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Pressure Effects on Dipalmitoylphosphatidylcholine Bilayers Measured by ^2H Nuclear Magnetic Resonance†

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ABSTRACT: The effects of pressure, up to 5 kbar, on multilamellar vesicles of 1,2-dipalmitoyl-*sn*-phosphatidylcholine perdeuterated in the acyl chains (DPPC-d_{62}) were examined by using high-pressure NMR techniques. A deuterium probe was built, and the quadrupole splitting was measured against pressure at various temperatures. The experiments were performed on pure lipid bilayers in the liquid-crystalline state and on bilayers in the liquid-crystalline state containing the local anesthetic tetracaine. The results show that the order parameter of all segments of the acyl chains increases with pressure in the liquid-crystalline state. The more highly ordered regions of the chains are affected slightly more than the regions near the methyl ends. The addition of tetracaine increases the disorder of the chains, and pressure reverses the effect of anesthetic on the lipid as seen by the reversal of the changes in line shape and the measured order parameter.

The thermodynamic understanding of any system depends on the measurement of changes caused by the variation in the classic parameters of temperature and pressure. Temperature studies of biochemical systems are easy to perform and have been the mainstay of the thermodynamic measurements in the past. Pressure studies of protein and membrane systems, while technically more difficult, have been initiated in the last decade or so in the interest of a more complete physicochemical understanding of these systems.

Of particular relevance to this study are the high-pressure investigations of membranes and their model systems that have been performed with a variety of techniques including IR and Raman spectroscopy (Wong, 1987a,b), NMR[1] spectroscopy (Jonas et al., 1988, 1990), ESR (Trudell et al., 1974), X-ray diffraction (Stamatoff et al., 1978), neutron scattering (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989), light transmission (Prasad et al., 1987), fluorescence spectroscopy (Chong & Weber, 1983; Chong, 1988), volumetric measurements (Tosh & Collings, 1986), and others.

Of particular physiological interest has been the effect of pressure on anesthetic action both in vivo (Lever et al., 1971) and in vitro (Mountcastle et al., 1978). In most cases pressure reverses anesthetic action. The mechanism of this action is still uncertain; however, the site of action is generally presumed to be the cellular membrane of the neuron. Whether anesthetics have a direct action on the membrane proteins, membrane lipids, or both is uncertain, but the correlation of anesthetic potency with membrane solubility is well-known. The reversal and antagonism of anesthetic action by pressure have been regarded as a possible key to the mechanism of action of anesthetics. Anesthetics increase the volume of the bilayer (Miller et al., 1973) and its fluidity (Trudell et al., 1973), and pressure is presumed to reverse both of these effects. It should be noted, however, that there are several reported cases (Halsey & Wardley-Smith, 1975; Kending & Cohen, 1977; Smith et al., 1984; Lodge, 1985) where pressure does not reverse the anesthetic effect.

To further investigate the effects of pressure on a model membrane system, we decided to use ^2H NMR to probe the pressure effects on DPPC multilamellar vesicles. Previously, we showed the feasibility of using high-pressure ^{13}C NMR as a method of studying pressure-induced phase changes in sonicated DPPC vesicles (Jonas et al., 1988). We have also used high-pressure one- and two-dimensional ^1H NMR to study pressure-induced changes in the spectra of DMPC and POPC sonicated vesicles (Jonas et al., 1990). In the latter paper, our results were consistent with an increase in the order parameter of the acyl chains in the liquid-crystalline state as pressure was increased.

In this study, ^2H NMR is used to study the effects of pressure on multilamellar vesicles of chain-perdeuterated DPPC. ^2H NMR has proven to be a very useful nonperturbing method for quantitatively measuring order and dynamics in lipid bilayer systems in both the gel and liquid-crystalline states

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[1] Abbreviations: NMR, nuclear magnetic resonance; DPPC 1,2-dipalmitoyl-*sn*-phosphatidylcholine; DPPC-d_{62}, chain-perdeuterated DPPC; DMPC, 1,2-dimyristoyl-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine.

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(Seelig & MacDonald, 1987; Davis, 1983; Smith & Oldfield, 1984; Smith, 1984). Perdeuterated lipids have the advantage of low cost and increased sensitivity at the expense of resolution; still, much information can be obtained, especially in the liquid-crystalline state.

To our knowledge, this is the first high-pressure ^{2}H NMR study performed on any biochemical system. Specifically, we set out to investigate how volume changes affect the order in the acyl-chain region of a pure phospholipid bilayer and a bilayer containing a dissolved local anesthetic.

Experimental Procedures

Sample. Chain-perdeuterated DPPC (DPPC-d_{62}) was purchased from Avanti Polar lipids, Birmingham, AL. Thin-layer chromatography (TLC) analysis of the lipid gave only one spot upon development in appropriate solvents. For samples without anesthetic, DPPC-d_{62} was dispersed in warm distilled water above the phase-transition temperature of the perdeuterated lipid (37 °C) and vortexed several times until an even dispersion was formed. The sample was pipetted directly into the sample cell and transferred to the spectrometer.

The samples containing anesthetic were prepared in a buffer containing 0.02 N citrate, 0.1 N NaCl, 0.02 N phosphate, and 0.017 N borate, adjusted to pH 5.5 with concentrated HCl. Perdeuterated DPPC was dispersed in buffer at a final concentration of 0.2 M. Tetracaine (from Sigma Chemical Co.) was added to give a final concentration of 0.1 M. The effective concentration of tetracaine in the DPPC bilayers was estimated to be 0.08 M on the basis of the partition coefficient of 22 (grams of anesthetic per gram of each phase) determined by Bulanger et al. (1980) at pH 5.5. The sample was vortexed several times while it was being kept above the transition temperature, until a smooth dispersion was formed. As above, the sample was transferred directly to the sample cell and then to the spectrometer.

NMR Probe and Spectrometer. In order to perform NMR experiments up to 5 kbar, a specially designed high-pressure vessel was used. The titanium pressure vessel, described elsewhere (Jonas et al., 1981), fits into a wide-bore (13 cm) Oxford superconducting magnet operating at a field strength of 4.2 T and at a deuterium frequency of 27.6 MHz. The pressure was varied inside the vessel by using CS_2 pressurizing fluid. The sample was isolated from the pressurizing fluid by a movable Teflon piston that transmits pressure. A solenoid coil was used to increase sensitivity (as compared to a Helmholtz coil). Temperature was controlled by means of an ethylene glycol/water bath flowing through coils wound around the high-pressure vessel and was monitored via a copper/constantan thermocouple placed inside the vessel.

A GE computer and a Nicolet 1280 data system were interfaced to a home-built spectrometer (Grandinetti, 1989) system; 130 W of power was delivered to the deuterium probe, giving a 90° pulse length of 4.5 μs for a 5 × 12 mm coil. The probe gave a signal-to-noise ratio of 8:1 (500 scans) for natural abundance deuterium (0.016%) in water.

Spectral Acquisition Procedures. In the liquid-crystalline phase, spectra were measured by using the quadrupole echo technique (Davis et al., 1976) with quadrature phase detection and four-step phase cycling. In most cases, the basic quadrupole echo sequence, $\pi/2_x$–τ_1–$\pi/2_y$–τ_2–acquire, was replaced with a composite pulse sequence (Levitt et al., 1984) with $\pi/2 = [(3\pi/4)_0(\pi/2)_\pi(\pi/4)_0]$. The conditions for a representative spectrum were as follows: a delay time of 60 μs was used; data acquisition was initiated prior to the solid echo by adjustment of T_2; the dwell time was 7 μs; the FID was left shifted to the top of the echo; and 1000 scans were taken at a rate of 4 s^{-1} for each condition of pressure and temperature. The sample was kept in the liquid-crystalline state as much as possible when pressures were changed to new experimental conditions.

Analysis of Data. FIDs were phased to produce a maximum value in the real portion of the quadrature signal, producing a symmetric line shape upon Fourier transformation. Lorentzian broadening of 30–100 Hz was used in the liquid-crystalline state.

Results and Discussion

The application of high pressure to a liquidlike system can be thought of as a probe of the intermolecular interactions in that system. Thus, the use of high pressure to study the "fluidlike" liquid-crystalline state of the DPPC model membrane provides information on the acyl-chain interactions that occur in DPPC-d_{62} bilayers. An increase of pressure in a liquid-crystalline bilayer can be qualitatively thought to decrease the "fluidity" of the membrane by a simple volume effect that increases molecular interactions and order in the bilayer (Chong & Weber, 1983). Although membrane fluidity has no precise definition, it can be described by several different properties of the lipids, including diffusion, order, packing, and permeability. In this study, we are interested in the order of the membrane lipids as expressed by the order parameter measured by ^{2}H NMR. Since, to our knowledge, there have been no measurements of the relationship between order parameter and volume in the hydrocarbon chains of the lipid bilayers, the application of high pressure combined with ^{2}H NMR seems to be suitable for this purpose.

^{2}H NMR has been widely used as a probe of membrane structure and motions because it is a nonperturbing probe that gives easily interpretable, quantitative insights into the order and dynamics of the lipids. Experimentally, the order and dynamics are assessed from the NMR spectra by the order parameter and relaxation measurements, respectively. In this study, we will concern ourselves only with the order parameter. The order parameter, S_{CD}, of a carbon–deuterium bond in the hydrocarbon-chain region of the phospholipid bilayer is given by (Seelig & Seelig, 1974) $S_{CD} = (4/3)(\Delta\nu)/k$, where k is the quadrupole coupling constant, $k = e^2qQ/h = 167$ kHz, and $\Delta\nu$ is the measured quadrupole splitting from the powder pattern. Note that S_{CD} is dependent upon the time-averaged orientation of the particular C–D bond with respect to the bilayer normal. If θ is the instantaneous angle of the C–D bond with respect to the normal, then $S_{CD} = (1/2)(3\langle\cos^2\theta\rangle - 1)$, where the brackets denote an average over the time scale of the experiment ($\sim 10^{-5}$ s). Thus, if one measures the experimental quadrupole splitting, one can say something about the average orientation of a particular C–D bond. In general, however, we will interpret the changes in order parameter caused by a perturbation of the system as changes in "order": $\Delta S_{CD} > 0$ gives an increase in order (the C–D bond spends more of its time as part of a translike segment), and $\Delta S_{CD} < 0$ gives a decrease in order. A more exact interpretation of changes in order parameter requires statistical mechanical modeling (Pastor et al., 1988; Meraldi & Schlitter, 1981; Jahnig, 1974; Schindler & Seelig, 1975).

Pure DPPC Bilayers. In this section we describe the use of chain-perdeuterated samples of DPPC to study the effects of pressure on the order of the model membrane lipids. Clearly, the use of a perdeuterated sample cannot provide the same quantitative information that specifically labeled lipids could; however, the NMR spectra of perdeuterated DPPC have been previously studied under ambient pressure conditions, providing many quantitative results. Here, we extended those

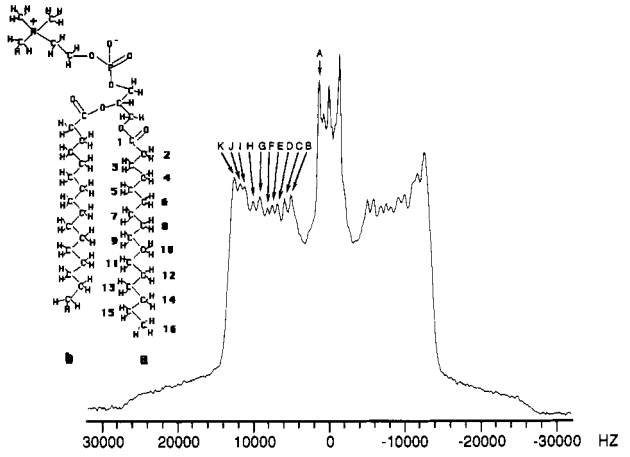

FIGURE 1: Structure of DPPC; the carbons in both acyl chains are numbered and labeled. Spectrum of DPPC-d_{62} at 45 °C, 1 bar, measured with a composite 90° quadrupole echo sequence with an echo delay, τ_1 = 70 μs, and 1200 scans. The resolved peaks are labeled, and the corresponding assignments are given in Table I.

Table I: NMR Peak Assignments for DPPC-d_{62} in the Liquid-Crystalline State at 45 °C and 1 bar[a]

peak[b]	$\Delta\nu$[c] (kHz)	assignment (carbon no. and chain a or b)[d]
A	2.69	16 (CD_3)
B	10.10	15a
C	11.73	15b, $2b_2$
D	13.66	14a
E	15.05	14b
F	16.26	13a
G	18.21	13b, 12a
H	19.97	12b, 11a
I	22.20	11b, 10a, $2b_1$
J	23.32	10b, 9a,b
K	25.01	8–3, 2a

[a] Taken from Davis (1979). [b] See the spectrum in Figure 1b. [c] $\Delta\nu = 2(\nu')$, where ν' is the distance from ν_0 to the peak. [d] See the DPPC-d_{62} structure in Figure 1a; $2b_1$ and $2b_2$ refer to nonequivalent deuterons.

studies to high-pressure conditions.

The structure of DPPC is shown in Figure 1. Since the phospholipid chains at 45 °C and 1 bar are in the disordered anisotropic liquid-crystalline state, an averaged axially symmetric powder pattern composed of many overlapping peaks corresponding to the various C–D bonds is seen in the DPPC spectrum (Figure 1). All of the peaks resolved in earlier studies at ambient pressure (Davis, 1979; Paddy et al., 1985) are resolved with our high-pressure probe and can be clearly identified (see Table I). Although we cannot resolve every deuterium in the spectrum, we can follow several individual peaks (corresponding to deuterium atoms on carbons 16, 15a,b, 14a,b, and 13a) and monitor regions of the phospholipid chains whose peaks are not independent of each other (carbons 9–13b and the "plateau" region of higher order at the edge of the spectrum).

The effect of pressure in the liquid-crystalline state on the order parameter, S_{CD}, of various peaks is seen in Figure 2a: a steady linear increase in the order parameters is produced by pressure. At this point, we cannot explain the slight upward curvature of the order parameter with pressure seen in this figure; but since it is more prominent in the plateau region, which is a region of several overlapping peaks, perhaps it is just an artifact of the differential effects of pressure on residues with nearly the same splitting. It appears that the slope increases gradually from the methyl end of the chains toward the ester bonds. Figure 2b shows more clearly that pressure has a slightly greater effect on regions of higher order within

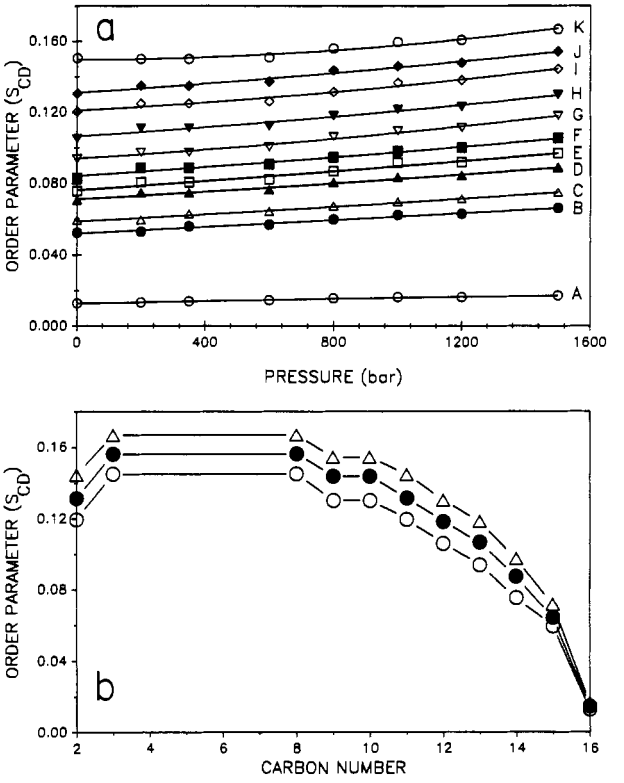

FIGURE 2: (a) Order parameter S_{CD} vs pressure at 75 °C for various splittings of DPPC-d_{62}. The peaks are labeled A–K as in Table I. (b) Order parameter S_{CD} vs carbon number for the *sn* 1 acyl chain a of DPPC-d_{62} at (○) 1 bar, (●) 600 bar, and (△) 1200 bar.

the chain. This effect has been seen and is even more marked in T_1 relaxation studies of perdeuterated bilayers (D. A. Driscoll, unpublished results), where pressure has a much greater effect on T_1 of high- vs low-order segments of the chain. It is interesting to compare these results to other pressure studies of phospholipid bilayers. ESR studies with spin-labeled egg PC vesicles indicate a linear increase in order with pressure (Trudell et al., 1973). High-pressure volumetric measurements (Tosh & Collings, 1986) of DPPC model membranes have shown a linear decrease in volume with pressure in the liquid-crystalline state. Neutron diffraction studies of DMPC (Braganza & Worcester, 1986b) show a linear increase in bilayer thickness with pressure in the liquid-crystalline state. High-pressure Raman spectroscopic studies of DPPC show several interesting features in the liquid-crystalline state (Wong & Mantsch, 1985). The data in the latter paper indicate that at constant temperature the peak height ratio of the CH_2 antisymmetric mode to the corresponding CH_2 symmetric mode, which is a qualitative measure of interchain interactions, increases with pressure. However, the parameter that is used to measure the relative population of gauche isomers in the chain and also the parameter that is used to measure the large angle interchain reorientational mobility both remain relatively unchanged by pressure but are changed markedly with temperature. The Raman method measures average changes throughout the acyl chain.

In an early statistical mechanical interpretation of order parameter results for the DPPC bilayer (Seelig & Seelig, 1974; Schindler & Seelig, 1975), the differences in order parameter along the chain (order parameter profile) and its temperature dependence were predicted by a model in which the hydrocarbon chain remained perpendicular to the bilayer surface and trans–gauche isomerizations were viewed as the main source of differences in the order parameter from one segment

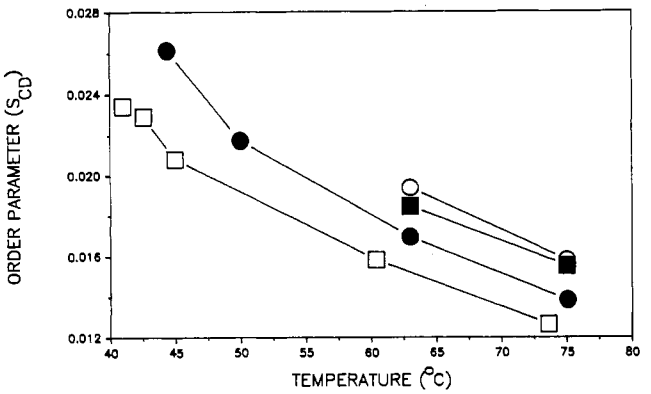

FIGURE 3: Order parameter S_{CD} of the methyl (CD_3) deuterium atoms of DPPC-d_{62} vs temperature at (□) 1 bar, (●) 350 bar, (■) 800 bar, and (○) 1000 bar.

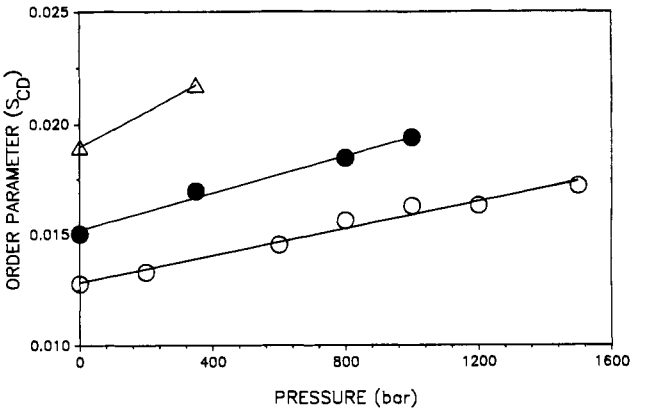

FIGURE 4: Order parameter S_{CD} of the methyl (CD_3) deuterium atoms in DPPC-d_{62} vs pressure for (△) 50 °C, (●) 63 °C, and (○) 75 °C.

of the chain to another. It is tempting to speculate that changes in the number of trans–gauche isomers might explain the increases in order parameter induced by pressure. A decrease in gauche isomers with increasing pressure could explain our results and the neutron diffraction and volumetric measurement results as mentioned briefly above.

However, Raman studies show no clear change in the number of overall gauche isomers with pressure. One need not interpret these data exclusively in terms of changes in trans–gauche ratio, however. As was pointed out by Peterson and Chan (1977), the measured order parameter, S'_{CD}, is composed of two components: S_0, which is the order parameter of the large angle fluctuation of the chain, and S_{CD} as defined earlier, corresponding to intrachain fluctuations. Thus, the measured order parameter is $S'_{CD} = S_0 S_{CD}$. In our experiments, the applied pressure may be changing either S_0 or S_{CD} to cause a measured change in S'_{CD}.

One possible model for the observed change in order parameter with pressure in the liquid-crystalline state of DPPC-d_{62} is as follows. Increasing pressure causes a decrease in overall volume available to the phospholipid molecules by "squeezing" them closer together, with an increase in overall intermolecular interaction. Since there is an increase in the thickness of the bilayer with pressure, a decrease in the overall cross-sectional area of the phospholipid molecule is needed to explain the decreased volume. The average surface area of the headgroup thus decreases. With pressure, the headgroup (which is normally oriented perpendicular to the bilayer normal) may change its orientation, thus causing the measured increase in bilayer thickness. The acyl chains would no longer have as much area for motion, because of decreased cross-sectional area per lipid molecule. The acyl-chain average orientation angle would be decreased, giving the measured increase in order parameter with pressure.

Figure 3 shows the constant-pressure isobars for changes in the order parameter of the methyl group at varying temperatures. The decrease in slope with increasing temperature is well-known at 1 bar and is observed here at various pressures. A Boltzman distribution of conformational disorder with temperature explains these curves. Figure 4 shows the effect of pressure on the order parameter of the methyl group, along isotherms in the liquid-crystalline state. The effect of pressure seems to be greater at lower temperatures; that is, $\Delta S_{CD}/\Delta P \propto 1/T$. Pressure changes the order of the system more markedly when the overall order is higher. This seems to be also true in the lipid layers containing anesthetic. Perhaps this may be related to the orientational and conformational energies of the chain: when more conformations are readily available (at higher temperatures), the chains can more readily respond elastically to an increase in interchain interactions by changing their intrachain conformations.

Bilayers Containing Anesthetic. The early observation that anesthetic potency is related to the relative solubility of the anesthetic in lipids has led to the view that anesthetics affect the nervous system through their action at the neuronal lipid membrane. Whether they directly affect the lipid and/or the protein components of the membrane is still controversial. If anesthetics do act physiologically via the lipid bilayer, their effect may depend on any of the following factors (Roth, 1979): (1) concentration of anesthetic in the lipid, (2) increased membrane volume, or (3) membrane expansion (increased surface area per lipid molecule).

The reversal of anesthetic action by pressure is regarded as strong support for the volume and membrane expansion hypotheses of anesthetic action. Pressure causes a decrease in the volume of liquid-crystalline membranes and increases the thickness of the membrane while reducing the surface area per lipid molecule.

The local anesthetic tetracaine has been used to demonstrate the effect of anesthetics on model membranes. For example, Boulanger et al. (1981) using ^{2}H NMR of perdeuterated and specifically labeled DPPC have shown tetracaine causes an overall disordering effect in the membrane and a specific decrease in the region of high order (plateau region). Tetracaine in increasing concentrations results in a decreased intensity of the plateau region and a buildup in intensity in regions of smaller order parameters. In addition, the quadrupole splitting decreases linearly for the acyl-chain region. The calculated width of the bilayer decreases with increasing tetracaine concentration, and the calculated area per lipid molecule increases, supporting the hypotheses stated above. Charged tetracaine was shown to rest at the interface between lipid and water, while the uncharged form was located deeper in the hydrophobic region of the bilayer (Boulanger et al., 1980).

Tetracaine is known to induce interdigitated gel forms of DPPC, presumably because the interfacial location of the molecule leads to separation of headgroups, allowing for interdigitation (Simon et al., 1986). The formation of an interdigitated tetracaine-induced gel phase was seen by using high-pressure FTIR spectroscopy on DMPC bilayers (Auger et al., 1988). This anesthetic was reported to be excluded from the DMPC membrane bilayer at 4.6 kbar in the absence of cholesterol at pH 5.5 (Auger et al., 1987), and a similar expulsion seems to occur in phosphatidylserine membranes at high pressures (Auger et al., 1990). These pressure studies of tetracaine-containing membranes do not directly address the effect of pressure on the reversal of anesthetic action, which

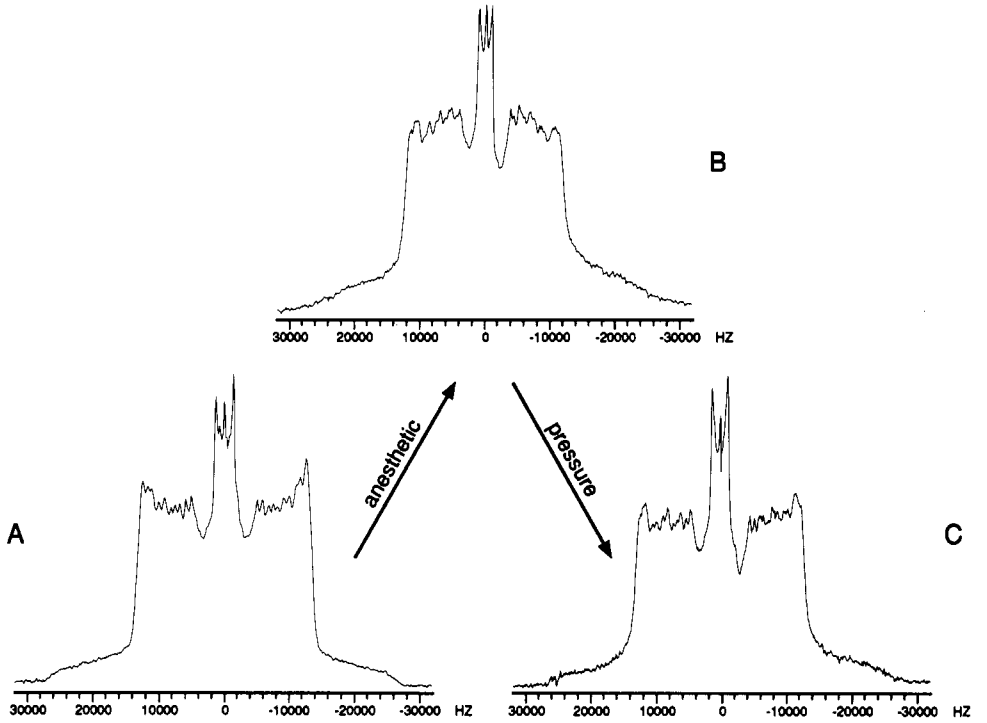

FIGURE 5: Effect of anesthetic and pressure on the line shape of DPPC-d_{62} in the liquid-crystalline state at 45 °C. (A) Pressure of 1 bar with 0.2 M DPPC and no tetracaine. (B) Pressure of 1 bar with 0.2 M DPPC and 0.1 M tetracaine. (C) Pressure of 600 bar with 0.2 M DPPC and 0.1 M tetracaine. The effective concentration of tetracaine in DPPC is 0.08 M. All spectra have 100-Hz line broadening.

occurs at much lower pressures.

Other investigators have reported that binding or partition of anesthetics into phospholipid bilayers and monolayers decreases with pressure. Kamaya et al. (1981) showed that the partition coefficient of inhalation anesthetics decreases in DPPC bilayers subjected to pressure (about a 24% decrease in partition coefficient from 1 to 300 bar). Seelig (1987) used monolayers of palmitoyloleoylphosphatidylcholine to assess the binding of dibucaine as a function of surface pressure. Her results indicate that with increasing monolayer pressure less anesthetic binds to the lipid and that dibucaine is completely excluded from the monolayers at a pressure near 39.5 mN/m, comparable to the hydrostatic pressure of 100 bar. Evidently, there is equilibration between the anesthetic in the lipid phase and in the aqueous phase, and there are pressure effects on the equilibrium. The observed effective pressures for complete expulsion depend on the physical state of the aggregated lipid, the location of the anesthetic in the bilayer, and the presence of other lipid components.

We have used ^{2}H NMR to study the effects of pressure on tetracaine-containing DPPC model bilayers in the liquid-crystalline state subjected to pressures from 1 to 600 bar.

The pressure reversal of the effect of tetracaine on our model bilayers can be directly seen by cursory examination of lineshape changes in the ^{2}H NMR spectra shown in Figure 5. As shown by other investigators, tetracaine causes a general disordering effect on the bilayer as seen by an overall decrease in the order parameter throughout the acyl chain. The plateau region seems to be affected the most, as the intensity of this region decreases compared to the end of the chains; also, a general decrease in resolution of the peaks is noted. When pressure is applied to the system, a reversal in the effect of the anesthetic is seen: the resolution of the peaks increases, the plateau region regains intensity, and the overall order becomes larger again.

Our results are quantitatively illustrated in Figure 6. Several points can be made: (1) Addition of tetracaine decreases the order parameter of the labeled group (in this case, C16 is most easily measured). (2) The phase-transition pressure increases from 350 to 600 bar at 45 °C, again pointing to the overall disordering of the membrane. This effect on the main phase transition has been noted (Trudell et al., 1975) and has been used as one hypothesis of the anesthetic effect on biological membranes. The reasoning is that anesthetic action depends on the gel to liquid-crystalline phase equilibrium, which is shifted by addition of anesthetic to the cell membrane. (3) The relationship between pressure and the order parameter is linear in both tetracaine-containing and pure membranes. (4) Pressure has a greater effect on the order parameter in the pure membranes, as judged by the slope of the line, than in the anesthetic-containing membranes.

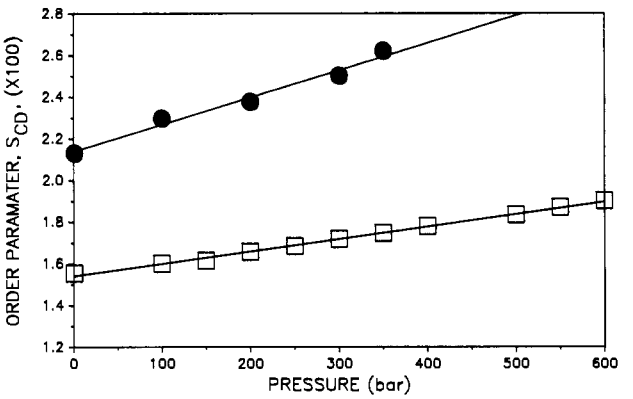

FIGURE 6: Change in the order parameter S_{CD} of the methyl group (CD_3) as a function of pressure in DPPC-d_{62} (0.2 M) with tetracaine (0.1 M) (the effective concentration in DPPC is 0.08 M) (□) and without tetracaine (●).

The data clearly indicate that pressure has a direct antagonistic effect on the perturbation of these DPPC bilayers by anesthetic, even though under our experimental conditions pressure never fully counteracts its effects. If the assumption is made that pressure does not expel tetracaine from the bilayer

until much higher pressures are reached (Auger et al., 1987, 1990), our results could be interpreted to mean that pressure reverses the increase in the volume and expansion of the membrane due to the anesthetic. The anesthetic may partition into a different portion of the bilayer with pressure, as demonstrated with the fluorescent probe PRODAN in a study by Chong (1988). On the other hand, the possibility that the concentration of tetracaine may decrease in the bilayer with increasing pressure cannot by dismissed. In fact, the observed change in the order parameter of CD_3 (Figure 6) for the bilayer containing tetracaine, in the pressure interval from 1 to 600 bar, is around 50%. This increase in order parameter is very similar to the extrapolated decrease in the partition coefficient of tetracaine (43%) in DPPC bilayers over the same range of pressures (Kamaya et al., 1981). Measurements with specifically labeled lipids and tetracaine may resolve these questions.

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