High-Pressure Infrared Study of Phosphatidylserine Bilayers and Their Interactions with the Local Anesthetic Tetracaine†

Michèle Auger,‡ Ian C. P. Smith,‡ Henry H. Mantsch,§ and Patrick T. T. Wong*,§
Divisions of Biological Sciences and of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6
Received July 20, 1989; Revised Manuscript Received October 5, 1989

ABSTRACT: High-pressure Fourier-transform infrared (FT-IR) spectroscopy was used to study the barotropic behavior of phosphatidylserine bilayers and their interactions with the local anesthetic tetracaine. The model membrane systems studied were multilamellar aqueous dispersions of 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS) and 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) in the absence and the presence of tetracaine at pH 5.5 and 9.5. The infrared spectra were measured at 28 °C in a diamond anvil cell as a function of pressure up to 25 kbar. The results show that the barotropic behavior of the negatively charged phosphatidylserine bilayers is very similar to that observed for zwitterionic phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, with corresponding acyl chains. The results also indicate that the local anesthetic partitions into phosphatidylserine bilayers in an environment close to the membrane–water interface and interacts electrostatically with the lipid head group. Application of high hydrostatic pressure on the lipid–anesthetic systems results in the pressure-induced expulsion of the anesthetic from a membrane to an aqueous environment. The pressures required for expulsion of anesthetic from bilayers are much higher for the unsaturated lipid (DOPS) than for the saturated lipid (DMPS) (≈6 kbar vs ≈2 kbar, respectively). Whereas incorporation of the anesthetic into DOPS bilayers does not affect significantly the structural and dynamic properties of the disordered acyl chains in the liquid-crystalline phase, it orders the DMPS acyl chains in the gel phase.

The molecular mechanism of local anesthetic action on nerve membranes has been the subject of many studies but is still poorly understood. Local anesthetics are known to exert their action by blocking the sodium channels of nerve membranes. However, whether this blocking is the result of direct anesthetic–protein interaction (Strichartz, 1973) or perturbation by the anesthetic of the lipid matrix surroundings the channels (Seeman, 1975) is still unclear. The interaction of the local anesthetic tetracaine with model membranes has been widely studied by a variety of techniques including electron spin resonance (Butler et al., 1973; Neal et al., 1976), X-ray diffraction (Coster et al., 1981), high-resolution nuclear magnetic resonance (Cerbon et al., 1972), and deuterium nuclear magnetic resonance (Auger et al., 1988a, 1989; Smith & Butler, 1985; Boulanger et al., 1980, 1981; Kelusky & Smith, 1983, 1984). All of these studies suggest that the anesthetic intercalates partially into the lipid bilayer. Moreover, ^{2}H NMR studies have shown that the location of the anesthetic and its effects on the orientational and motional properties of the lipid are dependent on the charge of the local anesthetic and on the charge and shape of the lipid studied.

Recently, high-pressure Fourier-transform infrared (FT-IR)[1] spectroscopy has been used to study the interaction of tetracaine (Figure 1) with phosphatidylcholine model membranes and with myelinated and unmyelinated nerves (Auger et al., 1987, 1988b). This technique has proven to be very useful in the study of structural and dynamic properties of phospholipid bilayers (Wong, 1987a–c; Wong et al., 1988). Two different aspects of the anesthetic–membrane interaction were investigated with this technique. One aspect was to study the effects of the local anesthetic on the structural and dynamic properties of the lipid. On the other hand, it was shown that the application of high hydrostatic pressure to DMPC bilayers can induce expulsion of the charged form of the anesthetic (pH 5.5) while the uncharged form (pH 9.5) is not expelled by pressures up to 25 kbar (Auger et al., 1987). These results have been interpreted in terms of location of the charged form of the anesthetic close to the lipid–water interface due to electrostatic interaction with the lipid head group, with the uncharged form deeper in the acyl chain region of the membrane (Auger et al., 1987). In the presence of a physiological concentration of cholesterol (30 mol %), both the charged and uncharged forms of the anesthetic were expelled by pressures lower than those necessary in the absence of cholesterol. A similar pressure-induced expulsion was also observed in two different nerves, the myelinated frog sciatic nerve and the unmyelinated nerve from the lobster walking leg. The expulsion pressures observed in the nerves at physiological pH (pH 7.4) were, however, higher than those obtained in the cholesterol-containing systems. It is therefore essential to investigate if the high expulsion pressures required for excitable nerve membranes are due to the presence of proteins or of particular lipids.

High phosphatidylserine (PS) levels are found in several preparations of excitable membranes (Camejo et al., 1969; Chacko et al., 1976); data obtained from different tissues suggest that PS is present in greater proportion in excitable tissues (Ritchie & Rogart, 1977) than in nonexcitable ones (Lazdunski et al., 1980). The structure and thermotropic properties of phosphatidylserine bilayers have been shown to be sensitive to the presence of divalent cations such as Ca^{2+}

† Issued as NRCC Publication No. 30760.
‡ Division of Biological Sciences.
§ Division of Chemistry.

[1] Abbreviations: FT-IR, Fourier-transform infrared; PS, phosphatidylserine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; TTC, tetracaine; ^{2}H NMR, deuterium nuclear magnetic resonance; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

0006-2960/90/0429-2008$02.50/0

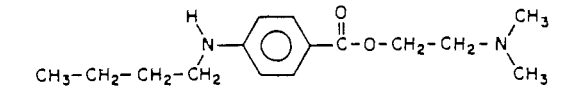

FIGURE 1: Structure of the local anesthetic tetracaine.

and Mg^{2+} (Hauser et al., 1982; Papahadjopoulos et al., 1977). In fact, it has been suggested that negative groups of phospholipid molecules might serve as binding sites for Ca^{2+} as well as for local anesthetic on membranes (Papahadjopoulos, 1970). We have therefore investigated the utility of infrared spectroscopy to monitor the electrostatic interaction between the local anesthetic and the serine head group and to follow the pressure-induced expulsion of the anesthetic from charged lipid bilayers.

Interactions between phosphatidylserine bilayers and monovalent and divalent ions have recently been studied by Fourier-transform infrared spectroscopy (Casal et al., 1987a–c). These studies have concentrated on the thermotropic behavior of the phosphatidylserine bilayers. However, with the addition of high pressure as a variable, new information on lipid structure and dynamics can be gained from studies of their barotropic behavior. In this study, we have monitored the barotropic behavior of several infrared features of fully hydrated phosphatidylserine bilayers containing saturated [dimyristoylphosphatidylserine (DMPS)] and unsaturated [dioleoylphosphatidylserine (DOPS)] acyl chains and compared the results with those obtained previously for the zwitterionic lipids phosphatidylcholine and phosphatidylethanolamine (Wong, 1987a–c; Wong & Mantsch, 1988a; Wong et al., 1986, 1988; Siminovitch et al., 1987a,b). The interaction between the two phosphatidylserine species and the local anesthetic tetracaine was then examined from different points of view, namely, the pressure-induced expulsion of the anesthetic from the membrane, the interaction of the anesthetic with the phospholipid head group, and the effects of the anesthetic on the structural and dynamic properties of the lipids. Finally, the results are compared with those obtained for tetracaine incorporated into the mixed system DOPS/DOPC (1:4 molar ratio), a situation more relevant to that encountered in excitable membranes.

Materials and Methods

Materials. DMPS-Na^+, DOPS-Na^+, and DOPC were obtained from Avanti Polar Lipids (Birmingham, AL), and tetracaine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). All other materials were analytical grade.

Sample Preparation. To prepare lipid dispersions for study by FT-IR, the lipids were hydrated with 50 wt % of D_2O. When the local anesthetic tetracaine was used, the lipids were hydrated with 50 wt % of a borate–phosphate–citrate buffer (Kelusky & Smith, 1984) made with D_2O and containing about 10 wt % of tetracaine with respect to solid lipids (which corresponds to a lipid:anesthetic molar ratio of about 4:1). To ensure the complete equilibration of the anesthetic in the lipid bilayers, the dispersions were subjected to at least five freeze-thaw cycles (Kelusky & Smith, 1984). Small amounts (typically 0.01 mg) of the homogeneous dispersions resulting from the freeze–thaw cycles were then placed at room temperature, together with powdered α-quartz, in a 0.37-mm-diameter hole in a 0.23-mm-thick stainless-steel gasket mounted on a diamond anvil cell, as described previously (Wong et al., 1985).

FT-IR Spectroscopy. Infrared spectra were measured at 28 °C on a Bomem Model DA3.02 Fourier-transform spectrophotometer with a liquid nitrogen cooled mercury cadmium telluride detector. The infrared beam was condensed by a sodium chloride lens system onto the diamond anvil cell. For each spectrum, 512 scans were co-added, at a spectral resolution of 4 cm^{-1} (corresponding to a total measuring time per spectrum of about 10 min). Pressures were determined from the 695 cm^{-1} phonon band of α-quartz (Wong et al., 1985). The frequency of this band was obtained from third-order derivative spectra using a breakpoint of 0.3 in the Fourier domain, and pressures were calculated according to the equation $P = (1.2062)\Delta\nu + (0.015164)\Delta\nu^2$. In order to separate unresolvable infrared band contours, Fourier derivation techniques (Moffatt et al., 1986) were applied. Frequencies associated with the methylene scissoring and rocking modes were obtained from third-order derivative spectra, using a breakpoint of 0.95 in the Fourier domain while those associated with the carbonyl and carboxylate stretching modes were also obtained from third-order derivative spectra but using a breakpoint of 0.3 in the Fourier domain.

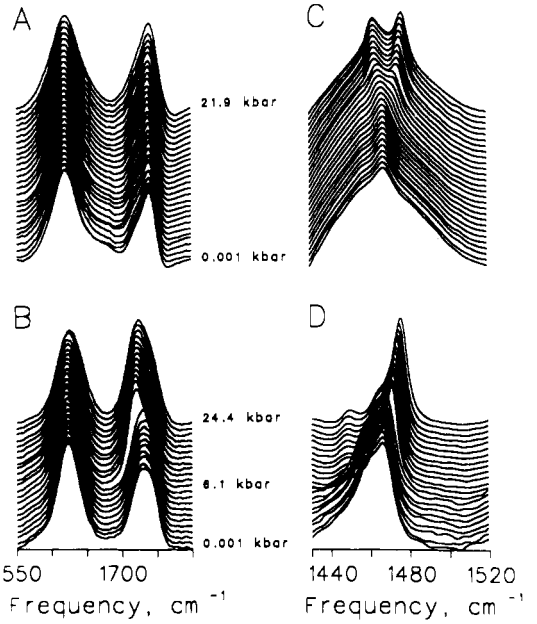

FIGURE 2: Stacked contour plots of infrared spectra of DMPS (A) and DOPS (B) in the region of the C=O and antisymmetric CO_2^- stretching bands and of DMPS (C) and DOPS (D) in the region of the CH_2 scissoring bands. Spectra were recorded at increasing hydrostatic pressure.

Results and Discussion

Pressure Dependence of Infrared Spectra of Pure DMPS and DOPS. In this study, we have compared the pressure dependence of several infrared bands for DMPS-Na^+ and DOPS-Na^+ bilayers for pressures from 0.001 to ≈25 kbar (1 bar ≈ 1 atm). Since the same salt forms were used for both lipids, DMPS-Na^+ and DOPS-Na^+ will be referred to as DMPS and DOPS, respectively, throughout this discussion. Due to the presence of a rigid cis double bond in the middle of each hydrocarbon chain of di-cis-monounsaturated lipids such as DOPS, the structural and dynamic properties of these lipid and their response to external hydrostatic pressure are expected to be different from those of lipids with saturated acyl chains, such as DMPS.

A comparison of the pressure dependence of the infrared spectra of DMPS and DOPS bilayers in the spectral region 1550–1800 cm^{-1} is shown in Figure 2A,B for pressures up to ≈25 kbar. The gel ($L_{\beta'}$) to liquid-crystalline (L_α) phase transition temperature for DMPS is 39 °C (Casal et al., 1987b) while this transition occurs at much lower temperature

for DOPS (−11 °C) (Casal et al., 1987c). At ambient temperature (28 °C) and pressure, DMPS is therefore in the gel phase while DOPS is in the liquid-crystalline phase. The infrared spectra of DMPS and DOPS in the region from 1550 to 1800 cm^{-1} show two bands at approximately 1740 and 1620 cm^{-1}. The first band is associated with the ester carbonyl stretching mode of the lipid acyl chains, which has been shown to give characteristic bands in the spectral region 1725–1745 cm^{-1}. On the other hand, the band at 1620 cm^{-1} is associated with the antisymmetric stretching vibration of the serine carboxylate (CO_2^-) group (Casal et al., 1987a–c). The symmetric CO_2^- stretching vibrations occur between 1390 and 1420 cm^{-1}.

The ester C=O stretching band of DMPS does not show significant variation in shape and intensity as a function of pressure. From the third-order derivative spectra (results not shown), it was determined that the band is in fact composed of two bands at 1741 and 1716 cm^{-1}, which are due to the non-hydrogen-bonded and hydrogen-bonded ester carbonyl group, respectively (Wong & Mantsch, 1988b; Blume et al., 1988; Casal et al., 1987b,c). The two ester carbonyl stretching bands are quite dissimilar in intensity; the band at high frequency is much more intense than that at low frequency. This type of different relative intensities of the two C=O stretching bands has been observed in the spectra of many other lipids in the gel phase (Wong & Mantsch, 1988b; Casal et al., 1987b). Neither the intensity nor the frequency of the two C=O bands varies significantly, indicating that pressures up to 25 kbar do not induce significant conformational changes of the C=O group of DMPS in the gel phase.

On the other hand, the C=O stretching band contour of DOPS shows a dramatic decrease in frequency ($\Delta\nu \approx 10$ cm^{-1}) at a pressure of 6.1 kbar, followed by a steady-increase with increasing pressure. In addition, the half-width of the band is decreased by about 5 cm^{-1} at 6.1 kbar. The pressure at which these changes occur is the critical pressure of the liquid-crystalline to gel transition. The pressure dependences of various vibrational spectral parameters can be used to determine the critical pressures of structural phase transition in lipid bilayers (Wong, 1984, 1986; Wong & Mantsch, 1985a). In particular, the critical pressure of the liquid-crystalline to gel phase transition at ambient temperature in the sample compartment of the FT-IR instrument (28 °C) can be determined for example from discontinuities in the pressure dependences of the infrared frequencies of the ν_{as}, CH_2 and $\nu_s CH_2$ bands (Wong & Mantsch, 1985b). Assuming that this transition is first-order, as it is in DPPC, it follows from the Clausius–Clapeyron relationship that the critical temperature, T_m, can be raised by applying external pressure. Since DOPS is in the liquid-crystalline phase at the ambient temperature of the diamond anvil cell (28 °C), elevation of pressure should induce the transition to the gel phase.

Monitoring the frequency of the ν_s CH_2 mode at 2850 cm^{-1}, we observed an abrupt drop in frequency at 6.1 kbar in DOPS (results not shown), indicating that this is the critical pressure at 28 °C. The changes observed in the C=O stretching band of DOPS at 6.1 kbar are therefore associated with the transition from the liquid-crystalline to the gel phase. Similar behavior has been observed for dioleoylphosphatidylcholine (DOPC), with a transition at 5.2 kbar (Siminovitch et al., 1987a, 1988; Wong & Mantsch, 1988a). The slightly higher critical pressure observed for DOPS compared to DOPC (6.1 and 5.2 kbar, respectively) is somewhat surprising since the phase transition temperature for DOPS is slightly higher than that of DOPC (−11 and −16 °C, respectively). However, these results may be rationalized in terms of the higher repulsion between the charged PS head groups compared to that for the zwitterionic PC head group. This would imply that for the charged lipid, a higher pressure is required to achieve the highly ordered packing observed in the gel phase (vide infra).

The spectra of DOPS in the C=O stretching region (Figure 2B) show that at all pressures, there are at least two components in the C=O band contour of DOPS. In the liquid-crystalline phase, the broad band can be resolved into two components at 1743 and 1724 cm^{-1}, which are due to the C=O modes of the non-hydrogen-bonded and hydrogen-bonded C=O groups, respectively (Wong & Mantsch, 1988b; Blume et al., 1988). In fact, it has been suggested that the high-frequency component originates mostly from the stretching vibration of the *sn*-1 C=O groups while the low-frequency component originates from the *sn*-2 C=O groups (Wong & Mantsch, 1988b). Above 6.1 kbar in the gel phase, the intensity of the high-frequency C=O band is considerably reduced, and the low-frequency band C=O band is shifted from 1724 to 1718 cm^{-1}. These results suggest that above the critical pressure, the environment of the *sn*-1 C=O group changes and becomes similar to that of the *sn*-2 C=O group, the *sn*-1 C=O group moving closer to the bilayer interface (Wong & Mantsch, 1988a). This conformational change of the glycerol backbone therefore permits adjacent packing of the double bonds of the *sn*-1 and *sn*-2 chains.

The spectral parameters and their pressure dependences observed for DOPS suggest that at high pressure, the cis double bond of the *sn*-1 oleoyl chain has to move and align itself with that of the *sn*-2 oleoyl chain. This is achieved by rotation of the C–C bond in the glycerol moiety. Spectral parameters other than the C=O stretching band which confirm this packing (Wong & Mantsch, 1988a; Siminovitch et al., 1987a) have also been observed for DOPS. The similar results obtained for both DOPC and DOPS indicate that the special packing properties observed in 18:1 unsaturated lipids are relatively independent on the nature of the lipid head group.

In contrast to the C=O stretching band, the barotropic behavior of the antisymmetric stretching vibration of the serine carboxylate group (ν_{as} CO_2^-) of both DMPS and DOPS is very similar, as shown in the spectra of Figure 2A,B. The DMPS spectrum shows a band at 1619 cm^{-1}, a frequency value characteristic of a well-hydrated carboxylate group in the gel phase (Casal et al., 1987b). The frequency of this band does not vary significantly with pressure up to ≈25 kbar, suggesting that the carboxylate group is not significantly altered by the application of hydrostatic pressure on gel-state DMPS bilayers. For DOPS, the frequency of the ν_{as} CO_2^- band is 1623 cm^{-1} in the liquid-crystalline phase and decreases to 1620 cm^{-1} in the gel phase, at pressures above 6.1 kbar. These frequencies for the antisymmetric CO_2^- stretching mode are again indicative of a well-hydrated carboxylate group, in both the gel and liquid-crystalline phase of DOPS (Casal et al., 1987c).

A comparison of the pressure dependences of the methylene scissoring mode δCH_2 in the spectra of DMPS and DOPS shown in Figure 2C,D reveals that the barotropic behavior of this band is very different in these two lipids. On one hand, in the spectrum of DMPS, we clearly observe a pressure-induced correlation field splitting at pressures above 3.7 kbar. The pressure is similar to that at which the correlation field splitting is observed for DMPC (3.2 kbar) (Auger et al., 1988b). This pressure-induced correlation field splitting of the CH_2 scissoring band is the result of interchain interactions between the lipid hydrocarbon chains (Snyder, 1961). These

interactions can be intramolecular or intermolecular, the former being predominant at lower pressure. The observation of only a single CH_2 scissoring band at atmospheric pressure reflects the fact that under those conditions of temperature and pressure, the orientation of the methylene chains is highly disordered due to significant reorientational fluctuations of the acyl chains. Increasing pressure leads to a damping of these reorientational fluctuations and an increase in interchain interactions, which give rise to the observed correlation field splitting.

On the other hand, the correlation field splitting band of the CH_2 scissoring mode, δCH_2, is absent in the spectrum of DOPS (Figure 2D), although the band shifts to higher frequencies and intensifies at the critical pressure of 6.1 kbar. The absence at high pressure of a correlation field splitting of the δCH_2 mode was also observed in the spectra of DOPC (Siminovitch et al., 1987a) and dioleoylphosphoethanolamine (DOPE) (Wong et al., 1986) bilayers. These results can only be consistent with an equivalent parallel orientation of the acyl chains within each of the lipid molecules, which are packed in a highly ordered and rigid lattice. In this highly ordered gel phase, the reorientational fluctuations of the acyl chains are completely damped, and the chains are highly extended with a bent conformation at the cis double bond (Wong & Manstch, 1988a). As was observed for the C=O stretching region, the similarities between the barotropic behavior of the CH_2 scissoring region for different dioleoyl glycerolipids indicate that the packing properties of these chains are not significantly dependent on the head-group structure.

Interactions of Tetracaine with Phosphatidylserine Bilayers. (A) Pressure-Induced Expulsion of the Local Anesthetic Tetracaine from Phosphatidylserine Bilayers. We have first monitored the pressure-induced expulsion of tetracaine from pure DMPS and DOPS bilayers, for both the charged (pH 5.5) and the uncharged form (pH 9.5) of the local anesthetic. A previous study from our laboratory has shown that the shape and intensity of the carbonyl band of tetracaine can be used to monitor the changes in the environment of the anesthetic, from the bilayer environment to the aqueous media, as a function of the hydrostatic pressure applied on the system (Auger et al., 1987). Specifically, it was demonstrated that in a bilayer environment, the tetracaine carbonyl stretching band is very weak and broad. The expulsion of the anesthetic by pressure from a hydrophobic to an aqueous environment results in a dramatic increase of the band intensity and decrease in bandwidth. Moreover, the band is shifted to lower frequency, from 1696 cm^{-1} in the bilayer environment to 1685 cm^{-1} in the aqueous media.

Following this study, it was found that another infrared band, namely, the tetracaine aromatic band at $\approx$1605 cm^{-1}, can be used to monitor the pressure-induced expulsion of the local anesthetic. Specifically, the frequency of this aromatic band is shifted from $\approx$1605 cm^{-1} to $\approx$1600 cm^{-1} when tetracaine goes from a hydrophobic to an aqueous environment. Whereas the shift in frequency observed for the anesthetic carbonyl stretching band was accompanied by a dramatic change in shape and intensity, the shape and intensity of the tetracaine aromatic band do not vary significantly as a function of pressure. A detailed analysis of these results will be published elsewhere.

Comparison of the infrared spectra in the spectral region 1550–1800 cm^{-1} for pure DMPS (Figure 2A) and DMPS in the presence of charged tetracaine at pH 5.5 (Figure 3A) indicates that at a pressure of 2.1 kbar, the anesthetic is expelled by pressure from the bilayer. This is indicated by the

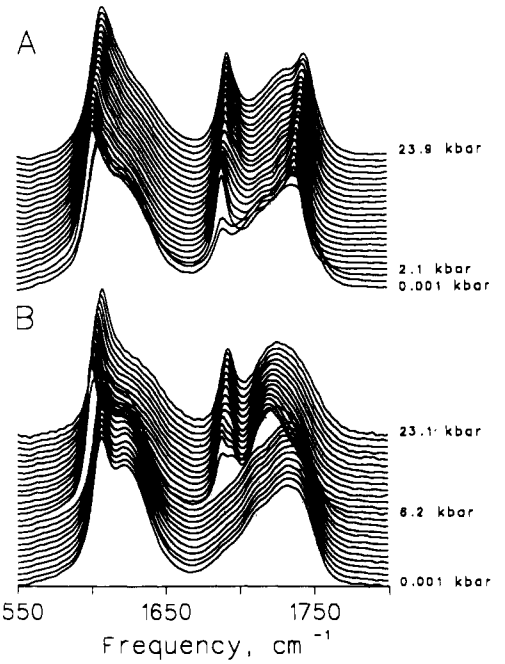

FIGURE 3: Stacked contour plots of infrared spectra recorded at increasing hydrostatic pressure of DMPS (A) and DOPS (B) in the presence of charged tetracaine at pH 5.5 in the region of C=O and $\nu_{as.}$ CO_2^- stretching bands.

abrupt increase in spectral intensity of the anesthetic carbonyl band at 1685 cm^{-1} and by the shift to lower frequency of the aromatic band. A similar behavior is observed at higher pH (pH 9.5), in which case the expulsion pressure is 2.7 kbar (results not shown). At pH 9.5, the local anesthetic tetracaine ($pK_a \approx 8.5$) is mostly in the uncharged form. For uncharged tetracaine incorporated into DMPC bilayers, it was shown that application of pressure up to $\approx$25 kbar could not expel the anesthetic from the bilayer. These results were interpreted in terms of location of the uncharged form deeper in the membrane than the charged form.

The expulsion of tetracaine from DMPS bilayers at relatively low pressure is somewhat surprising since it was expected that the interaction between the negatively charged lipid and the positively charged anesthetic would result in a higher expulsion pressure compared to those obtained for tetracaine in phosphatidylcholine bilayers. However, if the electrostatic interactions between the anesthetic and the serine head group result in location of the anesthetic close to the aqueous interface of the bilayer, a relatively low expulsion pressure could result. The similarities between the pressure-induced expulsion of the charged and uncharged forms of the anesthetic can be rationalized by the fact that the uncharged form can most likely still interact via hydrogen bonding with the serine head group (vide infra). In fact, it was demonstrated that the partition coefficient of the uncharged anesthetic in PS bilayers is still very high, although smaller than that of the charged form (Kelusky et al., 1986), suggesting strong lipid–anesthetic interactions.

In the case of the unsaturated phosphatidylserine (DOPS) bilayers, it was demonstrated earlier that at ambient temperature and pressure in the diamond anvil cell, DOPS is in the liquid-crystalline phase, as opposed to DMPS which is in the gel phase. Inspection of the infrared spectra in the region 1550–1800 cm^{-1} for DOPS in the presence of charged tetracaine (Figure 3B) clearly reveals that at atmospheric pressure, tetracaine is incorporated into the bilayer. A similar behavior was observed for the uncharged form of the anesthetic (results not shown). Spectral features characteristic of the pressure-

induced expulsion of the anesthetic only become apparent at much higher pressure. For the charged form of tetracaine, it appears that the expulsion pressure is in fact the liquid-crystalline to gel phase transition pressure (6.2 vs 6.1 kbar for the expulsion and phase transition pressure, respectively). In the case of the uncharged form at pH 9.5, the expulsion pressure is also very close to the critical pressure, although slightly smaller (5.6 vs 6.1 kbar).

It was demonstrated in the previous study on the interaction of tetracaine with DMPC bilayers (Auger et al., 1987) that the presence of gel phase lipid [pressure-induced (Wong, 1987a)] is not sufficient to exclude the anesthetic, the uncharged form of tetracaine not being expelled from DMPC bilayers at pressures up to 25 kbar. However, as was mentioned previously, the pressure-induced gel phase of DOPS is highly ordered. It therefore appears that this highly ordered phase cannot accommodate the presence of the local anesthetic molecule and that the liquid-crystalline to gel phase transition thus results in the complete expulsion of the anesthetic from the bilayer.

(B) Electrostatic Interactions between Tetracaine and Phosphatidylserine Bilayers. We have investigated whether infrared spectroscopy can be used to probe, at a molecular level, the electrostatic interaction between the phosphatidylserine head group and the local anesthetic tetracaine, and to examine how this interaction is modulated by pressure. At both pH values employed in this study, phosphatidylserine is in the anionic form. Several regions of the serine head group and interfacial zone could therefore interact with tetracaine, in particular the carbonyl groups of the lipid acyl chains close to the membrane–water interface and the negatively charged carboxylate and phosphate groups of the head group. In this study, we have investigated the effects of the incorporation of tetracaine on the two carbonyl stretching bands of the acyl chains as well as on the antisymmetric carboxylate stretching band at $\approx$1625 cm^{-1}.

Comparison of the carbonyl stretching band for DMPS and DOPS in the absence (Figure 2A,B) and in the presence (Figure 3) of charged tetracaine at pH 5.5 indicates that in the presence of anesthetic, the band is broader on the low-frequency side, at pressures below the expulsion pressure of the anesthetic. This is in part due to the presence of the broad tetracaine band at $\approx$1696 cm^{-1}. However, the third-order derivative spectra of these systems (results not shown) indicate that the C=O band associated with the hydrogen-bonded C=O groups is shifted to lower frequency in the absence of tetracaine. These results are illustrated in Figure 4 where the pressure dependences of the frequencies of the two C=O bands for DMPS (Figure 4A) and DOPS (Figure 4B) are shown, both in the absence and in the presence of tetracaine. From this figure, it is clear that at atmospheric pressure, the frequency of the C=O band associated with hydrogen-bonded C=O groups is decreased in the presence of tetracaine while that associated with the non-hydrogen-bonded C=O groups is not significantly altered. Specifically, the frequency of the hydrogen-bonded C=O band in DMPS decreases from 1716 to 1710 cm^{-1} in the presence of tetracaine while the C=O band in DOPS decreases from 1723 to 1710 cm^{-1} under the same conditions. It has been shown that when ester carbonyl groups such as those of lipid acyl chains form strong hydrogen bonds, the frequency of the C=O stretching mode is below 1716 cm^{-1} (Wong et al., 1988; Mushayakarara et al., 1986; Mantsch et al., 1985, 1987, 1989). The decrease in frequency of the hydrogen-bonded C=O band of phosphatidylserine bilayers in the presence of tetracaine can therefore be rationalized in

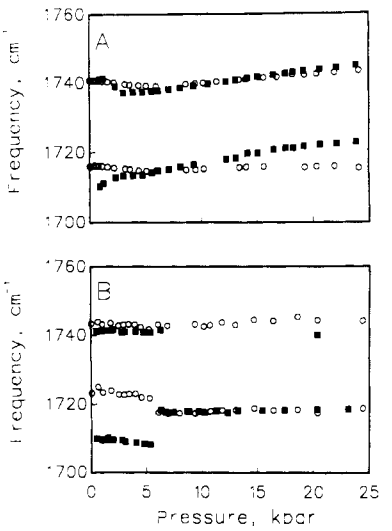

FIGURE 4: (A) Pressure dependence of the frequencies of the C=O stretching mode for DMPS (○) and DMPS + TTC, pH 5.5 (■). (B) Pressure dependence of the frequencies of the C=O stretching mode for DOPS (○) and DOPS + TTC, pH 5.5 (■).

terms of strong hydrogen bonding between the anesthetic molecule and the lipid C=O groups. The hydrogen bonding with tetracaine appears to be stronger than that with water and thus results in a larger shift to lower frequency. A possible candidate in tetracaine for hydrogen bonding with the acyl chain C=O groups is the NH group linking the butyl chain to the aromatic ring. Above the expulsion pressure, the frequency of the hydrogen-bonded C=O stretching band of DOPS returns to its value in the absence of anesthetic while that of the C=O stretching band of DMPS is slightly increased, suggesting that tetracaine expelled into the aqueous environment cannot interact via hydrogen bonding with the lipid carbonyl groups. The small increase in frequency observed for DMPS suggests that the interaction of the expelled anesthetic with the charged carboxylate gorup (vide infra) induces a small change in the conformation of the lipid hydrogen-bonded C=O groups, for which the frequency is very sensitive to conformational changes.

A different picture is obtained for the negatively charged carboxylate group of the serine head group. Inspection of the carboxylate band at $\approx$1620 cm^{-1} of DMPS and DOPS in the absence (Figure 2A,B) and in the presence (Figure 3) of the charged form of tetracaine at pH 5.5 suggests that at atmospheric pressure, the $\nu_{as.}$ CO_2^- band is shifted to higher frequency in the presence of anesthetic. This result is better illustrated in Figure 5 where are shown the pressure dependences of the frequencies of the $\nu_{as.}$ CO_2^- band for DMPS (Figure 5A) and DOPS (Figure 5B) without and with tetracaine. This figure indicates that the presence of the anesthetic results in a frequency shift of the $\nu_{as.}$ CO_2^- band from 1619 to 1629 cm^{-1} in DMPS and from 1623 to 1629 cm^{-1} in DOPS. However, unlike the observation for the C=O stretching band, the frequencies of the $\nu_{as.}$ CO_2^- band do not return to their values for the pure lipid systems after the expulsion of the local anesthetic from the bilayers, but are slightly lower than those observed below the expulsion pressure. An increase in the frequency of PS carboxylate bands has been observed for PS–Li^+ complexes and has been interpreted in terms of loss of water of hydration from the carboxylate group in the presence of Li^+ (Casal et al., 1987b,c). The increase in the frequency of the $\nu_{as.}$ CO_2^- band in the presence of tetracaine may therefore suggest that the trialkylammonium moiety of tetracaine interacts with the PS CO_2^- group and that this

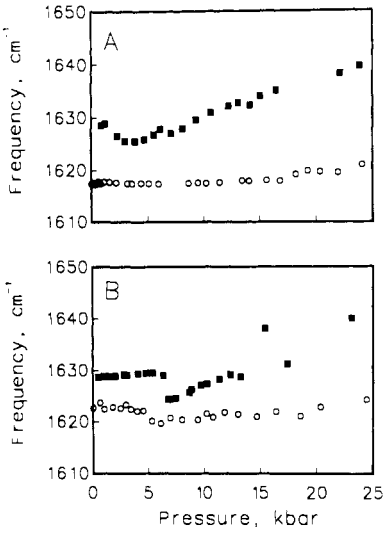

FIGURE 5: (A) Pressure dependence of the frequencies of the ν_{as} CO_2^- stretching band of DMPS (○) and DMPS + TTC, pH 5.5 (■). (B) Pressure dependence of the frequencies of the ν_{as} CO_2^- stretching band of DOPS (○) and DOPS + TTC, pH 5.5 (■).

interaction is still possible after the expulsion of the anesthetic from the lipid bilayer. Additional experiments would however be necessary to confirm this speculation.

(C) Effects of Tetracaine on the Structural and Dynamic Properties of Phosphatidylserine Bilayers. We have demonstrated in the preceding sections that at ambient temperature and pressure, the local anesthetic tetracaine is partitioned into DMPS and DOPS bilayers and interacts with the phospholipid head group. We have also investigated whether this interaction induces any changes in the structural and dynamic properties of the lipid acyl chains. A previous study of the interaction between DMPC bilayers and tetracaine has shown that the uncharged anesthetic disorders the lipid acyl chains while the charged form induces the formation of an interdigitated gel phase (Auger et al., 1988b). The uncharged form was found to be incorporated much deeper in the bilayer than was the charged form.

Comparison of the infrared spectra in the CH_2 scissoring region of pure DMPS (Figure 2C) and DMPS in the presence of both the charged and uncharged forms of the anesthetic (Figure 6A,B) indicates in all cases a pressure-induced correlation field splitting of the δCH_2 mode, which at low pressure gives rise to only one band at $\approx$1470 cm^{-1}. The pressure dependences of the frequencies of the methylene scissoring mode components of DMPS in the absence and presence of tetracaine were also examined (Figure 7). From this figure, the pressures at which the pressure-induced correlation field splittings become apparent can easily be determined. A well-defined correlation field component band $\delta'CH_2$ becomes apparent at 3.7 kbar for pure DMPS and at 2.8 kbar for both DMPS + TTC, pH 5.5, and DMPS + TTC, pH 9.5. The lower pressure required for splitting in the presence of anesthetic indicates that the incorporation of tetracaine into DMPS bilayers increases the orientational ordering of the DMPS acyl chains in the gel phase, and thus a lower pressure is necessary to stop the acyl chain reorientational fluctuations and induce a correlation field splitting. For the pure lipid, further increase in pressure then results in a relatively rapid, nonlinear increase in the magnitude of this splitting while a sudden discontinuous with slight increase thereafter is observed in the presence of anesthetic. This suggests that in the pure lipid, the pressure-induced ordering of the acyl chains is gradual while the presence of tetracaine results in a large

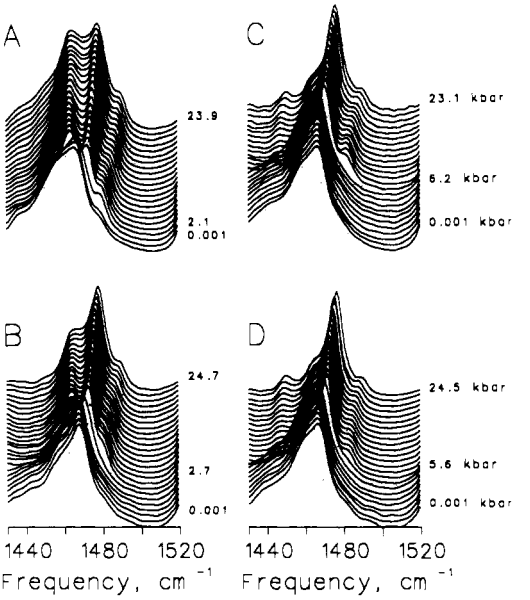

FIGURE 6: Stacked contour plots of infrared spectra in the CH_2 scissoring region of DMPS + TTC, pH 5.5 (A), DMPS + TTC, pH 9.5 (B), DOPS + TTC, pH 5.5 (C), and DOPS + TTC, pH 9.5 (D).

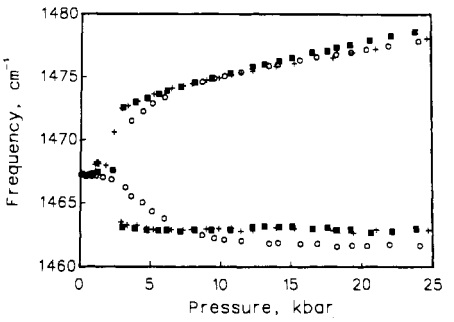

FIGURE 7: Pressure dependences of the frequencies of the δCH_2 mode for DMPS (○), DMPS + TTC, pH 5.5 (■), and DMPS + TTC, pH 9.5 (×).

increase in ordering which then remains relatively constant with further increase in pressure.

It is also interesting to note that the intensity of the correlation field component band $\delta'CH_2$ is not much greater than that of the δCH_2 band for pure DMPS and DMPS in the presence of charged tetracaine while the ratio $I_{\delta'}/I_{\delta}$ is much higher than 1 in the presence of uncharged anesthetic. The value of this ratio can be related to the packing of the lipid acyl chains, the absence of a pressure-induced correlation field splitting being associated with an equivalent, parallel orientation of the acyl chains, while a ratio $I_{\delta'}/I_{\delta}$ equal to unity indicates a perpendicular packing of the chains (Wong et al., 1988; Siminovitch et al., 1987a). It therefore appears that the presence of the charged and uncharged forms of the anesthetic may induce different packings of the DMPS acyl chains. Further experiments would, however, be necessary to confirm this result.

A completely different picture is obtained with DOPS. The infrared spectra in the CH_2 scissoring region of pure DOPS (Figure 2D) and DOPS in the presence of both the charged and uncharged forms of tetracaine (Figure 6C,D) indicate that the presence of tetracaine does not affect significantly the structural and dynamic properties of the lipid acyl chains. Moreover, the pressure dependences of the frequencies of the CH_2 scissoring mode components of DOPS are essentially identical in the absence and presence of tetracaine (results not shown). These results indicate that below the liquid-crystalline to gel phase transition pressure ($\approx$6.1 kbar), the incorporation

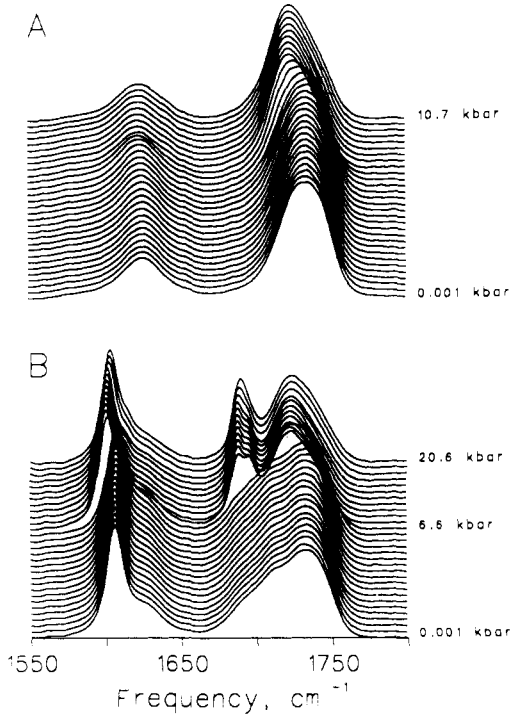

FIGURE 8: Stacked contour plots of infrared spectra in the 1550–1800 cm^{-1} region of DOPS/DOPC (1:4 molar ratio) (A) and DOPS/DOPC (1:4 molar ratio) + TTC, pH 5.5 (B).

of tetracaine into DOPS bilayers does not significantly affect the interchain interaction, or the chain mobility of the highly disordered DOPS acyl chains. After the expulsion of the anesthetic from the bilayer, only one CH_2 scissoring mode component is observed in all cases, indicating that the acyl chains are packed parallel in a highly ordered and rigid lattice.

Interactions of Tetracaine with DOPS/DOPC (1:4) Bilayers. We have discussed the interactions of the local anesthetic tetracaine with bilayers of pure phosphatidylserine. However, the concentration of phosphatidylserine found in excitable membranes is usually near 20 mol % of the total lipids (Camejo et al., 1969; Chacko et al., 1976). We have thus examined the interactions of the charged form of tetracaine at pH 5.5 with DOPS/DOPC (1:4 molar ratio) bilayers, a situation which more closely resembles that observed in biological membranes. The unsaturated phosphatidylserine was examined since high levels of unsaturated lipid are also found in excitable membranes.

The infrared spectra of DOPS/DOPC (1:4 molar ratio) bilayers in the absence and in the presence of charged tetracaine are shown in Figure 8 as a function of the hydrostatic pressure applied on the systems. From this figure, it is clear that, as was observed in the pure DOPS system, the anesthetic is expelled by pressure from the bilayer at the phase transition pressure of the lipid system. The fact that the expulsion pressure still corresponds to the liquid-crystalline to gel phase transition pressure even in the presence of only 20 mol % of phosphatidylserine indicates that chain unsaturation rather than negative head-group charge is responsible for the high expulsion pressure in this system.

It is also interesting to note that for the mixed lipid system in the absence of anesthetic, the spectral changes associated with the liquid-crystalline to gel phase transition are not sudden, as was observed for pure DOPS, but span a pressure range of about 1 kbar. However, in the presence of tetracaine, the shift of the C=O stretching contour band is similar to that observed for the pure lipid. These results are better illustrated in Figure 9 where are shown the pressure dependences of the

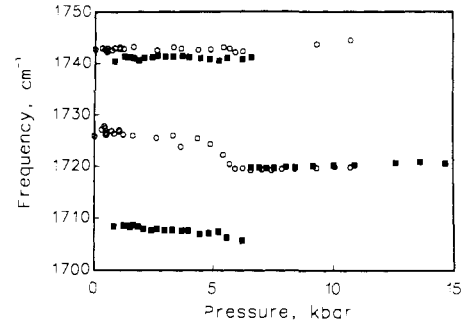

FIGURE 9: Pressure dependences of the frequencies of the C=O stretching band of DOPS/DOPC (1:4 molar ratio) (○) and DOPS/DOPC (1:4 molar ratio) + TTC, pH 5.5 (■).

frequencies of the non-hydrogen-bonded and hydrogen-bonded C=O bands for DOPS/DOPC (1:4 molar ratio) in the absence and presence of tetracaine at pH 5.5. In the absence of anesthetic, the frequency of the hydrogen-bonded C=O band gradually decreases from 1726 to 1719 cm^{-1} between ≈5 and 6 kbar, indicating that the liquid-crystalline to gel phase transition is occurring gradually at intermediate pressures between the phase transition pressures of the pure lipids (6.1 and 5.2 kbar for DOPS and DOPC, respectively). Other spectral parameters, such as the ν_s mode at 2850 cm^{-1}, also confirm this observation. However, in the presence of anesthetic, the phase transition occurs in a sudden fashion at slightly higher pressure (6.5 kbar). This suggests that the interactions between the anesthetic and the phospholipid molecules may result in a more homogeneous system compared to the mixed lipid system in the absence of tetracaine.

The pressure dependences of the frequencies of both the C=O stretching bands (Figure 9) and the ν_{as} CO_2^- band of the serine head group (results not shown) indicate that similar shifts in frequencies are obtained in the mixed and the pure lipid systems, the hydrogen-bonded C=O stretching band being shifted to lower frequency while the frequency of the serine carboxylate band slightly increases in the presence of anesthetic. It should be noted here that only one band is observed for the hydrogen-bonded C=O group, both below and above the expulsion pressure. This suggests that tetracaine also interacts via hydrogen bonding with the carbonyl groups of phosphatidylcholine and that this interaction is not specific to phosphatidylserine bilayers but only requires that the anesthetic is located close to the lipid–water interface region. However, the shift in frequency of the phosphatidylserine carboxylate group clearly indicates that even in the mixed lipid systems, tetracaine interacts with the PS head group.

CONCLUSIONS

The present study demonstrates that high-pressure FT-IR spectroscopy is a valuable technique to study the structural and dynamic properties of lipid bilayers as well as the effects of exogenous agents, such as local anesthetics, on these properties. The data indicate that the barotropic behavior of both saturated and unsaturated phosphatidylserine bilayers is comparable to that observed for zwitterionic phospholipids such as phosphatidylcholine and phosphatidylethanolamine, with corresponding acyl chains. Moreover, it is shown that tetracaine partitions into phosphatidylserine bilayers at the membrane–water interface and interacts via hydrogen bonding with the lipid molecules. The results also demonstrate that the local anesthetic is expelled by pressure from both DMPS and DOPS bilayers, the expulsion pressure being much higher for the unsaturated lipid. This suggests that a negative charge on a major lipid cannot solely explain the high pressure for

anesthetic expulsion observed in nerve membranes, but rather that a combination of negative charge and unsaturated chains results in expulsion pressures comparable to those required in nerve systems (Auger et al., 1987).

ACKNOWLEDGMENTS

We thank Douglas Moffatt for valuable technical assistance and Dr. Harold C. Jarrell for valuable discussions. We also thank the Natural Sciences and Engineering Research Council of Canada for the award of a postgraduate scholarship to M. Auger.

REFERENCES

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

Auger, M., Jarrell, H. C., & Smith, I. C. P. (1988a) *Biochemistry 27*, 4660–4667.

Auger, M., Jarrell, H. C., Smith, I. C. P., Siminovitch, D. J., Mantsch, H. H., & Wong, P. T. T. (1988b) *Biochemistry 27*, 6086–6093.

Auger, M., Smith, I. C. P., & Jarrell, H. C. (1989) *Biochim. Biophys. Acta 981*, 351–357.

Blume, A., Hübner, W., & Messner, G. (1988) *Biochemistry 27*, 8239–8249.

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.

Butler, K. W., Schneider, H., & Smith, I. C. P. (1973) *Arch. Biochem. Biophys. 154*, 548–555.

Camejo, G., Villegas, G. M., Barnola, F. V., & Villegas, R. (1969) *Biochim. Biophys. Acta 193*, 247–259.

Casal, H. L., Mantsch, H. H., Paltauf, F., & Hauser, H. (1987a) *Biochim. Biophys. Acta 919*, 275–286.

Casal, H. L., Mantsch, H. H., & Hauser, H. (1987b) *Biochemistry 26*, 4408–4416.

Casal, H. L., Martin, A., Mantsch, H. H., Paltauf, F., & Hauser, H. (1987c) *Biochemistry 26*, 7395–7401.

Cerbon, J. (1972) *Biochim. Biophys. Acta 290*, 51–57.

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

Coster, H. G. L., James, V. J., Berthet, C., & Miller, A. (1981) *Biochim. Biophys. Acta 641*, 281–285.

Hauser, H., Paltauf, F., & Shipley, G. G. (1982) *Biochemistry 21*, 1061–1067.

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.

Lazdunski, M., Balerna, M., Barhanin, J., Chicheportiche, R., Fossett, M., Frelin, C., Jacques, Y., Lombert, A., Poyssegur, J., Renaud, J. F., Romery, G., Schweitz, H., & Vincent, J. P. (1980) *Ann. N.Y. Acad. Sci. 358*, 169–182.

Mantsch, H. H., Madec, C., Lewis, R. N. A. H., & McElhaney, R. N. (1985) *Biochemistry 24*, 2440–2446.

Mantsch, H. H., Madec, C., Lewis, R. N. A. H., & McElhaney, R. N. (1987) *Biochemistry 26*, 4045–4049.

Mantsch, H. H., Madec, C., Lewis, R. N. A. H., & McElhaney, R. N. (1989) *Biochim. Biophys. Acta 980*, 42–49.

Moffatt, D. J., Kauppinen, J. K., Cameron, D. G., Mantsch, H. H., & Jones, R. N. (1986) *N.R.C.C. Bull. No. 18*.

Mushayakarara, E. C., Wong, P. T. T., & Mantsch, H. H. (1986) *Biochim. Biophys. Acta 857*, 259–264.

Neal, M. J., Butler, K. W., Polnaszek, C. F., & Smith, I. C. P. (1976) *Mol. Pharmacol. 12*, 144–155.

Papahadjopoulos, D. (1970) *Biochim. Biophys. Acta 211*, 467–477.

Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.

Ritchie, J. M., & Rogart, R. B. (1977) *Rev. Physiol. Biochem. Pharmacol. 79*, 1–50.

Seeman, P. (1975) *Prog. Anesthesiol. 1*, 243–251.

Siminovitch, D. J., Wong, P. T. T., & Mantsch, H. H. (1987a) *Biochemistry 26*, 3277–3287.

Siminovitch, D. J., Wong, P. T. T., & Mantsch, H. H. (1987b) *Biophys. J. 51*, 465–473.

Siminovitch, D. J., Wong, P. T. T., Berchtold, R., & Mantsch, H. H. (1988) *Chem. Phys. Lipids 46*, 79–87.

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

Snyder, R. G. (1961) *J. Mol. Spectrosc. 7*, 116–141.

Strichartz, G. R. (1973) *J. Gen. Physiol. 62*, 37–57.

Wong, P. T. T. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 1–24.

Wong, P. T. T. (1986) *Physica B + C 139, 140*, 847–852.

Wong, P. T. T. (1987a) in *High Pressure Chemistry and Biochemistry* (van Eldick, R., & Jonas, J., Eds.) pp 381–400, D. Reidel, Dordrecht, The Netherlands.

Wong, P. T. T. (1987b) in *Vibrational Spectra and Structure* (Durig, G. R., Ed.) pp 357–445, Elsevier, Amsterdam.

Wong, P. T. T. (1987c) in *Current Perspectives of Barobiology* (Jennasch, H. W., Marquis, R. E., & Zimmerman, A. M., Eds.) pp 288–314, Academic Press, New York.

Wong, P. T. T., & Mantsch, H. H. (1985a) *J. Chem. Phys. 83*, 3268–3274.

Wong, P. T. T., & Mantsch, H. H. (1985b) *Biochemistry 24*, 4091–4096.

Wong, P. T. T., & Mantsch, H. H. (1988a) *Biophys. J. 54*, 781–790.

Wong, P. T. T., & Mantsch, H. H. (1988b) *Chem. Phys. Lipids 46*, 213–223.

Wong, P. T. T., Moffatt, D. J., & Baudais, F. L. (1985) *Appl. Spectrosc. 39*, 733–735.

Wong, P. T. T., Weng, S. F., & Mantsch, H. H. (1986) *J. Chem. Phys. 85*, 2315–2319.

Wong, P. T. T., Siminovitch, D. J., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 947*, 139–171.