Interactions of the Local Anesthetic Tetracaine with Membranes Containing Phosphatidylcholine and Cholesterol: A ^2H NMR Study†

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ABSTRACT: The interactions of the local anesthetic tetracaine with multilamellar dispersions of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and cholesterol have been investigated by deuterium nuclear magnetic resonance of specifically deuteriated tetracaines, DMPC and cholesterol. Experiments were performed at pH 5.5, when the anesthetic is primarily charged, and at pH 9.5, when it is primarily uncharged. The partition coefficients of the anesthetic in the membrane have been measured at both pH values for phosphatidylcholine bilayers with and without cholesterol. The higher partition coefficients obtained at pH 9.5 reflect the hydrophobic interactions between the uncharged form of the anesthetic and the hydrocarbon region of the bilayer. The lower partition coefficients for the DMPC/cholesterol system at both pH values suggest that cholesterol, which increases the order of the lipid chains, decreases the solubility of tetracaine into the bilayer. For phosphatidylcholine bilayers, it has been proposed [Boulanger, Y., Schreier, S., & Smith, I. C. P. (1981) *Biochemistry 20*, 6824–6830] that the charged tetracaine at low pH is located mostly at the phospholipid headgroup level while the uncharged tetracaine intercalates more deeply into the bilayer. The present study suggests that the location of tetracaine in the cholesterol-containing system is different from that in pure phosphatidylcholine bilayers: the anesthetic sits higher in the membrane. An increase in temperature results in a deeper penetration of the anesthetic into the bilayer. Moreover, the incorporation of the anesthetic into DMPC bilayers with or without cholesterol results in a reduction of the lipid order parameters both in the plateau and in the tail regions of the acyl chains, this effect being greater with the charged form of the anesthetic.

The molecular mechanism of the action of local anesthetics on nerve membranes has been extensively studied for many years but is still unclear. Two theories have been proposed for local anesthesia. One is that the local anesthetic partitions into the lipid bilayer and indirectly causes the closure of the sodium channels responsible for nervous conduction (Seeman, 1975). This is referred to as the membrane expansion theory of anesthesia. The second theory proposes that the anesthetic may act to block the sodium channel directly or bind to the protein in such a way as to alter the protein conformation and close the channel (Boggs et al., 1976).

The location of the local anesthetic tetracaine in model membranes of phospholipids and its effects on the order and dynamics of the lipids have been the subject of several studies. Deuterium nuclear magnetic resonance (^2H NMR)[1] is a powerful technique to obtain information on both the degree of order and the molecular dynamics of liquid-crystalline media and has been extensively used on model and natural membranes (Seelig, 1977; Davis, 1983; Smith, 1984). Results obtained by ^2H NMR suggest that tetracaine interacts differently with different phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), depending mostly on the charge and the shape of the lipid studied (Smith & Butler, 1985). Moreover, it has been shown that the charged and uncharged forms of the anesthetic have different partition coefficients in the lipid bilayer. In PC and PE bilayers, the uncharged form is more lipid-soluble due to hydrophobic interactions between the anesthetic and the lipid molecules (Kelusky & Smith, 1983, 1984). For PS, which is negatively charged at the pH studied, the charged form of the anesthetic is more soluble in the lipid bilayer due to electrostatic interactions between the two opposite charges (Kelusky & Smith, 1986).

However, most plasma membranes, especially most excitable plasma membranes, contain a relatively large amount of cholesterol. For example, the cholesterol concentration of the excitable membranes of the garfish (*Lepisosteus osseus*) olfactory nerve is about 27% of the total lipids (Chacko et al., 1976). In the plasma membranes of nerve-ending particles (synaptosomes), the cholesterol concentration is about 29% of the total lipids (Breckenridge et al., 1971). Moreover, it has been shown that cholesterol acts as a regulatory agent with respect to the amplitude of the C–^2H angular fluctuations of the lipid fatty acyl chains: it orders the lipid above the transition temperature (T_c) and disorders it below T_c (Dufourc et al., 1984).

In this study, the interactions of the local anesthetic tetracaine with phosphatidylcholine membranes containing a physiological concentration of cholesterol have been investigated by ^2H NMR from the anesthetic, the lipid, and the cholesterol points of view. The results suggest a different location of the anesthetic in cholesterol-containing systems compared to that of pure PC bilayers, the tetracaine molecule being higher, closer to the aqueous interface of the lipid bilayer, for DMPC/cholesterol membranes. While the local anesthetic has little effect on the order of the cholesterol molecule, it

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TTC, tetracaine; BPC, borate–phosphate–citrate buffer; ^2H NMR, deuterium nuclear magnetic resonance; FT-IR, Fourier-transform infrared.

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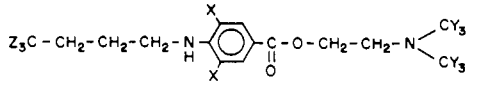
TETRACAINE (TTC)



TTC-$^2H_2$: X=^{2}H, Y= H, Z= H
TTC-$^2H_3$: X= H, Y= H, Z=^{2}H
TTC-$^2H_6$: X= H, Y=^{2}H, Z= H

FIGURE 1: Structures of the specifically deuteriated tetracaines.

decreases that of the lipid acyl chains, this effect being greater with the charged form of the anesthetic. Tetracaine only slightly alters the dynamics of DMPC acyl chains in cholesterol-containing systems as determined by ^{2}H spin–lattice relaxation times.

Materials and Methods

Materials. Tetracaine hydrochloride was purchased from Sigma Chemical Co. [$^2H_2$]TTC, [$^2H_3$]TTC, and [$^2H_6$]TTC were the generous gift of Dr. J. B. Giziewicz (NRCC, Ottawa, Canada). The structure of the labeled anesthetics is shown in Figure 1. Cholesterol was purchased from Steraloids Inc., and [$^2H_5$]cholesterol was a generous gift of Dr. E. J. Parish, Auburn University, Auburn, AL. DMPC was purchased from Sigma Chemical Co., and specifically deuteriated DMPC was synthesized according to published procedures (Perly et al., 1983). Deuterium-depleted water used for all samples was obtained from Aldrich Chemical Co.; all other materials were analytical grade.

Sample Preparation. DMPC and cholesterol (7:3 molar ratio, respectively) were dissolved in chloroform/methanol (2:1 v/v). The sample was dried under a stream of nitrogen and the solvent completely removed under vacuum. The resulting residue was dispersed in 1 mL of deuterium-depleted water and lyophilized overnight. The solid appeared as a fluffy white powder which was hydrated with 1 mL of buffer made with deuterium-depleted water and containing about 10 mg of anesthetic. The sample was then subjected to at least 10 freeze–thaw cycles to ensure complete equilibration of the anesthetic (Westman et al., 1982). Samples prepared in this manner gave very reproducible results. The buffer (BPC) consisted of sodium citrate (0.02 N), sodium phosphate (0.02 N), sodium borate (0.017 N), and sodium chloride (0.1 N) (Boulanger et al., 1980) in deuterium-depleted water in order to minimize the HDO signal. The samples were checked for degradation by thin-layer chromatography after the NMR experiments.

Partition Coefficients. The partition coefficients (K_p) were measured by dispersing the DMPC/cholesterol mixture (100 mg total) and the anesthetic (10 mg) in the BPC buffer (1 mL) at the pH of interest. The samples were heated to about 35 °C, vortexed, freeze–thawed, and centrifuged. The concentration of anesthetic in the supernatant was determined spectrophotometrically (λ_{max} for TTC = 285 nm), and the partition coefficient was calculated (Miller & Yu, 1977).

^{2}H NMR. ^{2}H NMR data were acquired at 30.7 MHz on a "home-built" Fourier-transform spectrometer operated by a Nicolet 1280 computer. Spectra were recorded by means of a modified quadrupolar echo sequence (Davis et al., 1976); quadrature detection was used to record the echo signals. Pulse spacing was typically 60 μs, the $\pi/2$ pulse length was 3.75 μs (10-mm coil), and the recycle time was greater than $5T_{1z}$. Longitudinal relaxation times (T_{1z}) were obtained by the standard inversion recovery sequence coupled with the quadrupolar echo sequence (Dufourc et al., 1984a). The frequency of the spectrometer was carefully set at the center of the quadrupolar powder patterns. Samples were enclosed in a glass jacket where the temperature was regulated to ±0.5 °C. Those spectra which approximated axially symmetric line shapes were "dePaked" according to Bloom et al. (1981) to obtain the 90°-oriented sample spectra from which the quadrupolar splittings were estimated.

Results and Discussion

Theoretical Background. For lipids in lamellar liquid-crystalline phases in which the bilayer normal is an axis of motional averaging, the ^{2}H NMR spectra of C–^{2}H fragments often have the shape corresponding to axially symmetric motion. In this case, the quadrupolar splitting, $\Delta\nu_Q$, between the peaks of the powder spectrum is related to the orientational order parameter, S_{C-^2H}, according to Seelig (1977):

$$\Delta\nu_Q = (3/4)A_Q S_{C-^2H} \quad (1)$$

The static deuterium quadrupolar coupling constant A_Q (where $A_Q = e^2qQ/h$) is 170 kHz for aliphatic C–^{2}H bonds (Burnett & Muller, 1971). Assuming axially symmetric motions of a given molecular subunit of the lipid, the S_{C-^2H} order parameter can be separated according to Seelig (1977) and Dufourc et al. (1983):

$$S_{C-^2H} = \left\langle \frac{3\cos^2\alpha - 1}{2} \right\rangle \frac{3\cos^2\gamma - 1}{2} = S_\alpha S_\gamma \quad (2)$$

where S_γ relates the average orientation of a given C–^{2}H bond of the subunit with respect to the axis of motional averaging and S_α monitors the angular fluctuations of the axis of motional averaging of the subunit with respect to the main axis of motion (usually the bilayer normal). In the present study, the segmental order parameter S_α will be referred to as S_{mol}. For a highly ordered environment, S_{mol} is 1, and it decreases to 0 for an isotropic environment.

Partition Coefficients. The partition coefficient K_p is the ratio of the anesthetic concentration in the lipid phase to that in the buffer (expressed in grams of anesthetic per gram of each phase). 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 in composition as possible to the NMR samples. The measured partition coefficients of tetracaine between DMPC/cholesterol (7:3 molar ratio) and the buffer at pH 5.5 and 9.5 are 8 and 110, respectively. For pure DMPC and egg PC bilayers (Boulanger et al., 1980), the partition coefficients are 21 and 22 at pH 5.5, respectively, and 200 and 600 at pH 9.5, respectively. For PC bilayers with and without cholesterol, the higher partition coefficients at pH 9.5 reflect the strong hydrophobic interactions between the primarily uncharged tetracaine and the hydrocarbon region of the lipids. For the cholesterol-containing systems, the smaller partition coefficients at both pH values indicate that cholesterol, which increases the order of the lipid acyl chains at temperatures above that of the gel to liquid-crystalline phase transition, T_C, decreases the solubility of tetracaine in the bilayer. These smaller K_p values are closer, however, to those found for tetracaine partitioned in bovine spinal cord and in nerves from the walking legs of lobster ($K_p \approx 40$ and 55, respectively) (M. Auger, K. W. Butler, and I. C. P. Smith, unpublished results).

^{2}H NMR of Specifically Deuteriated Tetracaines. The ^{2}H NMR spectra for [$^2H_2$]TTC and [$^2H_3$]TTC in multilamellar dispersions of DMPC/cholesterol (7:3 molar ratio) at pH 9.5 are shown in Figure 2. At high temperature, the spectra are

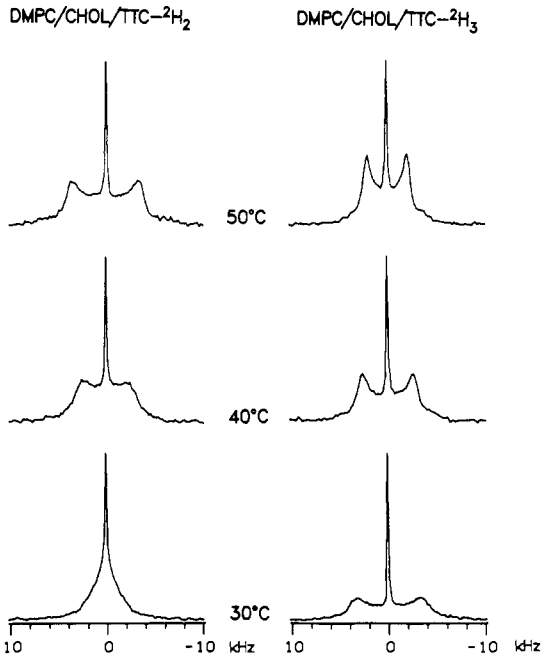


FIGURE 2: Temperature dependence of ^2H NMR spectra of [$^2H_2$]TTC and [$^2H_3$]TTC in multilamellar dispersions of DMPC/cholesterol (7:3 molar ratio), pH 9.5.

characterized by a superposition of a quadrupolar pattern resulting from tetracaine in the bilayer and the narrow line from tetracaine in solution. These spectra are characteristic of axially symmetric motions, and the quadrupolar splittings can be obtained from the dePaked spectra (vide supra). However, at low temperature, the line shapes for the spectra of both [$^2H_2$]TTC and [$^2H_3$]TTC do not reflect axial symmetry. This can result from several possibilities, such as nonaxially symmetric motions, or from an intermediate exchange between tetracaine in solution and tetracaine in the bilayer (exchange rate comparable to the difference in the quadrupolar splittings). However, the spectra do not show a pulse spacing dependence for τ values from 40 to 300 μs in the quadrupolar echo sequence (results not shown), indicating that the exchange between tetracaine in solution and in the bilayer is not intermediate (Wittebort et al., 1987). Quadrupolar splittings for the spectra at low temperature have been estimated from simulated spectra. Spectral simulation was done by using a narrow central resonance and a quadrupolar pattern. From these spectral simulations, it appeared that the intensity of the central resonance needed to reproduce the experimental spectra is too large to result only from tetracaine in the surrounding buffer, according to the partition coefficients (vide supra). However, because of the different spin–lattice relaxation times (T_{1z}) for the isotropic peak and the quadrupolar pattern (about 30 and 3 ms, respectively, at 30 °C), we have been able to use partially relaxed spectra of [$^2H_2$]TTC in DMPC/cholesterol bilayers at pH 9.5 to determine that the intensity of the isotropic peak is about 10% of the total spectrum, which corresponds to the amount of tetracaine in solution, as predicted by the partition coefficient.

The temperature dependence of the quadrupolar splittings, observed at pH 5.5 and 9.5 for [$^2H_2$]TTC in DMPC/cholesterol (7:3 molar ratio), is illustrated in Figure 3. The quadrupolar splittings for [$^2H_2$]TTC at pH 9.5 in pure DMPC bilayers are also shown. The quadrupolar splittings observed at both pH values in the cholesterol-containing system are relatively small and *increase* with an increase of temperature. For pure DMPC bilayers at pH 9.5, the quadrupolar splittings are much larger and *decrease* slightly with an increase of

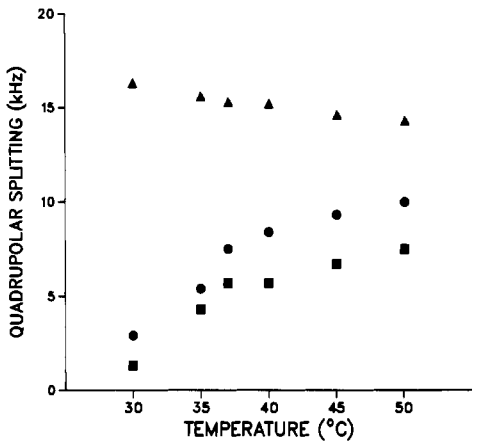


FIGURE 3: Temperature dependence of the quadrupolar splittings for [$^2H_2$]TTC in multilamellar dispersions of (▲) DMPC, pH 9.5, (●) DMPC/cholesterol (7:3 molar ratio), pH 5.5, and (■) DMPC/cholesterol (7:3 molar ratio), pH 9.5.

temperature. The decrease of the quadrupolar splittings for the cholesterol-containing system can be related to a change in the orientational order parameter S_{C-^2H} according to eq 1. In order to get such a change, two possibilities must be considered. The first implies a change of the angle γ between the C–^2H bond and the axis of motional averaging. Since the portion of the tetracaine molecule from the ester carbonyl to the *p*-amino group is rigid, only one quadrupolar splitting is observed for [$^2H_2$]TTC in both systems, with and without cholesterol, which confirms that the angle between the C–^2H bond and the axis of motional averaging is equivalent for both deuterons (Boulanger et al., 1980; Johanson & Lindbloom, 1981). A reduction in the quadrupolar splittings for [$^2H_2$]TTC in these systems can then be interpreted in terms of a reduction of the molecular order parameter S_{mol}. For physical reasons, it seems reasonable to assume that the axis of motional averaging for tetracaine in lipid bilayers passes through or near the 1,4 direction of the aromatic ring. This assumption has also been made in previous studies on the interaction of tetracaine with different phospholipid bilayers (Boulanger et al., 1980; Kelusky et al., 1986; Kelusky & Smith, 1983, 1984). By making this assumption, we can compare relative order parameter values for the different systems studied. The angle γ of eq 2 is 120°, and if the static quadrupolar coupling constant is set to 176 kHz (Barnes, 1974), then a combination of eq 1 and 2 can be rearranged to give

$$S_{mol} = (0.061)\Delta\nu_Q \quad (3)$$

where $\Delta\nu_Q$ is in kilohertz.

For [$^2H_2$]TTC in pure DMPC bilayers at pH 9.5, the values of S_{mol} calculated according to eq 3 vary from 1.00 to 0.86 for temperatures from 30 to 50 °C, respectively. These values are very large in part because of the rigid nature of the TTC aromatic ring but are also higher than the value of 0.93 obtained for [$^2H_2$]TTC in egg PC at pH 9.5 (Kelusky & Smith, 1984). This can be explained by the higher degree of saturation of the DMPC acyl chains compared to egg PC. However, for [$^2H_2$]TTC in cholesterol-containing DMPC bilayers, the values of S_{mol} vary from 0.18 to 0.61 at pH 5.5 for temperatures from 30 to 50 °C, respectively, and from 0.17 to 0.48 at pH 9.5 for the same temperature range. The values at low temperature (30 °C) are much smaller than those obtained for all the phospholipids previously studied (Kelusky & Smith, 1983, 1984; Kelusky et al., 1986) which suggests that this portion of the tetracaine molecule is in a less ordered environment in the cholesterol-containing systems. Moreover, an

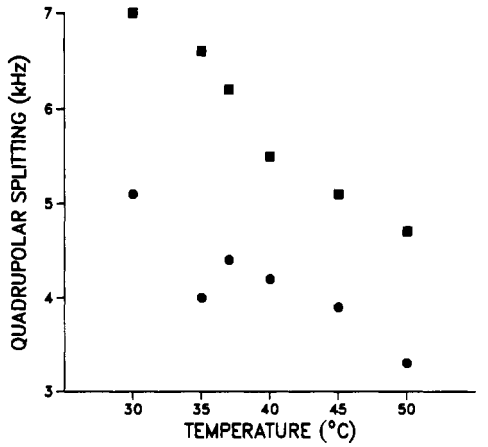


FIGURE 4: Temperature dependence of the quadrupolar splittings for [$^2H_3$]TTC in multilamellar dispersions of DMPC/cholesterol (7:3 molar ratio) at (●) pH 5.5 and (■) pH 9.5.

increase of temperature for these systems induces a change in the environment of the aromatic ring of tetracaine, resulting in increased order. These results may be rationalized as follows. At low temperature, the lipid acyl chains are highly ordered in the cholesterol-containing DMPC bilayers (vide infra) and thus exclude the tetracaine molecule from the hydrophobic region to one near the aqueous interface. An increase in temperature, which decreases the order of the lipid acyl chains (vide infra), would then allow a deeper penetration of the anesthetic in the bilayer, where the aromatic ring of tetracaine would be located closer to the ordered "plateau region" of the lipid bilayer. According to this hypothesis, comparison of the results at pH 5.5 and 9.5 for [$^2H_2$]TTC in DMPC/cholesterol bilayers suggests that the positively charged form of the anesthetic at pH 5.5 is located slightly deeper in the bilayer than the uncharged form, probably because of electrostatic interaction between the phospholipid phosphate and the anesthetic dimethylammonium moieties.

In order to confirm these results obtained with [$^2H_2$]TTC, we have monitored the ^{2}H NMR spectra of [$^2H_3$]TTC in DMPC bilayers in the presence and absence of cholesterol as a function of temperature, from 30 to 50 °C. Figure 2 displays the ^{2}H NMR spectra for [$^2H_3$]TTC in the DMPC/cholesterol (7:3 molar ratio) system at pH 9.5 for three temperatures, from 30 to 50 °C. The temperature dependence of the quadrupolar splittings observed at pH 5.5 and 9.5 for [$^2H_3$]-TTC in this system is also illustrated in Figure 4. At both pH values, the quadrupolar splittings are relatively large and decrease with an increase of temperature. For pure PC bilayers at both pH values, no quadrupolar splittings are observed for [$^2H_3$]TTC (Boulanger et al., 1981; Kelusky & Smith, 1984). According to the model proposed by Boulanger et al. (1981), the terminal methyl group of the anesthetic would be located close to the plateau region of the lipid bilayer. For the cholesterol-containing system, the plateau region is more ordered (vide infra) which could explain the larger quadrupolar splittings obtained for [$^2H_3$]TTC. Moreover, the large decrease in the quadrupolar splittings with increasing temperature suggests a change to a less ordered environment of the terminal methyl group of TTC. This can be explained by a tetracaine molecule sitting higher in the cholesterol-containing bilayers, for which an increase of temperature results in a deeper penetration of the anesthetic in the bilayer into the hydrophobic region which is below the plateau in acyl chain order.

Since the terminal methyl group of tetracaine is in the acyl chain region of the lipid bilayer, an estimate of the molecular order parameter S_{mol} would be of interest. This parameter (S_{mol}) has been calculated by using

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

according to the convention of Stockton et al. (1976). Values of the S_{C-^2H} can be found from eq 1. The values of S_{mol} for [$^2H_3$]TTC calculated from eq 4 vary from 0.35 to 0.21 at pH 5.5 for temperatures from 30 to 50 °C, respectively, and from 0.44 to 0.29 at pH 9.5 for the same temperature range. The relatively large S_{mol} values reflect the constrained environment of the terminal methyl group of the tetracaine molecule in DMPC/cholesterol bilayers. This confirms that the anesthetic is located higher in cholesterol-containing PC layers. The differences in the S_{mol} values for the two pH values also confirm that the charged form of the molecule at pH 5.5 is located slightly deeper in DMPC/cholesterol bilayers than its uncharged form at pH 9.5.

Table I: Quadrupolar Splittings (kHz) for [$^2H_6$]TTC in Different Systems

	pH	$\Delta\nu_Q$
egg PC	5.5	1.8
	9.5	0.0
DMPC/chol (7:3)	5.5	1.4
	9.5	0.8
spinal cord	7.0	1.5

The values of the quadrupolar splittings obtained for [$^2H_6$]TTC in egg PC and DMPC/cholesterol (7:3 molar ratio) at pH 5.5 and 9.5 are summarized in Table I, together with those obtained in spinal cord at pH 7.0 (Smith & Butler, 1985). The values are very small and are essentially independent of the systems studied. This suggests that there is no significant change in the average orientation of this portion of the tetracaine molecule in PC bilayers in the presence and absence of cholesterol.

The differences in the location of the charged and uncharged form of tetracaine in DMPC bilayers in the presence and absence of cholesterol have been confirmed by high-pressure FT-IR studies on these systems (Auger et al., 1987). These studies confirm that the local anesthetic is located close to the aqueous interface of the lipid bilayer in cholesterol-containing DMPC bilayers and that in pure DMPC bilayers the uncharged form of the anesthetic intercalates more deeply than the charged form.

^{2}H NMR of DMPC. (A) Membrane Organization. The effects of the local anesthetic tetracaine on the order and the dynamics of the acyl chains of DMPC in DMPC/cholesterol (7:3 molar ratio) and in pure DMPC bilayers have been studied by using DMPC, ^{2}H-labeled at positions 4 and 14 on the *sn*-2 chain ([4′,4′,14′,14′,14′-$^2H_5$]DMPC). This allows the simultaneous investigation of the effects of the anesthetic on the plateau and the tail region of the lipid acyl chains. The ^{2}H NMR spectrum and the dePaked spectrum of [$^2H_5$]DMPC (Figure 5) exhibit two quadrupolar patterns. On the basis of the spectrum obtained for [4′,4′-$^2H_2$]DMPC, the larger quadrupolar pattern is assigned to the 4′-deuterons (plateau region) while the smaller one is assigned to the three deuterons of the terminal methyl group (tail region). The central resonance is due to residual HDO.

For both the plateau and the tail regions, the quadrupolar splittings have been measured at 30 and 50 °C for seven different systems. The influence of cholesterol on pure DMPC bilayers was investigated and compared with previously re-

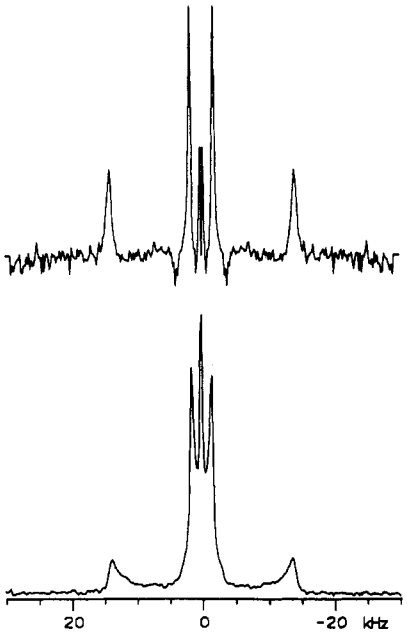


FIGURE 5: ^2H NMR spectrum (bottom) and dePaked spectrum (top) of [4′,4′,14′,14′,14′-$^2H_5$]DMPC at 30 °C.

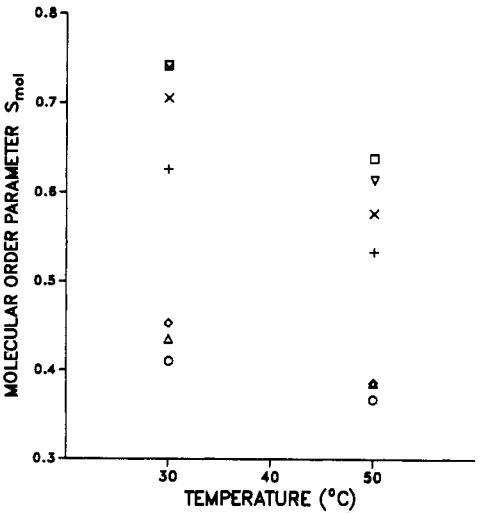


FIGURE 6: Temperature dependence of the molecular order parameter S_{mol} for (◇) [4′,4′,14′,14′,14′-$^2H_5$]DMPC (plateau region) alone and in mixtures of (□) DMPC + cholesterol, (○) DMPC + TTC, pH 5.5, (×) DMPC + cholesterol + TTC, pH 5.5, (+) DMPC + cholesterol + TTC +, pH 5.5, (△) DMPC + TTC, pH 9.5, and (▽) DMPC + cholesterol + TTC, pH 9.5.

ported results (Dufourc et al., 1984b). The effect of both the charged and uncharged forms of tetracaine on the order of the plateau and the tail regions of DMPC bilayers in the presence and absence of cholesterol has also been investigated. For the studies with the charged form of the anesthetic at pH 5.5, the same amount of tetracaine (≈10 mg) was added to pure DMPC and to the cholesterol-containing DMPC bilayers. However, because of the differences in the partition coefficients of the tetracaine in the two systems (vide supra), the experiment for the cholesterol-containing system was repeated under conditions where the equivalent amount of anesthetic was actually partitioned into the lipids. This is referred to as the (DMPC + chol + TTC +) system in Figures 6 and 7.

For DMPC deuteriated at C-4′ (plateau region), the average orientation of a chain methylene C–^2H bond with respect to the axis of motional averaging can be taken to be 90°; i.e., the C–^2H bond angular reorientations are equiprobable around

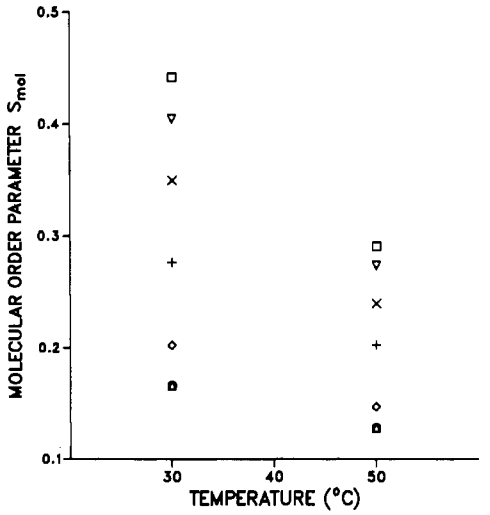


FIGURE 7: Temperature dependence of the molecular order parameter S_{mol} for (◇) [4′,4′,14′,14′,14′-$^2H_5$]DMPC (tail region) alone and in mixtures of (□) DMPC + cholesterol, (○) DMPC + TTC, pH 5.5, (×) DMPC + cholesterol + TTC, pH 5.5, (+) DMPC + cholesterol + TTC +, pH 5.5, (△) DMPC + TTC, pH 9.5, and (▽) DMPC + cholesterol + TTC, pH 9.5.

$\gamma = 90°$ (Seelig & Niederberger, 1974). In this case, the molecular order parameter S_{mol} can be expressed as

$$S_{mol} = |2S_{C-^2H}| \quad (5)$$

where the orientational order parameter S_{C-^2H} is calculated from the quadrupolar splitting values, according to eq 1. The S_{mol} values for the plateau region of DMPC in the different systems studied are shown in Figure 6.

Addition of cholesterol (30 mol %) to pure DMPC bilayers results in a large increase of the molecular order parameter S_{mol} although the temperature dependence remains the same. These results are in agreement with those obtained by Dufourc et al. (1984b) and have been interpreted in terms of a reduction by the cholesterol molecule of the angular fluctuations of the lipids in the plateau region. When tetracaine is added to pure DMPC bilayers at pH 5.5, the S_{mol} values for the C-4′ position are reduced by about 10% at 30 °C and 5% at 50 °C. At pH 9.5, when tetracaine is primarily uncharged, the S_{mol} values are reduced by about 5% at 30 °C but do not change at 50 °C. The effect of tetracaine on the acyl chain plateau region is then to increase the amplitude of motion of the C–^2H segment, which is reflected in a decrease of the order parameters S_{mol}. Moreover, this effect is greater for the charged form of the anesthetic. The same behavior was observed with tetracaine partitioned in deuteriated DPPC bilayers (Boulanger et al., 1981). These results can be rationalized as follows: the electrostatic interaction between the phosphate group of the phospholipid and the dimethylammonium moiety of the anesthetic leads to a higher location of the charged form of tetracaine, greater space between the phospholipid molecules, and a consequent increase in the amplitude of motions for the C-4′ position of the acyl chains. At high pH, the tetracaine molecule partitions more deeply into the bilayer (vide supra) with a concomitantly smaller effect on the motion of the plateau region.

For cholesterol-containing DMPC bilayers, addition of the same amount of tetracaine results in a reduction of the order parameters, S_{mol}, compared to that of the DMPC/cholesterol system. This effect is of the same order of magnitude as in the absence of cholesterol and is less for the uncharged form of the anesthetic. This again can be explained by the electrostatic interactions between the DMPC headgroups and the

charged anesthetic. For the uncharged form of the anesthetic, results obtained with deuteriated tetracaines (vide supra) and by high-pressure FT-IR spectroscopy (Auger et al., 1987) suggest that the uncharged tetracaine is located close to the aqueous interface in cholesterol-containing DMPC bilayers. The "condensing" effect of cholesterol prevents a deeper penetration of the anesthetic in the membrane. This higher location can be responsible for the smaller effect of the uncharged form of the anesthetic on the amplitude of motion of the lipid acyl chain. If more tetracaine is added to the DMPC/cholesterol system in order to have the same amount partitioned into the membrane (compared to the pure DMPC bilayers), S_{mol} for the C-4′ position of DMPC is reduced by about 15% at pH 5.5, which is greater than the reduction observed in pure DMPC bilayers. For the same ratio of lipid to anesthetic, the effect of the charged form of tetracaine is greater in cholesterol-containing DMPC bilayers. The "ordering" effect of cholesterol is partially compensated by the "fluidizing" effect of the local anesthetic in the plateau region of the bilayer.

For DMPC deuteriated at C-14′ (tail region), the S_{mol} values at 30 and 50 °C are represented in Figure 7. These S_{mol} values have been calculated according to Stockton et al. (1976) where the motions of the chain methylene group and the terminal methyl group are related to the same motional axis, i.e., the bilayer normal. To do so, S_{mol} for [14′,14′,14′-$^2H_3$]DMPC can be defined as

$$S_{mol} = |6S_{C-^2H}| \quad (6)$$

In the absence of cholesterol, the S_{mol} values for DMPC deuteriated at C-14′ are very small and decrease by about 18% at 30 °C and 12% at 50 °C on addition of both the charged form and uncharged form of the anesthetic. The presence of cholesterol results in a large increase of S_{mol} as was observed in the plateau region. The S_{mol} values obtained for DMPC/cholesterol (7:3 molar ratio) are in agreement with those obtained by Dufourc et al. (1984b) and confirm that cholesterol decreases the amplitude of motion of the DMPC acyl chains, both in the plateau and in the tail region.

Addition of tetracaine to the DMPC/cholesterol system results in a reduction of the order parameter S_{mol} at C-14′ by about 20% for the charged anesthetic at pH 5.5 and 7% for the uncharged form at pH 9.5. If more charged tetracaine is added in order to have the same amount partitioned into the lipids, the S_{mol} values are reduced by about 35%. The differences observed between the effects of the two forms of the anesthetic in the tail region of the lipid bilayer confirm that the uncharged form is located close to the aqueous interface and does not perturb the acyl chains of DMPC as much as the charged form. Moreover, as observed in the plateau region, the effect of the same amount of charged tetracaine partitioned into the lipids is greater in cholesterol-containing DMPC bilayers. On the other hand, the effect of tetracaine on lipid headgroup conformation has been investigated by Siminovitch et al. (1984) and Browning and Akutsu (1982). These studies suggested that the anesthetic interacts with the polar head group of phosphatidylcholine bilayers.

(*B*) *Membrane Dynamics.* As discussed previously, the deuterium quadrupolar splitting $\Delta\nu_Q$ gives structural information on the average orientation of a particular C–^{2}H bond and on the amplitude of the angular fluctuations of that segment. ^{2}H NMR relaxation rates, on the other hand, yield complementary information about the dynamics of the lipid molecule. A detailed description of relaxation of spin 1 systems in anisotropic media has been given by Jeffrey (1981) and Brown (1982).

Table II: Relaxation Times (T_{1z}, ms) of DMPC in DMPC + chol and DMPC + chol + TTC as a Function of Temperature and Position of Labeling[a]

temp (°C)	DMPC + chol	DMPC + chol + TTC at pH 5.5	DMPC + chol + TTC at pH 9.5
	Plateau Region		
30	18.3 ± 1.5	15.9 ± 0.9	
37	21.6 ± 1.8	20.2 ± 0.6	22.1 ± 1.8
50	25.9 ± 2.3	26.0 ± 0.9	
	Tail Region		
30	189 ± 3	166 ± 4	
37	223 ± 6	196 ± 7	188 ± 6
50	286 ± 8	244 ± 9	

[a] Errors are obtained from the fitting procedure.

Longitudinal (spin–lattice) deuterium relaxation times (T_{1z}) were measured for [4′,4′,14′,14′,14′-$^2H_5$]DMPC in DMPC/cholesterol and DMPC/cholesterol/TTC at pH 5.5 and 9.5. Results for the plateau and the tail regions are presented in Table II. Due to the superposition of two powder spectra, the entire set of partially relaxed spectra was dePaked to facilitate spectral integrations. T_{1z} values were extracted by fitting the equation $M(\tau_1) = M(0)[1 - A\exp(\tau_1/T_{1z})]$ to the integrated areas of the dePaked spectra corresponding to the plateau and the tail regions. $M(\tau_1)$ and $M(0)$ represent the longitudinal magnetization at times τ_1 and $\tau_1 = 0$, respectively. The adjustable parameter A was used to account for imperfections of the 180° inverting pulse. In all T_{1z} measurements, the magnetization varied exponentially with time, within experimental error.

For DMPC/cholesterol systems in the presence and absence of tetracaine, the relaxation times T_{1z} increase with temperature, for both the plateau and the tail regions, suggesting that the relevant molecular motions of DMPC in the temperature range studied are in the short correlation time regime ($\omega_0^2\tau_c^2 \ll 1$) (Brown et al., 1979). The larger T_{1z} values obtained for the tail region relative to those of the plateau region can be rationalized in terms of the faster motion of the terminal methyl group of the acyl chain of the lipid. Addition of tetracaine does not induce significant variations in the relaxation time T_{1z} for the plateau region of cholesterol-containing DMPC bilayers. The T_{1z} values are, however, slightly decreased in the tail region upon addition of tetracaine. If T_{1z} for a specifically deuteriated position of a molecule increases exponentially with temperature, the activation energy E_a^i for the motion dominating the longitudinal relaxation at the frequency and temperature studied can be estimated from the plot of ln T_{1z}^{-1} as a function of $1/T$. The E_a^i values obtained from the data of Table II are 13.9 ± 2.5 and 16.7 ± 0.8 kJ mol^{-1} for the plateau and tail regions, respectively, of [$^2H_5$]-DMPC/cholesterol and 19.6 ± 3.0 and 15.3 ± 1.5 kJ mol^{-1} for the plateau and tail regions of [$^2H_5$]DMPC/cholesterol/TTC, pH 5.5. Within the errors of our measurements, there is no dependence of the activation energies on the position of the deuteriated chain segment. This was also observed by Brown et al. (1979) for deuteriated DPPC. The average activation energy obtained for DPPC was 14.6 ± 1.2 kJ mol^{-1}, which is relatively close to the average obtained in this study for DMPC in a cholesterol-containing system. Moreover, the addition of tetracaine to DMPC/cholesterol bilayers does not induce a significant change in the activation energy.

The results presented here suggest that tetracaine affects the amplitude of the acyl chain motion of cholesterol-containing DMPC bilayers without causing significant changes in the rates of motions with frequencies close to the Larmor frequency.

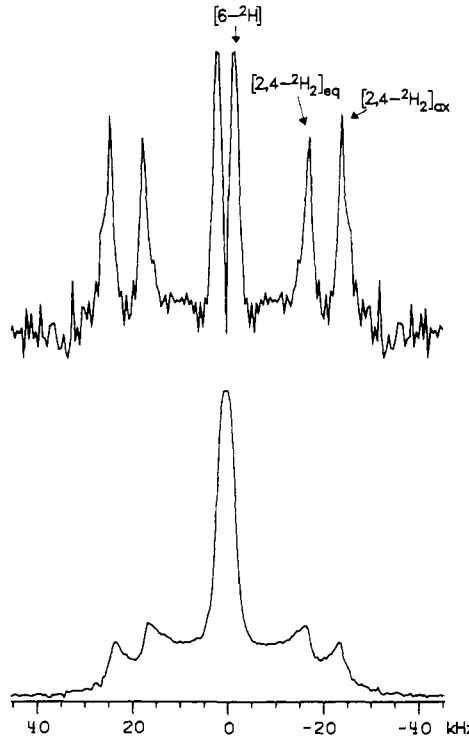


FIGURE 8: ^2H NMR spectrum (bottom) and dePaked spectrum (top) of [2,2,4,4,6-$^2H_5$]cholesterol 30 mol % in DMPC at 30 °C.

^2H NMR of Cholesterol. In order to elucidate the effect of the local anesthetic tetracaine on the orientation and the amplitude of motion of the cholesterol molecule in DMPC/cholesterol (7:3 molar ratio), we have monitored the spectra of [2,2,4,4,6-$^2H_5$]cholesterol in DMPC/cholesterol bilayers in the presence and absence of tetracaine, at pH 5.5 and 9.5, from 30 to 50 °C. The ^2H NMR spectrum and the dePaked spectrum of [2,2,4,4,6-$^2H_5$]cholesterol/DMPC bilayers are shown in Figure 8. For all temperatures studied, the spectra are characteristic of axially symmetric motion. The assignment of each peak has been done according to Taylor et al. (1981). The different quadrupolar splittings associated with deuterons on the rigid sterol nucleus reflect the common molecular order parameter, S_{mol}, of the fused ring system and the inequivalent geometrical orientations of the C–^2H bonds within the molecular frame.

The quadrupolar splitting of about 2.7 kHz associated with the C-6 deuteron of cholesterol does not change significantly with temperature or upon addition of tetracaine, at either pH 5.5 or pH 9.5. The small value of this quadrupolar splitting indicates that the angle, γ, between the C–^2H bond and the axis of motional averaging is close to the "magic angle" of 54.7° for which the 3 $\cos^2 \gamma - 1$ dependence of the quadrupolar splitting collapses. As a result, a very small variation in the orientation of this C–^2H bond with respect to the axis of motional averaging results in a large change of the quadrupolar splitting. Since no significant variation of the quadrupolar splitting for the C-6 deuteron of cholesterol is observed upon addition of tetracaine at both pH values, it seems that intercalation of tetracaine does not induce a change in the orientation of cholesterol in DMPC/cholesterol (7:3 molar ratio) bilayers.

The temperature dependence of the quadrupolar splittings for the (2,4-$^2H_2$)$_{eq}$ and (2,4-$^2H_2$)$_{ax}$ deuterons is represented in Figure 9 for DMPC/cholesterol, pH 5.5, DMPC/cholesterol/TTC, pH 5.5, and DMPC/cholesterol/TTC, pH 9.5. The smallest quadrupolar splittings, attributed to the 2,4-equatorial deuterons, are slightly reduced with increasing

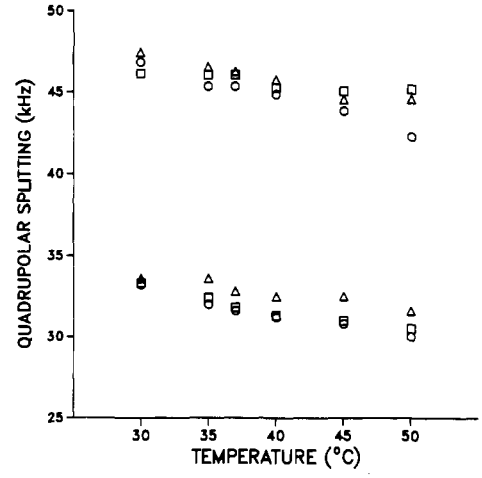


FIGURE 9: Temperature dependence of the quadrupolar splittings for [2,2,4,4,6-$^2H_5$]cholesterol in (△) DMPC + cholesterol, (○) DMPC + cholesterol + TTC, pH 5.5, and (□) DMPC + cholesterol + TTC, pH 9.5.

temperature, reflecting an increase in motional averaging of the quadrupolar interaction. In the presence of tetracaine at both pH values, these splittings are slightly reduced, this effect being greater at pH 5.5 when tetracaine is positively charged. This decrease in the quadrupolar splittings can be related to a change in the orientational order parameter S_{C-^2H}, which according to eq 2 may reflect a change in one or both angular terms. One possibility implies a change in the orientation of cholesterol relative to its motional axis, which would then result in a change of the angle γ (eq 2) for each deuteriated position. However, this possibility is unlikely since no change in the quadrupolar splitting for the C-6 deuteron of cholesterol is observed (vide supra). To confirm that the average orientation of cholesterol is unchanged, the ratios of the observed quadrupolar splittings for the (2,4-$^2H_2$)$_{ax}$ and (2,4-$^2H_2$)$_{eq}$ deuterons were calculated. Since S_{mol} is constant for the rigid steroid nucleus, this ratio, R_k, can be expressed as

$$R_k = \frac{\Delta\nu_i}{\Delta\nu_j} = \frac{3\cos^2\gamma_i - 1}{3\cos^2\gamma_j - 1}$$

assuming equal quadrupolar coupling constants for the various deuterons (Taylor et al., 1981). If the average orientation of the cholesterol molecule remains unchanged upon addition of tetracaine, the ratios R_k should stay constant for the different systems. This is in fact observed for the three systems studied, which confirms the hypothesis that the average orientation of cholesterol is unchanged in DMPC/cholesterol (7:3) bilayers in the presence and absence of tetracaine.

The reduction of the quadrupolar splittings observed in the presence of tetracaine can thus be related to a decrease of the molecular order parameter S_{mol}. In order to quantitate this effect, we will assume that for pure DMPC/cholesterol (7:3) bilayers the order parameter S_{mol} is equal to 0.79 ± 0.03 at 30 °C, as determined by Dufourc et al. (1984b), and decreases to 0.75 ± 0.03 at 45 °C. With these S_{mol} values, the angle between the C–^2H bond and the axis of motional averaging calculated from eq 2 would be approximately 36.3° for the axial deuterons and 41.7° for the equatorial. Assuming no change in the orientation of the cholesterol molecule upon addition of the anesthetic (vide supra), the molecular order parameter S_{mol} decreases by about 2% on average at pH 5.5 and by 1% at pH 9.5. These reductions are very small but constant over all the temperature range. The major effect of tetracaine on the cholesterol molecule in DMPC/cholesterol (7:3 molar ratio) bilayers is thus a small increase of the am-

plitude of angular fluctuations with respect to the bilayer normal.

Conclusions

The present studies demonstrate that the location of the local anesthetic tetracaine in DMPC bilayers in the presence and absence of cholesterol is dependent on the charge of the anesthetic. The uncharged form partitions deeply in pure DMPC bilayers while the presence of cholesterol (30 mol %) "squeezes" it closer to the aqueous interface of the bilayer. The charged form may interact electrostatically with the phospholipid headgroup and induce a greater perturbation of the amplitude of motion of the lipid acyl chains than does the uncharged form, both in the presence and in the absence of cholesterol. In contrast to its influence on acyl chain order, intercalation of anesthetic has little effect on the rates of acyl chain motion, or on the ordering of cholesterol, in the DMPC/cholesterol system.

The results obtained in this study are in agreement with the view that the charged form of local anesthetic is pharmacologically active at micromolar concentrations, as suggested by Aracava et al. (1984). This approach, in conjunction with other techniques, may afford a better understanding of the mechanism of local anesthesia. It is also applicable to the study of intact nerve systems.

Registry No. Tetracaine, 94-24-6; cholesterol, 57-88-5.

References

Aracava, Y., Ikeda, S. R., Daly, J. W., Brookes, N., & Albuquerque, E. T. (1984) *Mol. Pharmacol. 26*, 304–313.

Auger, M., Jarrell, H. C., Smith, I. C. P., Wong, P. T. T., Siminovitch, D. J., & Mantsch, H. H. (1987) *Biochemistry 26*, 8513–8516.

Barnes, R. G. (1974) *Adv. Quadrupole Res. 15*, 335–355.

Bloom, M., Davis, J. H., & MacKay, A. L. (1981) *Chem. Phys. Lett. 42*, 390–394.

Boggs, J. M., Roth, S. M., Yoong, T., Wong, E., & Hsia, J. C. (1976) *Mol. Pharmacol. 12*, 136–143.

Boulanger, Y., Schreier, S., Leitch, L. C., & Smith, I. C. P. (1980) *Can. J. Biochem. 58*, 986–995.

Boulanger, Y., Schreier, S., & Smith, I. C. P. (1981) *Biochemistry 20*, 6824–6830.

Breckenridge, C., Combos, G., & Morgan, I. G. (1972) *Biochim. Biophys. Acta 266*, 695–707.

Brown, M. F. (1982) *J. Chem. Phys. 77*, 1576–1599.

Brown, M. F., Seelig, J., & Haberlen, U. (1979) *J. Chem. Phys. 70*, 5045–5053.

Browning, J. L., & Akutsu, H. (1982) *Biochim. Biophys. Acta 684*, 172–178.

Burnett, L. J., & Muller, B. H. (1971) *J. Chem. Phys. 55*, 5829–5831.

Chacko, G. K., Villegas, G. M., Barnola, F. V., Villegas, R., & Goldman, D. E. (1976) *Biochim. Biophys. Acta 443*, 19–32.

Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.

Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.

Dufourc, E. J., Smith, I. C. P., & Jarrell, H. C. (1983) *Chem. Phys. Lipids 33*, 153–177.

Dufourc, E. J., Smith, I. C. P., & Jarrell, H. C. (1984a) *Biochemistry 23*, 2300–2309.

Dufourc, E. J., Parish, E. J., Chitrakorn, S., & Smith, I. C. P. (1984b) *Biochemistry 23*, 6062–6071.

Jeffrey, K. R. (1981) *Bull. Magn. Reson. 3*, 69–82.

Johansson, L. B. A., & Lindblom, G. (1981) *Biophys. J. 36*, 735–741.

Kelusky, E. C., & Smith, I. C. P. (1983) *Biochemistry 22*, 6011–6017.

Kelusky, E. C., & Smith, I. C. P. (1984) *Can. J. Biochem. Cell Biol. 62*, 178–184.

Kelusky, E. C., Boulanger, Y., Schreier, S., & Smith, I. C. P. (1986) *Biochim. Biophys. Acta 856*, 85–90.

Miller, K. W., & Yu, S. C. T. (1977) *Br. J. Pharmacol. 61*, 57–63.

Perly, B., Dufourc, E. J., & Jarrell, H. C. (1983) *J. Labelled Compd. Radiopharm. 21*, 1–13.

Roth, S., & Seeman, P. (1972) *Biochim. Biophys. Acta 255*, 207–219.

Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.

Seelig, J., & Neiderberger, W. (1974) *J. Am. Chem. Soc. 96*, 2069–2072.

Seeman, P. (1975) in *Molecular Mechanism of Anesthesia, Progress in Anesthesiology* (Fink, B. R., Ed.) Vol. 1, pp 243–251, Raven, New York.

Siminovitch, D. J., Brown, M. F., & Jeffrey, K. R. (1984) *Biochemistry 23*, 2412–2420.

Smith, I. C. P. (1984) *Biomembranes 12*, 133–168.

Smith, I. C. P., & Butler, K. W. (1985) in *Effects of Anesthesia* (Covino, B. G., et al., Eds.) pp 1–11, Waverly, Baltimore.

Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.

Taylor, M. G., Akiyama, T., & Smith, I. C. P. (1981) *Chem. Phys. Lipids 29*, 327–339.

Westman, J., Boulanger, Y., Ehrenberg, A., & Smith, I. C. P. (1982) *Biochim. Biophys. Acta 685*, 315–328.

Wittebort, R. J., Olejniczak, E. T., & Griffin, R. G. (1987) *J. Chem. Phys. 86*, 5411–5420.