow resonance observed in the ^2H NMR spectra of the deuterated anesthetics.

Partition Coefficients. Partition coefficients (K_p) were measured by dispersing egg PE (100 mg) and anesthetic (5–10 mg) in a BPC buffer (1 mL) at the pH of interest. Samples were heated to 65 °C, vortexed, freeze–thawed, and centrifuged. The concentration of anesthetic in the supernatant was determined spectrophotometrically (TTC, $\epsilon = 2.32 \times 10^4$ cm^{-1} M^{-1}, $\lambda_{max} = 285$ nm; PRC, $\epsilon = 1.87 \times 10^4$ cm^{-1} M^{-1}, $\lambda_{max} = 307$ nm), and the partition coefficient was calculated (Miller & Yu, 1977). Since K_p shows strong ionic strength and concentration dependence (Roth & Seeman, 1972), the values of K_p were determined at concentrations similar to those of the NMR experiments.

^2H NMR. The ^2H NMR spectra were obtained on a Bruker CXP-300 spectrometer operating at 46.063 MHz by using a 10 mm homebuilt probe (R. A. Byrd, unpublished results). Spectra were acquired by using the quadrupole echo sequence (Davis et al., 1976) with full phase cycling of the radio-frequency pulses. Pulse spacing was typically 50 μs, the $\pi/2$ pulse length was 5 μs (10-mm coil), and the recycle time was always greater than $5T_1$. Spectra were acquired on resonance by using quadrature detection but were folded in order to increase the signal-to-noise ratios. In each case the spectra were checked before folding to ensure that the folding did not introduce artifacts in the line shape. T_1 values were determined by using a modified inversion recovery sequence (180°_x–τ–echo). Spectral de-Paking (Bloom et al., 1981) was done on a Nicolet 1280 data station connected to the Bruker ASPECT-2000. Three iterations on 750 data points gave good convergence. Sample temperature was 20 °C unless otherwise indicated.

Results and Discussion

Partition Coefficients. The measured partition coefficients between water and egg PE are the following: TTC, pH 5.5 (46), pH 9.5 (71); PRC, pH 5.5 (3.3), pH 9.5 (4.1). The K_p values are considerably larger for TTC relative to those for PRC, consistent with studies in other lipids (Boulanger et al., 1980; Boulanger, 1980), and arising as a consequence of the hydrophobic butyl tail on TTC. The K_p values are considerably smaller than those observed for egg PC (Boulanger et al., 1980) but are very close to those found for erythrocyte ghosts (Roth & Seeman, 1972; E. C. Kelusky and I. C. P. Smith, unpublished results). Partitioning is also observed to be greater at higher pH, although the differences in K_p at pH 5.5 and 9.5 are smaller than are observed for PC, PS, and PC/PS mixtures (Boulanger, 1980). Because the partition coefficients for positively charged anesthetics decrease at higher free anesthetic concentrations and at higher ionic strength (Roth & Seeman, 1972), we have determined K_p on samples as close as possible in composition to the NMR samples. We have confirmed these values by determining the partition coefficient for several of the NMR samples.

^2H NMR of TTC. The ^2H NMR spectra observed for the specifically deuterated tetracaine species in egg PE are characterized by the presence of a doublet, with quadrupole splitting D_q, and a narrow central resonance. Figure 2 shows the ^2H spectra observed at pH 9.5 and Figure 3 at pH 5.5. The quadrupole splittings observed at pH 5.5, 7.0, and 9.5 are summarized in Table I.

In all cases, the area of the central resonance correlates well with the concentration of TTC in solution concentration predicted from the K_p values. This is in contrast to the studies in egg PC, where the area of the central resonance was significantly greater than predicted from the partition coefficients. As a further check, the free TTC concentration of the NMR

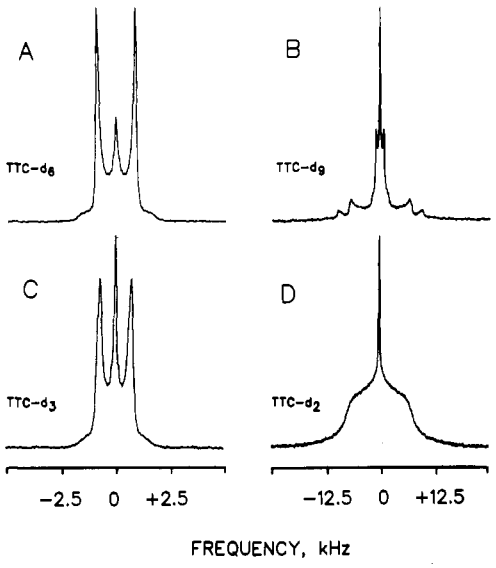


FIGURE 2: ^{2}H NMR spectra of the specifically deuterated tetracaines in egg PE at pH 9.5. (A) TTC-d_6 (48 mM) and egg PE (184 mM) in 0.75 mL of BPC buffer. (B) TTC-d_9 (25 mM) and egg PE (185 mM) in 0.75 mL of BPC buffer. (C) TTC-d_3 (50 mM) and egg PE (184 mM) in 0.5 mL of BPC buffer. (D) TTC-d_2 (24 mM) and egg PE (370 mM) in 0.4 mL of BPC buffer.

Table I: Quadrupole Splittings for Deuterated Tetracaines and Procaines in Semisynthetic Egg Phosphatidylethanolamine (in kHz)[a]

		pH 5.5	pH 7.0	pH 9.5
TTC-d_6		1.85	1.80	1.55
TTC-d_2		12.5	12.8	13.0
TTC-d_9:	α	19.8	20.1	19.9
	β	14.6	14.4	14.6
	γ	13.9	14.0	14.0
	δ	1.60	1.65	1.85
TTC-d_3		1.60	1.65	1.85
PRC-d_4		*b*		*b*
PRC-d_2		9.2		11.4

[a] All spectra show a narrow central resonance. [b] No quadrupole splitting was observed, only a narrow resonance.

samples was determined by spinning down these samples on a centrifuge, separating the aqueous layer, and measuring the TTC concentration spectrophotometrically. The agreement between the two results was good. The ^{2}H NMR spectra of the spun down sample showed the quadrupole doublet with the narrow line almost completely removed. The narrow signal that remained represents the anesthetic in the residual water.

The observed quadrupole splittings show only a weak pH dependence. In fact, it is only at the two end positions of tetracaine, TTC-d_6 and TTC-d_3, that the splittings vary by more than 10%. This is in contrast to the egg PC studies where these positions show a substantial pH dependence (Boulanger et al., 1980; Westman et al., 1982). However, data for TTC/egg PE at pH 5.5 and 7.0 are almost identical, suggesting that the observed pH dependence of the quadrupole splittings is a function of the charge on tetracaine. the pK_A of the tertiary amino group of tetracaine is 7.5 (Boulanger et al., 1980), indicating that the anesthetics are mostly charged at pH 5.5 and 7.0 while almost totally uncharged at pH 9.5.

The splittings for the alkyl chain of tetracaine were obtained by de-Paking (Bloom et al., 1981) the TTC-d_9 spectra. This procedure reduces the spectrum from a powder pattern to a simple doublet corresponding to one angle between the magnetic field and the motional axis. Figure 4 shows the spectrum of TTC-d_9 and the de-Paked result. The central doublet is somewhat distorted, due to the inability to subtract all the

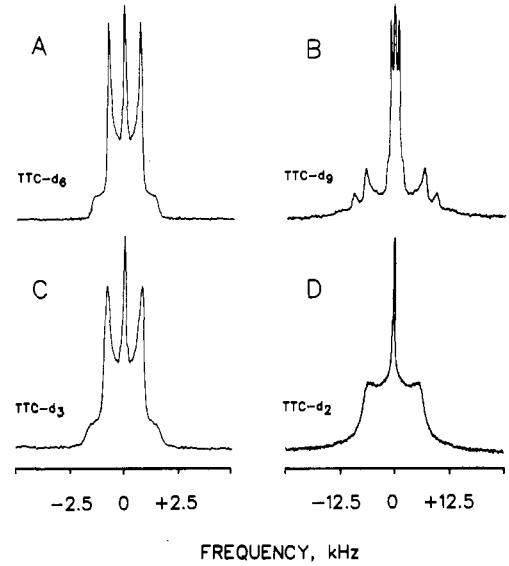


FIGURE 3: ^{2}H NMR spectra of the specifically deuterated tetracaines in egg PE at pH 5.5. (A) TTC-d_6 (48 mM) and egg PE (185 mM) in 0.75 mL of BPC buffer. (B) TTC-d_9 (25 mM) and egg PE (184 mM) in 0.75 mL of BPC buffer. (C) TTC-d_3 (45 mM) and egg PE (185 mM) in 0.75 mL of BPC buffer. (D) TTC-d_2 (24 mM) and egg PE (370 mM) in 0.4 mL of BPC buffer.

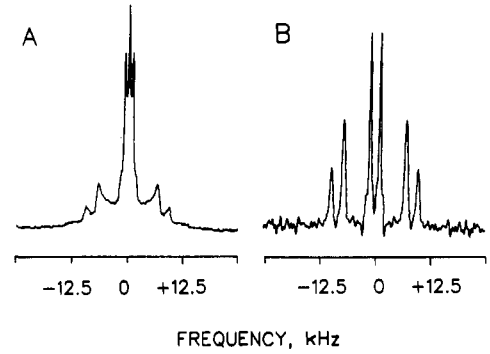


FIGURE 4: ^{2}H NMR spectra of TTC-d_9 in egg PE at pH 9.5. (A) Normal spectrum. (B) De-Paked version of A. The narrow doublet is distorted by an inability to remove the central line prior to de-Paking.

central resonance prior to de-Paking. However, the spectrum does show an approximate 3/4/2 ratio of peak areas, indicating that two of the methylene splittings overlap. We have tentatively assigned the splittings on the basis of a decrease in the quadrupole splitting with position down the butyl chain. The largest splitting (~20 kHz) is assigned to the methylene deuterons adjacent to the nitrogen, and the two, almost overlapping, splittings are assigned to the next two methylene groups. The narrow splitting, which integrates as three deuterons, is assigned to the methyl group, in agreement with the TTC-d_3 spectra. The observed splittings, while large, are less than those observed for specifically deuterated DMPE's in the plateau region (E. C. Kelusky and I. C. P. Smith, unpublished results; Marsh et al., 1983). This could result from the presence of the bulky benzenoid moiety which has a larger excluded volume than a free chain. The butyl group is thus free to undergo larger amplitude motions than the chains of the PE. A tilt in the butyl group could also reduce the splittings.

For TTC-d_2, where the aromatic ring is deuterated, the spectrum at 30 °C is broad, rather featureless, with a width of approximately 13 kHz. The broad nature of the spectra is the result of very large component line widths, corresponding to short T_{2e} values. Attempts to measure T_{2e} were complicated by diffusion, and the decay was not a simple exponential;

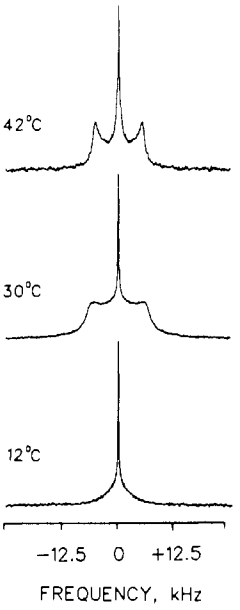


FIGURE 5: Temperature dependence of the ^2H NMR spectrum of TTC-d_2 in egg PE at pH 9.5.

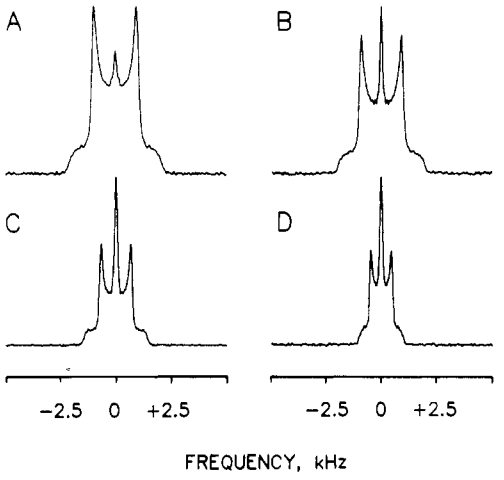


FIGURE 6: Effect of increasing the TTC-d_6/egg PE ratio at pH 9.5: (A) 0.06; (B) 0.13; (C) 0.23; (D) 0.40.

however, T_{2e} appeared to be in the range of 100–150 μs.

In order to explore the ring motion more fully a temperature study was performed (Figure 5). At 5 °C the signal from the quadrupole pattern has almost disappeared, and at −30 °C (not shown) *no* wide line spectrum can be seen. This is a consequence of the short T_{2e}, which is reduced further at lower temperatures to the point where the signal is no longer observed after the echo. At higher temperatures the T_{2e} is increased, resulting in a more clearly resolved quadrupole pattern. Also, at higher temperatures, the central resonance is increased in intensity relative to the quadrupole pattern. This is a result of a lower K_p and an increased water solubility for TTC. At temperatures above 50 °C the spectra are complicated by the presence of nonbilayer structures. These structures, as determined by ^{31}P NMR give rise to single resonances rather than a bilayer pattern (E. C. Kelusky and I. C. P. Smith, unpublished results), and this is reflected in an increase in the ^2H isotropic component.

The effect of varying the TTC concentration in the bilayer was also studied. Figure 6 shows the ^2H NMR spectra of varying ratios of TTC-d_6 and egg PE at pH 9.5. Increasing the anesthetic/lipid ratio, by using smaller quantities of egg PE with constant TTC-d_6 and buffer concentrations, results in a decrease in the quadrupole splitting and an increase in the magnitude of the narrow line. The increase in the narrow component merely reflects the greater relative quantity of aqueous tetracaine relative to tetracaine in the lipid phase. Calculation of K_p from the integrated areas of the spectra shows excellent agreement with each other, and with the spectrophotometrically determined partition coefficient. Since the line width of the narrow component does not increase with increasing anesthetic concentration, the reduction in the quadrupole splitting is just a reflection of increasing disorder in the bilayer introduced by the tetracaine. The lack of change in the line width suggests an exchange rate which is very slow, certainly much less than 10^3 s^{-1}, between bound and free anesthetic.

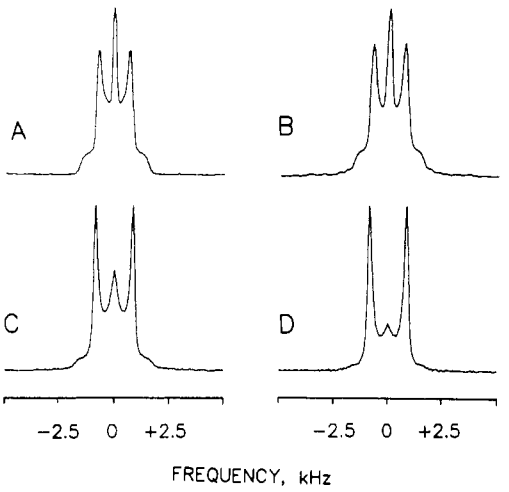


FIGURE 7: Effect of pulse spacing on the ^2H NMR line shape. (A, B) TTC-d_6 at pH 9.5 with pulse spacings of 50 and 1050 μs. (C, D) TTC-d_6 at pH 5.5 with pulse spacings of 50 and 1050 μs.

Perhaps the most noticeable difference in the spectra at different pH values is the observed dependence of T_{2e} on position within the quadrupolar powder pattern (in other words, T_{2e} varies with the angle between the axis of motional averaging and the applied magnetic field). This is manifest by lower intensity (due to incomplete refocusing by the echo pulse) in the central portion of the quadrupole pattern (55° orientation) and at the shoulders (0° orientation). At pH 5.5 all samples show a strong angular-dependent line width, while at pH 9.5 the line width is angular independent. The effects on the spectra vary strongly with the echo pulse spacing (see Figure 7). As the spacing is increased for TTC-d_6 at pH 5.5, the center and shoulders disappear almost completely, while at pH 9.5 the ratio of intensities for the 0°, 55°, and 90° orientations decrease equally.

Recent work (H. J. Jarrell, E. J. Dufourc, and I. C. P. Smith, unpublished results) from this laboratory indicates that the angular dependence of the line width, observed in many ^2H spectra, scales as sin (2θ), where θ is the angle between the axis of motional averaging and the applied magnetic field. This angular dependence may arise as a consequence of diffusion over a curved bilayer surface. It is also possible that TTC at pH 5.5 introduces local curvature in the PE dispersions or that it changes the average particle size.

The TTC-d_6, -d_3, and -d_2 spectra at pH 9.5 were simulated by assuming a two-site exchange model (Figure 8). The exchange between bound and free TTC is assumed to be slow on the ^2H time scale ($\leq 1 \times 10^3$ s^{-1}). This results in spectra which are merely a superposition of the individual free and bound TTC signals, with the populations determined from K_p.

The simulations are of the echo FID, with Lorentzian line shapes, and take into account pulse spacing distortions (Bloom

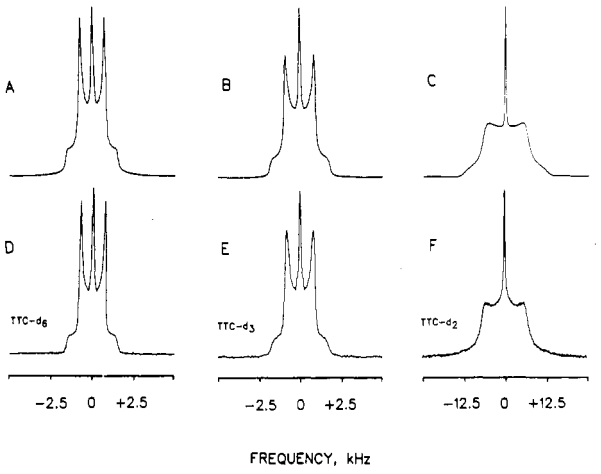


FIGURE 8: Simulations of the following: (A) TTC-d_6; bound $\Delta\nu_Q$ = 1.55 kHz, line width = 45 Hz; free line width = 50 Hz. (B) TTC-d_3; bound $\Delta\nu_Q$ = 1.85 kHz, line width = 125 Hz; free line width = 75 Hz. (C) TTC-d_2; bound $\Delta\nu_Q$ = 13.0 kHz, line width = 2500 Hz; free line width = 150 Hz. All spectra were simulated with 1024 data points, 150 angles, a total echo delay of 125 μs, and the same spectral width used in the experimental spectra. The experimental spectra are shown for (D) TTC-d_6, (E) TTC-d_3, and (F) TTC-d_2.

et al., 1980). For TTC-d_6 and TTC-d_3 the simulations show good agreement with the experimental spectra. However, for TTC-d_2 there is a small percentage of narrow line which is not accounted for. Its total area is 2%, but it requires a much larger line width than is found for TTC-d_2 in solution. This may simply be an artifact of trying to acquire a narrow line with too few data points and a large spectral width. It may be that there is a weak, isotropic association of the free TTC with the PE bilayer surface; however, this should certainly manifest itself in the TTC-d_3 spectrum. Finally it is also possible that it represents a nonbilayer PE structure which binds TTC. There is a small isotropic signal in the ^{31}P NMR of that sample; however, the presence of a phosphate buffer makes an exact determination uncertain.

Order Parameters. The quadrupole splitting observed for a deuteron attached to a rigid structure is given by

$$\Delta\nu_i = \frac{3}{4}\frac{e^2qQ}{h}\left(\frac{3\cos^2\gamma - 1}{2}\right)\frac{3\cos^2\alpha_i - 1}{2} \quad (1)$$

where e^2qQ/h is the static quadrupole coupling constant, α_i defines the fixed angle between the ith C–^{2}H bond and the director for rapid anisotropic diffusion, and γ is the angle which describes the reorientation of the director about its equilibrium position (Taylor et al., 1981). The parentheses denote a time or ensemble average. This former term, known as the molecular order parameter (S_{mol}), describes the anisotropic reorientation of the molecule.

A portion of the tetracaine molecule, from the ester carbonyl to the *p*-amino group, is rigid and can be treated with eq 1. The deuterons on the aromatic ring give rise to only one quadrupole splitting (Figure 5), confirming that the director passes through the 1,4-position of the aromatic ring (Boulanger et al., 1980; Johansson & Lindblom, 1981). The angle α (of eq 1) is therefore 120°, and if the static quadrupole coupling constant is set at 176 kHz (Barnes, 1974), then eq 1 can be rearranged to give

$$S_{mol} = 0.061\Delta\nu_Q \quad (2)$$

where $\Delta\nu_Q$ is in kilohertz.

For the aromatic deuterons of tetracaine, a quadrupole splitting of 13 kHz was obtained, giving S_{mol} of 0.79. This S_{mol} is comparable to those found for TTC-d_2 (Boulanger et al., 1980) and cholesterol (Taylor et al., 1982) in egg PC. It is much greater than that found for the plateau region of the DMPE bilayer (E. C. Kelusky and I. C. P. Smith, unpublished results; Marsh et al., 1983), in part because of the rigid nature of the TTC aromatic ring.

The butyl group of tetracaine will be in the acyl chain region of the PE bilayer, and an estimate of the segmental order parameter, S_{mol}, for each position would be of interest. The butyl chain is attached to the rigid aromatic unit through a nitrogen. In X-ray studies of similar structures such as procaine (Kashino et al., 1982), this nitrogen appears to be sp^2 hybridized, yielding an angle of approximately 120° between the director and the N–C_α bond. This imparts to the butyl group an overall tilt of 24.75° with respect to the director axis. With this tilt, the values of the chain segment molecular order parameter, S_{mol}, can be calculated according to the convention of Stockton et al. (1976).

$$S_{mol} = \frac{S_{CD_2}}{[\frac{1}{2}(3\cos^2 90 - 1)][\frac{1}{2}(3\cos^2 24.75 - 1)]} = 2.71S_{CD_2} \quad (3)$$

$$S_{mol} = S_{CD_3}/[[\tfrac{1}{2}(3\cos^2 109.5 - 1)][\tfrac{1}{2}(3\cos^2 35.25 - 1)][\tfrac{1}{2}(3\cos^2 24.75 - 1)]] = 8.15S_{CD_3} \quad (4)$$

Values of S_{CD} can be found from the equation

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}S_{CD} \quad (5)$$

and used to solve eq 3 and 4.

The values of S_{mol}, calculated for TTC at pH 9.5, are the following: TTC-d_2, 0.79; TTC-d_9, (α) 0.43, (β) 0.32, (γ) 0.30, (δ) 0.12. It is important to realize that these values of S_{mol} are based on the chain tilt arising from the sp^2-hybridized nitrogen. Anything more than sp^2-hybridized (i.e., approaching sp^3) will give a larger tilt and, hence, a greater S_{mol}. However, because of the aromatic ring adjacent to the nitrogen, the latter is likely to be very close to pure sp^2 hydridized. The proton and the butyl carbon α to the nitrogen will therefore be in the plane of the aromatic ring. The values of S_{mol} calculated with a tilt of 24.75° fall off quite rapidly with position and are smaller than those found for the plateau region of the acyl chains in DMPE (Marsh et al., 1983; E. Kelusky and I. C. P. Smith, unpublished results). This is likely a consequence of the larger cross section of the aromatic ring which allows the butyl group some extra freedom of motion.

^{2}H NMR of Procaine. The ^{2}H NMR spectra of PRC-d_2 and PRC-d_4 at pH 9.5 are shown in Figure 9. There is no quadrupole pattern observed for the PRC-d_4 samples, but there is a wide pattern, of low integrated intensity, for PRC-d_2. The low intensity of the quadrupole pattern of PRC-d_2 reflects the very low partition coefficients observed in these systems.

The absence of a quadrupole pattern for the PRC-d_4 samples, at both pH 5.5 and pH 9.5, suggests that procaine sits higher in the bilayer than does tetracaine, since the TTC-d_6 samples show quadrupole splittings. The line width of the PRC-d_4 is larger than the line width of the free anesthetic and of the narrow component of the TTC-d_6 spectra. This suggests a very disordered environment for the procaine "head-group" region relative to that of tetracaine.

The quadrupole splitting for PRC-d_2 shows the most pronounced pH dependence (Table I). The reduced splitting observed at pH 5.5 suggests that procaine sits slightly higher in the bilayer. At pH 9.5, where procaine is primarily uncharged (pK_A = 9.0) (Boulanger et al., 1980), it could penetrate more deeply into the bilayer.

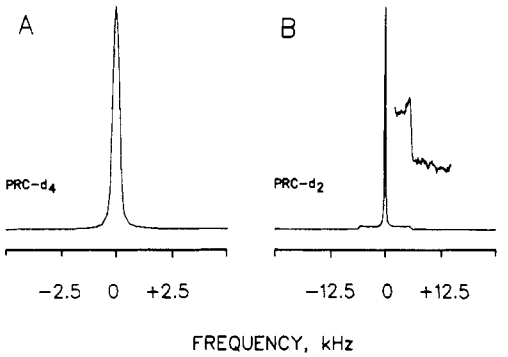


FIGURE 9: ^{2}H NMR spectra of specifically deuterated procaines in egg PE at pH 9.5. (A) PRC-d_4 (50 mM) and egg PE (185 mM) in 0.75 mL of BPC buffer. (B) PRC-d_2 (50 mM) and egg PE (185 mM) in 0.75 mL of BPC buffer.

Table II: T_1 Values for Specifically Deuterated Tetracaines in Semisynthetic Egg Phosphatidylethanolamine (ms)

		pH 5.5	pH 9.5
TTC-d_6	narrow line	60	112
	quadrupole pattern	51	44
TTC-d_2	narrow line	40	20
	quadrupole pattern	4.6	4.5
TTC-d_3	narrow line	140	94
	quadrupole pattern	122	105

^{2}H Spin–Lattice Relaxation Times. The quadrupole splittings observed for the deuterated local anesthetics provide information on the amplitude of the anisotropic motion. The spin–lattice relaxation times (T_1) can give information on the rates of these motions (Brown, 1982; Brown et al., 1979). In particular, T_1 is sensitive to rates of motion with frequencies of the order of 46 MHz, the frequency of the NMR experiments. The ^{2}H T_1 values for TTC-d_6, -d_3, and -d_2 were determined at both pH 5.5 and 9.5 (Table II).

The T_1 values of the narrow lines are slightly smaller than those observed for solutions of TTC alone, and this is likely just a reflection of a more viscous solution. The T_1 values are reduced for TTC in the membrane, with the most significant reductions found for TTC-d_2 at both pH 5.5 and pH 9.5 and for TTC-d_6 at pH 9.5. These smaller T_1 values indicate a generally slower motion in the membrane environment, relative to that of the anesthetic free in solution.

The observed relaxation times for TTC-d_2 in the bilayers are much shorter than those reported for the acyl chains of liquid-crystalline dipalmitoylphosphatidylcholine (Brown et al., 1979). This reflects an increase in the density of ring motions, which have frequencies near the deuterium resonance frequency, on entering the bilayer. In addition, the phospholipid acyl chains may exhibit fast trans–gauche isomerizations which are unavailable to the aromatic ring (Taylor et al., 1983). In the PE bilayer TTC-d_2 shows a T_1 very similar to the T_1 minimum observed for cholesterol, deuterated in the A ring, in egg PC (Taylor et al., 1981, 1982). This indicates similar slow motions for the aromatic ring and the sterol in the bilayer and suggests a correlation time close to the 3.5×10^{-9} s rad^{-1} (Taylor et al., 1982) found for cholesterol at 30 °C ($\omega_0\tau_c \simeq 1$ at the T_1 minimum).

For TTC-d_3 at both high and low pH the T_1 values are large relative to those of the aromatic deuterons. Furthermore, they changed by less than 15% from their solution values on entering the bilayer. This suggests an environment for the terminal methyl group which is deep in the bilayer (Brown & Davis, 1981) and which is not greatly constrained by the PE acyl chains. This likely results from the larger cross-sectional area of the aromatic ring, which pushes neighboring lipids apart, giving considerable freedom to the tetracaine butyl chain.

For TTC-d_6 the T_1 values at pH 9.5 show a substantial reduction on entering the bilayer, reflecting a more constrained environment for the methyl groups. When free in solution the TTC is unprotonated and will interact less with water than does protonated TTC at pH 5.5. The observed T_1 values for the membrane-bound species are larger than the 20–22 ms observed for the α and β deuterons of the ethanolamine head group (Kelusky & Smith, 1983) but are not unexpected for methyl groups. The T_1 for the choline methyl groups of DPPC is 85 ms (Seelig & Seelig, 1977) and that of egg lecithin (from egg PE by methylation) is 50 ms (Stockton et al., 1976).

Conclusions

We have studied the interaction of deuterated tetracaine and procaine with semisynthetic egg PE. The anesthetics are observed to exist in only two environments, strongly bound in the membrane and free in solution. The former is characterized by a doublet and the latter by a narrow line. The exchange of anesthetic between the two sites is very slow on the ^{2}H NMR time scale. The evidence indicates that tetracaine penetrates more deeply into the PE bilayer than does procaine.

There is no significant pH dependence to the quadrupole splittings of TTC, indicating a similarity in the depth of bilayer penetration at all pH values. This is not surprising if one considers the TTC–PE interaction in terms of the relative molecular shapes (Israelachvili et al., 1977; Israelachvili & Mitchell, 1975). Egg PE, because of the small cross-sectional size of the head group relative to the chains, has the shape of a cone. Tetracaine and procaine to a lesser extent act as wedges when they insert into the bilayer. It is this shape dependence that is suggested to be the source of the stabilization of PE bilayers by anesthetic against a transformation to a hexagonal structure (Hornby & Cullis, 1981). Therefore, it is not the charge but the general wedge shape of TTC (determined by the aromatic ring and the butyl chain) that dictates the depth of penetration, and this will be essentially independent of pH.

In the TTC–PC system (Boulanger et al., 1980; Westman et al., 1982), the PC molecules have an essentially cylindrical shape, because of a larger head group. Therefore, the shape of the anesthetic will have little or no bearing on the depth of penetration. Rather, it is the presence of the TTC charge, and its interaction with the PC head group, that appears to govern the anesthetic location. This gives rise to the strong pH dependence for the TTC quadrupole splittings observed in egg PC.

This molecular shape theory may also account for the apparently slow exchange of TTC from the strong binding site of the PE bilayer to the solution. The PE bilayer is stabilized by the presence of the TTC and thus is likely, for thermodynamic reasons, to bind TTC more strongly than the corresponding PC.

The significant difference found for the interaction of TTC and PRC with PE vs. PC or PS, as determined by ^{2}H NMR, is rather interesting. In view of the known preference of PE for the intracellular side of many biological membranes (Op den Kamp, 1979; Van Meer et al., 1981) and the observed increase in effectiveness of amine anesthetics applied to the inside surface of the nerve (Frazier et al., 1970; Hille et al., 1975; Strichartz, 1973), one is tempted to speculate on a TTC–PE interaction as an element in the mechanism of anesthesia. A recent paper on the effect of tetracaine, procaine,

and dibucaine on the topology of rat brain synaptosomes demonstrated a greater perturbation of PE than PS (Bradford & Marinetti, 1982). Recent ^2H NMR work on the effect of dibucaine (Browning & Akutsu, 1982), tetracaine, and procaine (Kelusky & Smith, 1983) on head-group-labeled PE shows a substantial change in the head-group orientation. The addition of dibucaine, and to a lesser extent tetracaine (only the charged form), results in ^2H NMR spectra of the labeled PE which indicate two types of lipids; anesthetic bound and free. This preferential interaction of local anesthetics with phosphatidylethanolamine represents an interesting alternative to the other lipid-based mechanisms of anesthesia; we are currently expanding our studies to examine the effect of tetracaine and procaine on specifically deuterated dipalmitoylphosphatidylethanolamines.

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Registry No. TTC-d_2, 87494-65-3; TTC-d_9, 87508-93-8; tetracaine, 94-24-6; procaine, 59-46-1.

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