Effects of the Local Anesthetic Tetracaine on the Structural and Dynamic Properties of Lipids in Model Membranes: A High-Pressure Fourier Transform Infrared Study†

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ABSTRACT: High-pressure Fourier transform infrared (FT-IR) spectroscopy was used to study the effects of a local anesthetic, tetracaine, on the structural and dynamic properties of lipids in model membranes. The model membrane systems studied were multilamellar aqueous dispersions of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (DHPC) in the absence and presence of a physiological concentration of cholesterol (30 mol %). The infrared spectra were measured at 28 °C in a diamond anvil cell as a function of pressure up to 25 kbar. The results indicate that the effects of tetracaine on the structure of pure DMPC bilayers in the gel state are dependent on the state of charge of the anesthetic. The uncharged tetracaine disorders the lipid acyl chains while the charged form induces the formation of an interdigitated gel phase. The presence of cholesterol in the latter system prevents the formation of the interdigitated phase, whereas in the former system it disorders the lipid acyl chains in the gel state. Moreover, it is shown that the addition of uncharged tetracaine to interdigitated DHPC bilayers does not alter the interdigitated state of the hydrocarbon chains.

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 a direct anesthetic–protein interaction (Boggs et al., 1976) or a perturbation by the anesthetic of the lipid matrix surrounding the channels (Seeman, 1975) is still unclear.

Many techniques have been used to study the interactions of local anesthetics with model and biological membranes, including high-resolution nuclear magnetic resonance (Cerbon et al., 1972), electron spin resonance (Butler et al., 1973; Neal et al., 1976; Giotta et al., 1974), and neutron diffraction (Coster et al., 1981). All of these studies suggest that the anesthetic intercalates partially into the lipid bilayer. Moreover, a recent infrared spectroscopic study has shown that the local anesthetic tetracaine is located close to the aqueous interface of the lipid bilayer in model membrane systems containing a physiological concentration (30 mol %) of cholesterol (Auger et al., 1987). This study also demonstrated that tetracaine, in both myelinated and unmyelinated nerves, is located in an environment similar to that in model systems.

On the other hand, information about the effects of the local anesthetic tetracaine on the order and dynamics of the lipid acyl chains has been obtained by ^{2}H NMR[1] spectroscopy. These studies indicated that the anesthetic reduces the order parameter of the entire lipid acyl chains in the liquid-crystalline phase. This disordering effect is larger in the presence of cholesterol (30 mol %) and for the charged form of the anesthetic (Boulanger et al., 1981; Auger et al., 1988). By comparison of ^{2}H spin–lattice relaxation times, these studies also showed that tetracaine alters only slightly the dynamics of DMPC acyl chains in cholesterol-containing systems.

Nevertheless, little is known about the effects of local anesthetics on the structural and dynamic properties of phospholipid bilayers in the gel state. In this study, we examine the effects of tetracaine on 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (DHPC) bilayers in the presence and absence of 30 mol % cholesterol, by using high-pressure FT-IR spectroscopy. This technique has proven to be very powerful in the study of structural and dynamic properties of phospholipid bilayers (Wong, 1987a,b). In particular, the correlation field splitting of the methylene scissoring mode, δCH_2, in both Raman and infrared spectra is very useful for the characterization of interchain packing (Wong, 1984; Boerio & Koenig, 1970). Moreover, the methylene rocking mode γCH_2 of polymethylene chains as well as its correlation field component are infrared active, and the infrared intensity of these components can be related to the relative orientation of the chains (Snyder, 1961).

Until recently, most investigations of phospholipid bilayers using vibrational spectroscopy have concentrated on their thermotropic behavior. With the addition of high pressure as a variable, new information on lipid structure and dynamics has been gained from studies of their barotropic behavior, as illustrated in this paper.

Materials and Methods

Materials. DMPC and tetracaine hydrochloride were

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[1] Abbreviations: FT-IR, Fourier transform infrared; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; TTC, tetracaine; NMR, nuclear magnetic resonance; ESR, electron spin resonance; ^{2}H NMR, deuterium nuclear magnetic resonance; DSC, differential scanning calorimetry; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; lyso-PC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; 1,3-DPPC, 1,3-dipalmitoyl-*sn*-glycero-3-phosphocholine.

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purchased from Sigma Chemical Co. (St. Louis, MO), and the ether-linked lipid DHPC was purchased from Calbiochem Behring Corp. (La Jolla, CA). Cholesterol was purchased from Steraloids Inc. (Pawling, NY). All other materials were of analytical grade.

Sample Preparation. To prepare lipid dispersions for study by FT-IR, 50 mg of lipid was hydrated with 50 μL of D_2O. When the local anesthetic tetracaine was used, the lipids were hydrated with 50 μL of a borate–phosphate–citrate buffer (Kelusky & Smith, 1984) made with D_2O and containing about 5 mg of tetracaine. To ensure 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 coadded, 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 with a breakpoint of 0.5 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, with a breakpoint of 0.95 in the Fourier domain.

Results and Discussion

Effects of Tetracaine on Pure DMPC Bilayers. Figure 1 compares the pressure dependence of the methylene scissoring mode δCH_2 of DMPC multilamellar dispersions in the presence and absence of tetracaine. The anesthetic was incorporated in either its charged form at pH 5.5 or its uncharged form at pH 9.5. For pure DMPC bilayers, the pressure dependence of the methylene scissoring mode band is very similar to that observed for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in multilamellar aqueous dispersions (Wong & Mantsch, 1985a; Siminovitch et al., 1987a). As the pressure is increased, one observes 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 first manifestation of this splitting with increasing pressure is a shoulder on the high-frequency side of δCH_2, which steadily gains intensity until a well-defined correlation field component is apparent. The observation of only a single CH_2 scissoring band at atmospheric pressure reflects the fact that under these 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.

The gel ($L_{\beta'}$) to liquid-crystalline (L_α) phase transition temperature for DMPC is 23.5 °C (Silvius, 1982), which means that at ambient temperature (28 °C) and at atmospheric pressure DMPC is in the liquid-crystalline phase. Assuming that the gel to liquid-crystalline phase transition for DMPC is a first-order transition, as it is in DPPC, the critical temperture T_m can be raised by applying external pressure (the Clausius–Clapeyron relationship). Elevation of pressure should therefore induce the transition to the gel phase. This pressure-induced increase in T_m has been described in detail (Wong et al., 1982; Wong & Mantsch, 1985b). The critical pressure for the transition from the liquid-crystalline phase to the gel phase in DMPC is 0.15 kbar at 28 °C (Wong & Mantsch, 1985b). Therefore, application of a very small pressure to DMPC in the liquid-crystalline phase in the diamond anvil cell will induce a transition to the gel state.

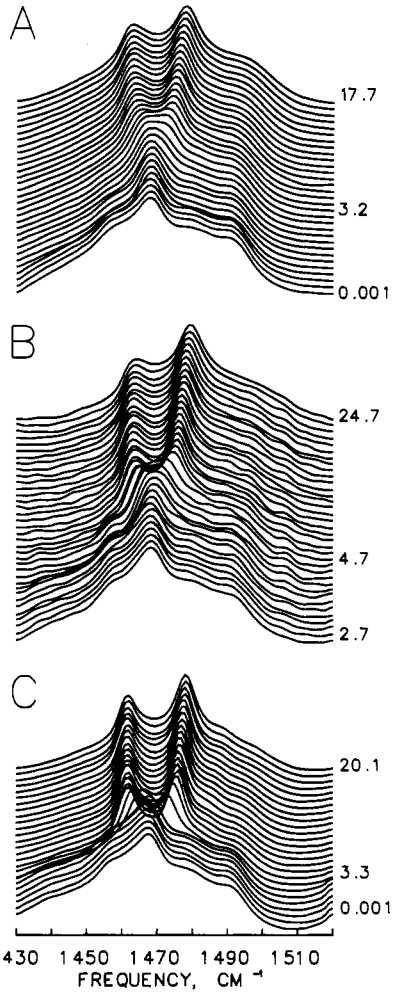

FIGURE 1: Stacked contour plots of the infrared spectra at increasing hydrostatic pressures of aqueous DMPC dispersions in the CH_2 scissoring region for (A) DMPC, (B) DMPC + TTC, pH 9.5, and (C) DMPC + TTC, pH 5.5. To the right of each contour plot, the lowest and highest pressures (in kilobars) are indicated, as well as the pressure at which the correlation field splitting first occurs.

When uncharged tetracaine (pH 9.5) is incorporated into DMPC bilayers, high-pressure FT-IR (Auger et al., 1987) and ^2H NMR studies (Auger et al., 1988; Boulanger et al., 1981) have shown that the anesthetic intercalates deeply into the bilayer. In order to elucidate the effects of the incorporation of uncharged tetracaine on the acyl chain interactions in DMPC, the pressure dependence of the CH_2 scissoring mode bands for DMPC in the presence of uncharged tetracaine at pH 9.5 (Figure 1B) was first compared with that observed for pure DMPC (Figure 1A). The pressure dependences of the frequencies of the methylene scissoring mode components for DMPC in the absence and presence of uncharged tetracaine were also examined (Figure 2A). From this figure, the

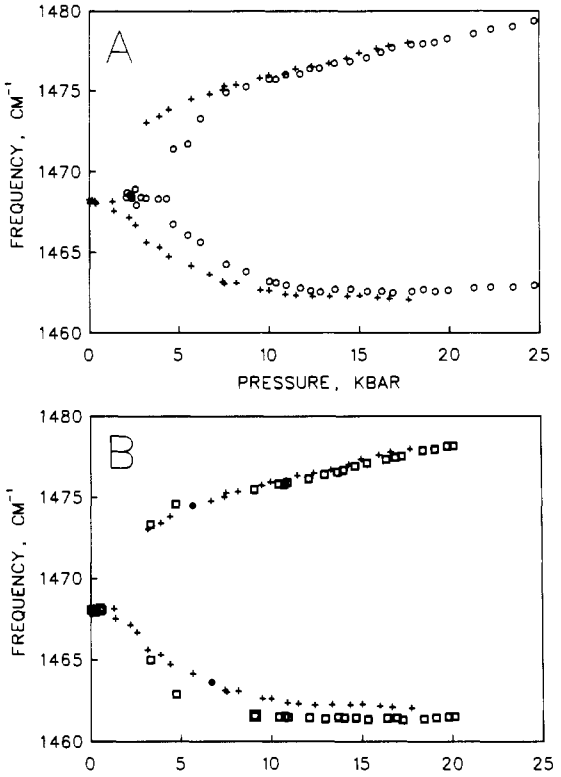

FIGURE 2: Comparison of the pressure dependence of the frequencies of the δCH_2 mode for (+) DMPC with that of (A) (○) DMPC + TTC, pH 9.5, and (B) (□) DMPC + TTC, pH 5.5.

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 3.2 kbar for pure DMPC and at 4.7 kbar for DMPC + TTC, pH 9.5. Further increase in pressure then results in a relatively rapid, nonlinear increase in the magnitude of this splitting. The pressure at which the correlation field component band becomes apparent is much higher for DMPC in the presence of uncharged tetracaine compared to that for pure DMPC, while the magnitude of the correlation field splitting is smaller for the former system.

The pressure-induced correlation field splittings of CH_2 scissoring bands are 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. For DMPC in the presence of uncharged tetracaine, the smaller correlation field splitting indicates smaller interchain interactions due to the increase in both the orientational and conformational disorders as a result of the intercalation of the anesthetic between the lipid acyl chains. Moreover, the higher pressure at which this splitting becomes apparent compared to that obtained for pure DMPC bilayers also indicates that in the presence of uncharged tetracaine the DMPC acyl chains are orientationally more disordered, and thus a higher pressure is necessary to stop the acyl chain reorientational fluctuations and induce a correlation field splitting.

The pressure dependence of the CH_2 scissoring mode bands for DMPC in the presence of charged tetracaine at pH 5.5 (Figure 1C) was also examined and compared with that obtained for pure DMPC (Figure 1A). A visual inspection of these spectra shows that the "valley" between the two component bands of the pressure-induced correlation field splitting is particularly pronounced for DMPC in the presence of charged tetracaine but much less so for pure DMPC. A pressure dependence of the δCH_2 mode similar to that observed for DMPC in the presence of charged tetracaine has been observed for various lipid bilayer systems, such as 1,2-DHPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine (lyso-PC), and 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine (1,3-DPPC) (Siminovitch et al., 1987a,b). These systems are known to form a lamellar phase with complete interdigitation of the hydrocarbon chains under the conditions of temperature and pressure at which the correlation field splitting is observed. The pressure dependence of the methylene scissoring mode obtained for DMPC in the presence of the charged anesthetic therefore suggests that the incorporation of charged tetracaine in DMPC bilayers induces the formation of an interdigitated lamellar gel phase.

The induction of an interdigitated gel phase in phospholipids by exogenous agents such as glycerol (McDaniel et al., 1983; McIntosh et al., 1983; O'Leary & Levin, 1984), protein (Boggs & Moscarello, 1982), ions (Cunningham & Lis, 1986), and tetracaine (McIntosh et al., 1983) has been extensively studied. It has been demonstrated that the ability to induce an interdigitated phase does not depend on the presence or absence of a charge on the molecule (Simon et al., 1986). However, Simon et al. (1986) suggested that the one requirement for the induction of the interdigitated phase is that the guest molecule must reside at the membrane–water interface and must not extend too deeply into the acyl chain region. This explains why there is no interdigitation in the presence of uncharged tetracaine, which penetrates too deeply into the hydrophobic region to allow a stable interdigitated phase.

High-pressure FT-IR (Auger et al., 1987) and ^2H NMR studies (Auger et al., 1988; Boulanger et al., 1981) have demonstrated that charged tetracaine is located close to the aqueous interface of DMPC bilayers. Moreover, X-ray diffraction studies have shown that, with incorporation of tetracaine into DPPC liposomes at 20 °C, the bilayer thickness is only 30 Å, which is about 20 Å smaller than two fully extended DPPC molecules (McIntosh et al., 1983). From these observations, we can therefore conclude that because charged tetracaine is located close to the aqueous interface of DMPC bilayers, the DMPC acyl chains in the pressure-induced gel phase are interdigitated. The stability of the interdigitated phase can be rationalized as follows. The intercalation of the charged anesthetic in DMPC bilayers tends to separate the lipid head groups. Since the gel-state acyl chains are rigid under the present conditions, this will tend to form defects between the chains which are energetically unfavorable (Israelachvili et al., 1980). The lowest energy phase is therefore the interdigitated phase (Simon et al., 1986).

The pressure dependences of the frequencies of the CH_2 scissoring mode components for pure DMPC and DMPC in the presence of charged tetracaine at pH 5.5 (Figure 2B) are very similar, the magnitude of the correlation field splitting being however slightly larger in the presence of tetracaine. These results support the conclusion of the formation of an interdigitated lamellar gel phase in DMPC in the presence of charged tetracaine.

It has been shown that at a pressure of 4.6 kbar the charged form of the anesthetic is expelled from DMPC bilayers (Auger et al., 1987). However, above that pressure, the pressure dependence of the methylene scissoring region remains characteristic of lipids in an interdigitated gel phase. This can be explained by the fact that under high hydrostatic pressure the energy required to revert to a noninterdigitated phase after the expulsion of the anesthetic would be very large. The mismatch between the lipid head group and the hydrocarbon region created by the expulsion of the anesthetic can however

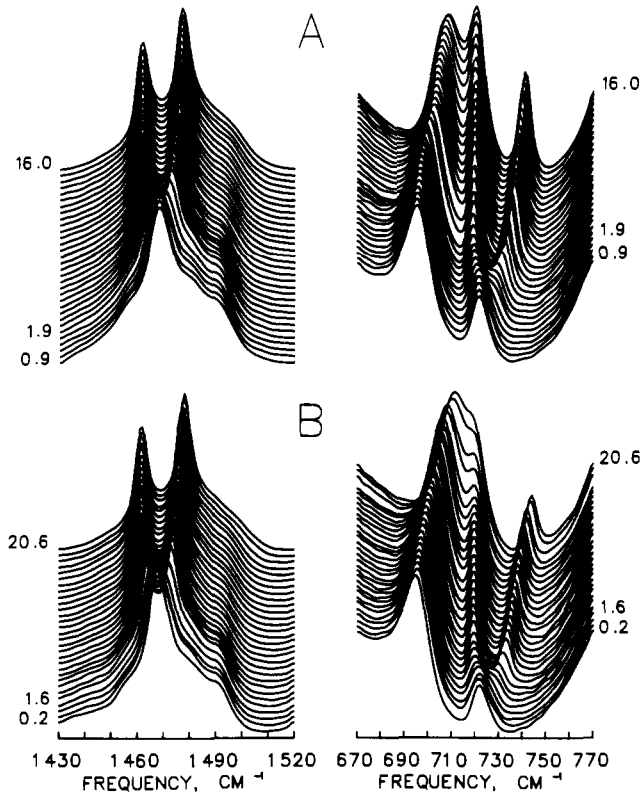

FIGURE 3: Stacked contour plots of the infrared spectra of aqueous DHPC dispersions in the CH_2 scissoring region (left spectra) and in the CH_2 rocking region (right spectra) for (A) DHPC and (B) DHPC + TTC, pH 9.5. The band at 695 cm^{-1} (right spectra) is the phonon band of α-quartz used for the pressure calibration.

be compensated by a complete interdigitation of the lipid acyl chains.

The pressure dependences of the methylene rocking mode γCH_2 of DMPC multilamellar dispersions in the absence and presence of tetracaine, both in its charged and uncharged form, have also been examined (data not shown). In the presence of charged tetracaine at pH 5.5, the pressure dependence of the CH_2 rocking mode is similar to that observed for systems known to form an interdigitated lamellar gel phase (Siminovitch et al., 1987a,b), which therefore supports the conclusion that the pressure-induced gel phase for DMPC bilayers in the presence of charged tetracaine is an interdigitated lamellar gel phase.

Effects of Tetracaine on DHPC Bilayers. Recent studies using differential scanning calorimetry (DSC), X-ray diffraction, ^{14}N, ^{31}P, and ^{2}H NMR, and high-pressure FT-IR spectroscopy (Ruocco et al., 1985a,b; Siminovitch et al., 1983, 1987a) have shown that the replacement of the fatty acyl chains with the corresponding alkyl chains has a significant effect on the structure and dynamics of lipids in the low-temperature gel phase. As mentioned previously, it has been demonstrated that 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (DHPC) forms an interdigitated lamellar gel phase at temperatures below the pretransition at 35 °C, whereas DPPC does not.

From the pressure dependence of the carbonyl stretching band of tetracaine incorporated in DHPC multilamellar dispersions at pH 9.5, it has been shown that the uncharged anesthetic intercalates deeply into DHPC bilayers in the gel state (Auger et al., 1987). In order to see the effects of the incorporation of uncharged tetracaine on the alkyl chain packing of DHPC in the gel phase, we have monitored the pressure dependence of the methylene scissoring and rocking

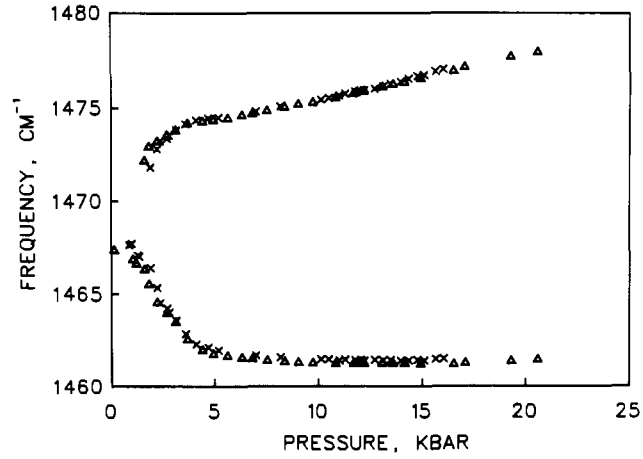

FIGURE 4: Pressure dependence of the frequencies of the δCH_2 mode for (×) DHPC and (Δ) DHPC + TTC, pH 9.5.

mode bands for DHPC in the presence of uncharged tetracaine and compared the results with those obtained by Siminovitch et al. (1987a) for pure aqueous DHPC multilamellar dispersions. The results are presented in Figure 3.

The pronounced "valley" between the methylene scissoring and rocking mode bands and their correlation field components for pure DHPC has been interpreted in terms of the formation of an interdigitated lamellar gel phase (vide supra). The DHPC head group surface area in the gel state is 79 $Å^2$ compared to 48 $Å^2$ for that of DPPC in noninterdigitated bilayers at 20 °C (Janiak et al., 1979). For both phospholipids however, the hydrocarbon chain cross-sectional area is approximately 40 $Å^2$ per molecule (i.e., 20 $Å^2$ per acyl chain). The mismatch between the DHPC head group and hydrocarbon chain surface area (79 $Å^2$ vs 40 $Å^2$, respectively) therefore favors the formation of an interdigitated bilayer phase.

The pressure dependences of the CH_2 scissoring and rocking mode bands for DHPC in the presence of uncharged tetracaine are very similar to those obtained for pure DHPC and are characteristic of lipids in an interdigitated gel phase. Moreover, the pressure dependences of the frequencies of the methylene scissoring mode band and its correlation field component (Figure 4) for DHPC do not vary significantly upon addition of uncharged tetracaine. These results therefore suggest that the incorporation of uncharged tetracaine into DHPC does not prevent the formation of an interdigitated lamellar gel phase.

The effects of the incorporation of an equimolar concentration of cholesterol on the lipid packing, conformation, and dynamics of DHPC multilamellar dispersions have been studied by X-ray diffraction and ^{2}H NMR spectroscopy (Siminovitch et al., 1987c). The results of these studies indicate that the addition of 50 mol % cholesterol to DHPC prevents the formation of an interdigitated phase at 22 °C since the introduction of cholesterol [surface area $\approx$ 38 $Å^2$ (Pethica, 1955; Small, 1977)] into the hydrophobic hydrocarbon portion of the bilayer creates a more favorable match between the head group and hydrocarbon surface areas (Siminovitch et al., 1987c). Moreover, the thermal phase behavior and structure of hydrated mixtures of DPPC and DHPC have been investigated by DSC and X-ray diffraction techniques (Lohner et al., 1987; Kim et al., 1987) and indicate that, at temperatures below the "subtransition" of DHPC, the incorporation of 50 mol % DPPC to DHPC prevents the formation of an interdigitated gel structure. However, when less than 50 mol % DPPC is added, DHPC remains in the interdigitated gel phase and shows full solubility for DPPC up to equimolarity without major structural changes.

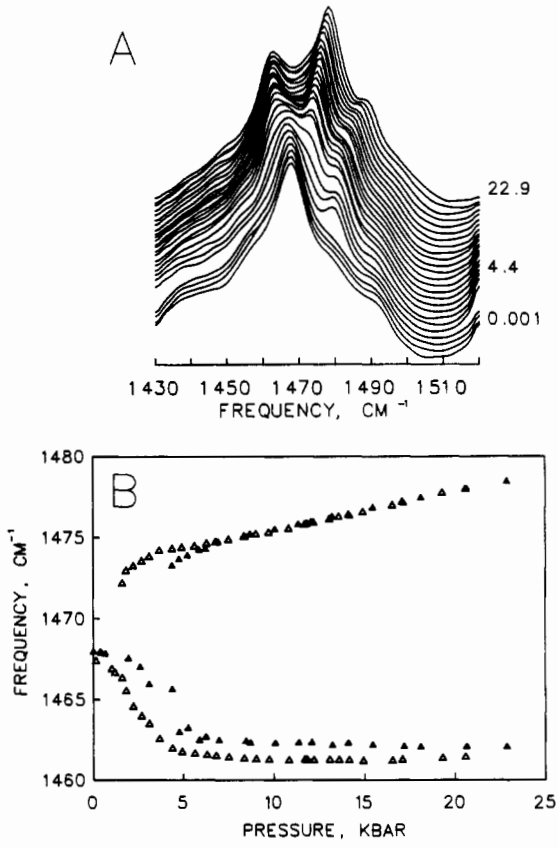

FIGURE 5: (A) Stacked contour plot of the infrared spectra in the CH_2 scissoring region for DHPC–cholesterol + TTC, pH 9.5, and (B) pressure dependence of the frequencies of the δCH_2 mode for (△) DHPC + TTC, pH 9.5, and (▲) DHPC–cholesterol + TTC, pH 9.5.

The amount of uncharged tetracaine incorporated in the DHPC bilayers corresponds to about 20 mol % of the total lipids, according to the very large partition coefficients determined for uncharged tetracaine in phosphatidylcholine bilayers (Boulanger et al., 1980). This amount is therefore less than the amount of cholesterol or DPPC (50 mol %) required to prevent the formation of interdigitated DHPC bilayers. This could explain why upon addition of 20 mol % of uncharged tetracaine DHPC bilayers maintain an interdigitated gel structure. However, the hydrophobic portion of the tetracaine molecule is much shorter than that of cholesterol and DPPC. Moreover, in the model proposed by Boulanger et al. (1981) for the location of uncharged tetracaine in phosphatidylcholine bilayers, the anesthetic penetrates relatively deeply in the bilayer, but because of the shortness of the butyl tail, the last few CH_2 segments of the phospholipid molecules are not in contact with the anesthetic. This model could also explain why DHPC maintains an interdigitated structure upon addition of uncharged tetracaine. However, whether the shorter hydrophobic portion of tetracaine or the concentration of anesthetic used in this study is responsible for this effect is still to be determined.

The pressure dependence of the methylene scissoring bands for DHPC–cholesterol + TTC, pH 9.5 (Figure 5A), was also examined in order to see if the presence of 30 mol % cholesterol can prevent the interdigitation of the DHPC alkyl chains in the presence of uncharged tetracaine. Upon addition of cholesterol, the valley between the two component bands of the pressure-induced correlation field splitting in DHPC is less pronounced than it is for the pure lipid in the absence and presence of uncharged anesthetic. Moreover, comparison of the pressure dependence of the frequencies of the CH_2 scissoring mode components for DHPC + TTC, pH 9.5, and DHPC–cholesterol + TTC, pH 9.5 (Figure 5B), shows that the magnitude of the correlation field splitting is smaller for the latter system and that the pressure at which the splitting becomes apparent is 4.4 kbar in the presence of cholesterol compared to 1.6 kbar for the system without cholesterol. These results indicate that the presence of cholesterol does prevent, to a certain extent, the interdigitation of DHPC alkyl chains in the presence of uncharged tetracaine. However, whether or not the interdigitation is totally or only partially prevented cannot be determined from our data. The pressure dependence of the CH_2 scissoring component for DHPC–cholesterol + TTC, pH 9.5, would have to be compared with that obtained for DHPC in the presence of 50 mol % cholesterol or DPPC, which are known to completely prevent the interdigitation of DHPC alkyl chains (vide supra).

Effects of Cholesterol (30 mol %) on Pure DMPC Bilayers. Many techniques have been used to study the structural and dynamical properties of cholesterol-containing membranes, such as ^2H NMR (Stockton & Smith, 1976; Oldfield et al., 1978; Taylor et al., 1981, 1982; Dufourc et al., 1984) and ESR (Schreier-Muccillo et al., 1973; Neal et al., 1976). These studies indicated that cholesterol has a "condensing" effect on the lipid fatty acyl chains at temperatures above that of the gel to liquid-crystalline phase transition T_m, and a disordering action below T_m. However, little detail is available on the effect of cholesterol on interchain packing of lipids in the gel phase.

A recent high-pressure FT-IR spectroscopic study on lipid–cholesterol interactions in the anhydrous solid state (Wong et al., unpublished results) has demonstrated that the magnitude of the correlation field splitting of δCH_2 is much smaller in dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) containing 20 mol % cholesterol than in pure DPPE and that the splitting is only apparent at higher pressure in the presence of cholesterol. These results suggested that insertion of cholesterol between DPPE molecules causes a reduction in the strong interchain coupling of the CH_2 scissoring modes in pure DPPE.

Prior to a study of the effect of tetracaine on DMPC bilayers containing a physiological concentration of cholesterol (30 mol %), we have monitored the effects of cholesterol on the pure lipid in the gel phase. The pressure dependence of the methylene bands for DMPC in the absence and presence of cholesterol are shown in panels A and B of Figure 6, respectively. In the presence of cholesterol, the methylene scissoring mode band and its correlation field component are much broader than those for pure DMPC, indicating increased conformational disorder of the acyl chains in the gel state due to the incorporation of cholesterol. Moreover, comparison of the pressure dependence of the frequencies of the CH_2 scissoring mode components for DMPC in the absence and presence of cholesterol (Figure 6C) indicates that the magnitude of the correlation field splitting is smaller in the presence of cholesterol and that this splitting only becomes apparent at higher pressure in this system. These results demonstrate that the intercalation of cholesterol between the lipid acyl chains in the gel state decreases the interchain interactions and increases the chain mobility, as was observed for DPPE in the solid state.

Incorporation of cholesterol (30 mol %) into DMPC bilayers in the gel phase has three main effects: a decrease in interchain interactions as reflected in the smaller correlation field splitting, an increase in reorientational fluctuations of the acyl chains as reflected in the higher pressure required for splitting, and an increased conformational disorder of the chains as

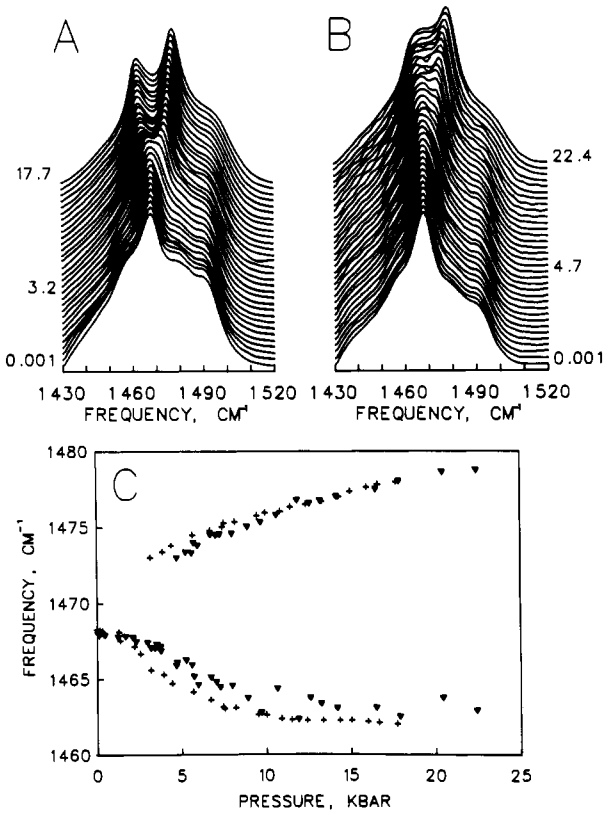

FIGURE 6: Stacked contour plots of the infrared spectra in the CH_2 scissoring region for (A) DMPC and (B) DMPC–cholesterol (7:3 molar ratio) and (C) pressure dependence of the frequencies of the δCH_2 mode for (+) DMPC and (▼) DMPC–cholesterol (7:3 molar ratio).

reflected in the broadening of the CH_2 scissoring mode bands. These results are in agreement with previous studies (vide supra) suggesting that cholesterol "disorders" the lipid acyl chains at temperatures below that of the gel to liquid-crystalline phase transition T_m.

Effects of Tetracaine on DMPC–Cholesterol Bilayers. Since many plasma membranes and most excitable plasma membranes contain a relatively large amount of cholesterol (≈30 mol % of the total lipids), we have investigated the effects of the incorporation of charged and uncharged tetracaine into DMPC bilayers containing a physiological concentration of cholesterol. As mentioned previously, ^2H NMR and high-pressure FT-IR studies have shown that the anesthetic is located close to the aqueous interface of cholesterol-containing bilayers (Auger et al., 1987, 1988).

The pressure dependences of the methylene scissoring bands for DMPC–cholesterol bilayers in the presence of uncharged and charged anesthetic are shown in panels A and B of Figure 7, respectively. Comparison of these contour plots with that obtained for DMPC–cholesterol bilayers in the absence of tetracaine (Figure 6B) indicates that incorporation of both charged and uncharged tetracaine into cholesterol-containing DMPC bilayers does not induce significant changes in the pressure dependences. The CH_2 scissoring mode band and its correlation field component remain very broad, indicating significant conformational disorder of the lipid acyl chains.

Figure 8A compares the pressure dependence of the δCH_2 mode frequencies for DMPC–cholesterol bilayers in the absence and presence of tetracaine. Upon addition of both charged and uncharged tetracaine, the magnitude of the correlation field splitting for DMPC–cholesterol bilayers is not significantly changed while the pressure at which this splitting first occurs is 4.8 kbar in the absence of anesthetic,

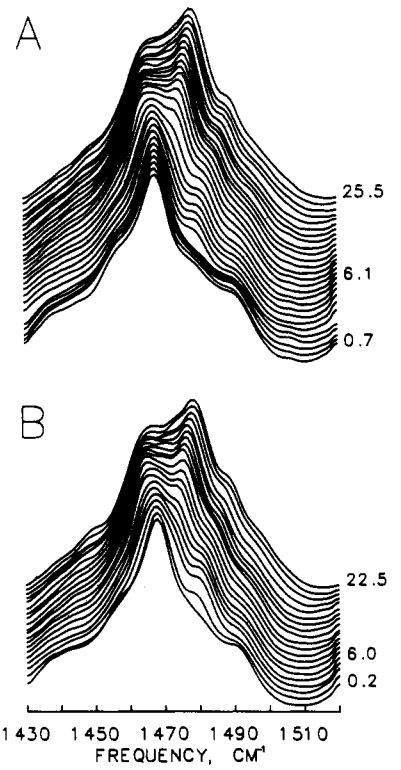

FIGURE 7: Stacked contour plots of the infrared spectra in the CH_2 scissoring region for (A) DMPC–cholesterol + TTC, pH 9.5, and (B) DMPC–cholesterol + TTC, pH 5.5.

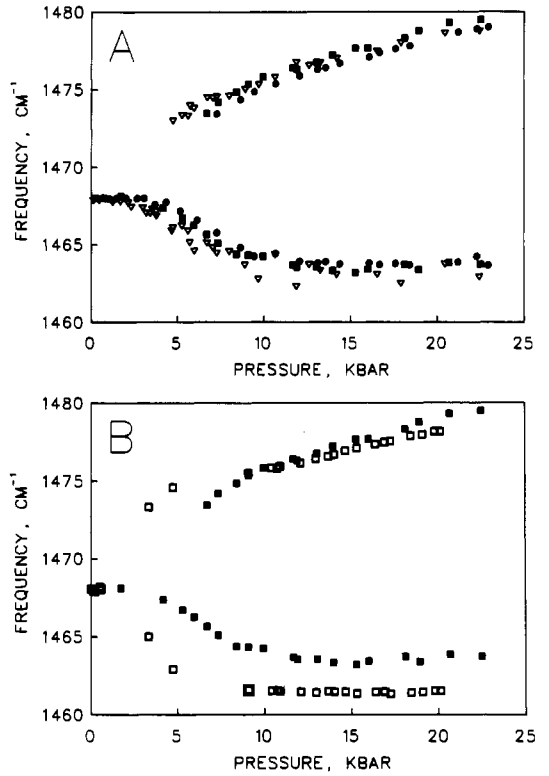

FIGURE 8: (A) Pressure dependence of the frequencies of the δCH_2 mode for (▽) DMPC–cholesterol, (●) DMPC–cholesterol + TTC, pH 9.5, and (■) DMPC–cholesterol + TTC, pH 5.5, and (B) pressure dependence of the frequencies of the δCH_2 mode for (□) DMPC + TTC, pH 5.5, and (■) DMPC–cholesterol + TTC, pH 5.5.

compared to 6.1 kbar in the presence of uncharged tetracaine and 6.0 kbar in the presence of charged tetracaine. These results suggest that the addition of the anesthetic only slightly increases the reorientational fluctuations of the lipid acyl chains in the gel state.

As mentioned previously, the addition of charged tetracaine to pure DMPC bilayers induces the formation of an interdigitated lamellar gel phase. In order to see if the presence of 30 mol % cholesterol in this system prevents the formation of the interdigitated phase, we have compared the pressure dependences of the methylene scissoring mode bands for DMPC + TTC, pH 5.5 (Figure 1C), and DMPC–cholesterol + TTC, pH 5.5 (Figure 7B). These figures show that the valley between the two component bands of the pressure-induced correlation field splitting is less pronounced in the presence of cholesterol. Moreover, the pressure dependence of the frequencies of the CH_2 scissoring bands for these two systems (Figure 8B) indicates that the magnitude of the correlation field splitting is smaller in the presence of cholesterol and that the first manifestation of this splitting in DMPC–cholesterol + TTC, pH 5.5, is at 6.0 kbar compared to 3.3 kbar for DMPC + TTC, pH 5.5. These results suggest that the presence of 30 mol % cholesterol does, to a large extent, prevent the interdigitation of DMPC acyl chains in the presence of charged tetracaine.

Conclusions

The present results indicate that high-pressure FT-IR spectroscopy is a valuable technique to study the effects of exogenous agents, such as local anesthetics, on lipid membranes. For all the systems studied, the infrared spectra at atmospheric pressure were very similar, but the pressure dependence of the methylene scissoring and rocking mode bands contains a great deal of information about the structural and dynamic properties of the lipid hydrocarbon chains. This information is complementary to that obtained by other spectroscopic techniques and demonstrates that high-pressure FT-IR is more than simply an alternative approach.

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Mapping of Caldesmon: Relationship between the High and Low Molecular Weight Forms†

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ABSTRACT: Caldesmon is a widely distributed contractile protein that occurs in both a high molecular weight [120–150-kilodalton (kDa)] and a low molecular weight (71–80-kDa) form, depending on the tissue. The structural relationship between these two forms was examined by mapping techniques. Partial cyanogen bromide cleavage in conjunction with sodium dodecyl sulfate gel electrophoresis was used to construct a map of the cleavage points and determine the relative position of the fragments in a high molecular weight caldesmon from chicken gizzard ($caldesmon_{125}$). By use of this map, markers for different regions of the protein were obtained: Antibodies directed toward certain areas were prepared by affinity purification, and specific ^{125}I-labeled tryptic peptides were found to originate from terminal cyanogen bromide fragments. Mapping of a lower molecular weight form of caldesmon ($caldesmon_{72}$ from chicken liver) revealed the presence of sequences located in both ends of $caldesmon_{125}$. A terminal 38-kDa fragment of both proteins was apparently identical on the basis of arrangement of cleavage sites, antibody reactivity, and iodopeptide mapping. Fragments from the other end of both proteins exhibited an identical pattern of peptides. These results show that it is sequences located in the central area of $caldesmon_{125}$ which are missing in $caldesmon_{72}$, indicating that the smaller molecule is not simply a proteolytic product of the larger. The two forms of caldesmon may be derived from separate genes or by alternative splicing from a single gene.

Caldesmon was originally identified as a major calmodulin- and actin-binding protein in smooth muscle (Sobue et al., 1981). Although its function in vivo is not yet certain, caldesmon has many properties suggestive of a contractile regulator. In the absence of calcium ion, the protein inhibits the myosin ATPase reaction and binds tightly to actin filaments (Sobue et al., 1982; Ngai & Walsh, 1984; Marston & Lehman, 1985; for reviews, see Kakiuchi & Sobue, 1983; Marston & Smith, 1985). When the calcium concentration rises, calcium–calmodulin decreases the affinity of caldesmon for actin and abrogates the inhibition of the ATPase. Hence, caldesmon may play a role in smooth muscle analogous to that of troponin I in skeletal muscle. Caldesmon is also subject to phosphorylation in vitro and in vivo, possibly indicating further levels of control (Ngai & Walsh, 1984; Marston & Lehman, 1985; Umekawa & Hidaka, 1985; Litchfield & Ball, 1987).

Caldesmon was first isolated as a doublet of 150K and 147K from smooth muscle, but it is now clear that it is widely distributed (Ngai & Walsh, 1985a; Owada et al., 1984; Sobue et al., 1985; Kakiuchi et al., 1983; Bretscher & Lynch, 1985). Antibodies to smooth muscle caldesmon recognize proteins in a variety of different tissues and cell lines. These cross-reactive caldesmons fall into two apparent classes on the basis of size, one of 120–150K and the other of 70–80K. Often both types are found in a single cell (Bretscher & Lynch, 1985). The lower M_r proteins share all the known properties of caldesmon including stability to heat. The different sizes of caldesmon are referred to by subscripting their relative molecular masses [e.g., $caldesmon_{120}$, Sobue et al. (1985)].

Details of the relationship between the two forms of caldesmon are currently not known. The most obvious possibility is that the smaller forms are proteolytically cleaved fragments of the larger (Ngai & Walsh, 1985a). Caldesmon is known to be very sensitive to proteases. There is, however, evidence to suggest that the smaller form exists prior to cell lysis: inclusion of protease inhibitors does not influence the profile of reactive proteins (Owada et al., 1984), lower molecular weight caldesmon is not generated from higher molecular weight caldesmon added to extracts (Bretscher & Lynch, 1985), and disruption of cells in 10% TCA[1] or hot SDS still allows subsequent detection of lower M_r forms (Bretscher & Lynch, 1985). In order to investigate this question more directly, we have used mapping techniques to compare $caldesmon_{125}$ and $caldesmon_{72}$ isolated from chicken tissues. Mapping of cyanogen bromide cleavage sites by partial cleavages and SDS gel electrophoresis allowed ordering of the fragments and delineation of markers for both ends of the molecule. The results indicate that the lower M_r form cannot be a simple

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[1] Abbreviations: SDS, sodium dodecyl sulfate; kDa, kilodalton; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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