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High-Pressure ^{31}P NMR Study of Dipalmitoylphosphatidylcholine Bilayers[†]

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ABSTRACT: High-pressure ^{31}P NMR was used for the first time to investigate the effects of pressure on the structure and dynamics of the phosphocholine headgroup in pure 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) multilamellar aqueous dispersions and in DPPC bilayers containing the positively charged form of the local anesthetic tetracaine (TTC). The ^{31}P chemical shift anisotropies, $\Delta\sigma$, and the ^{31}P spin-lattice relaxation times, T_1, were measured as a function of pressure from 1 bar to 5 kbar at 50 °C for both pure DPPC and DPPC/TTC bilayers. This pressure range permitted us to explore the rich phase behavior of DPPC from the liquid–crystalline (LC) phase through various gel phases such as gel I (P_{β}'), gel II (L_{β}'), gel III, gel IV, gel X, and the interdigitated, Gi, gel phase. For pure DPPC bilayers, pressure had an ordering effect on the phospholipid headgroup within the same phase and induced an interdigitated Gi gel phase which was formed between the gel I (P_{β}') and gel II (L_{β}') phases. The ^{31}P spin-lattice relaxation time measurements showed that the main phase transition (LC to gel I) was accompanied by the transition between the fast and slow correlation time regimes. Axially symmetric ^{31}P NMR lineshapes were observed at pressures up to ~3 kbar but changed to characteristic axially asymmetric rigid lattice lineshapes at higher pressures (3.1–5.1 kbar). As expected, the addition of the positively charged form of TTC increased the absolute value of the ^{31}P chemical shift anisotropy, $|\Delta\sigma|$, and brought about a change in the conformation of the headgroup which swung toward the bilayer normal, from its usual orientation parallel to the membrane surface. The addition of TTC also raised the critical pressure of the main phase transition and induced the formation of an interdigitated Gi gel phase directly from the LC phase. Interestingly the expressions for ^{31}P, $\Delta\sigma$, introduced by Scherer and Seelig (1989) to determine the effect of electric surface charge on the polar headgroup of phospholipid bilayers were found applicable for the LC phase at high pressures and also for the gel phases at pressures in excess of 3.5 kbar.

There has been a growing interest in pressure as an experimental variable in studies of membranes by a variety of experimental techniques, including IR and Raman spectroscopy (Wong, 1987a,b), fluorescence spectroscopy (Chong, 1988; Chong & Weber, 1983), X-ray diffraction (Stamatoff et al., 1978), neutron scattering (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989; Winter et al., 1991), NMR[1] (Jonas et al., 1988; Driscoll et al., 1991a,b), and light transmission (Prasad et al., 1987). Pressure variation at constant temperature in studies of membranes allows the identification of pure volume effects, and in addition, it also allows us to change continuously the intermolecular interaction in the system studied. In contrast, temperature changes result in simultaneous thermal and volume effects which are very difficult to separate. By applying high pressure to membrane systems, one can not only observe volume effects but produce new pressure-induced phases (Wong et al., 1988). Temperature–pressure phase diagrams have been generated for several phospholipid systems (Braganza & Worcester, 1986a,b; Prasad et al., 1987; Wong et al., 1988).

In our laboratory, we have initiated systematic high-pressure NMR studies on model phospholipid membranes (Jonas et al., 1988, 1990; Driscoll et al., 1991a,b). Of particular relevance to the present study are two of our recent high-pressure NMR studies (Driscoll et al., 1991a,b) of perdeuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC-d_{62}). One series of experiments (Driscoll et al., 1991a) was performed on pure lipid bilayers of DPPC-d_{62} in the liquid–crystalline (LC) state and on bilayers in the LC state containing the charged form of the local anesthetic tetracaine (TTC). The results showed that the order parameters of all segments of the acyl chains increased with pressure in the LC state. The more highly ordered regions of the chains were affected slightly more than the regions near the methyl ends. The addition of tetracaine increased the disorder of the chains, and pressure reversed the effect of anesthetic on the lipid as seen by reversal of the changes in lineshape and the measured order parameters. The second study (Driscoll et al., 1991b) concentrated on the pressure effects on the gel states of DPPC-d_{62} and used the high-pressure ^{2}H NMR techniques to detect various gel phases and to generate a pressure–temperature phase diagram for this phospholipid. Gel state spectra were observed at several temperatures and pressures up to 5 kbar. The gel state is in reality made up of several phases, each with a particular structure. At ambient pressure, the most often studied are the P_{β}' (pretransition) and L_{β}' phases (Cevc & Marsh, 1987). Also present is a subtransition (L_c) phase which appears upon prolonged refrigeration of the L_{β}' phase. No attempt will be made to review at this point the structure and properties of these phases (Cevc & Marsh, 1987). In contrast to the above well-studied phases, much less is known about the existence and structure of other high-pressure/low-temperature phases. In particular, Wong et al. (1988) have done extensive work in this area using vibrational spectroscopy and have reported the existence of at least five high-pressure-induced gel phases which they named GI through GV, corresponding to the increasing transition pressures; GI and GII refer to P_{β}' and L_{β}', respectively, but the structure and dynamics of the GIII, GIV, and GV gel phases are not as well characterized. In addition

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[1] Abbreviations: NMR, nuclear magnetic resonance; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; TTC, tetracaine; DPPC-d_{62}, chain perdeuterated DPPC.

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to Wong's studies, small angle neutron diffraction (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989; Winter et al., 1991) studies have shown the existence of a pressure-induced interdigitated phase, Gi, in DPPC bilayers at pressures above 1 kbar and temperatures above 40 °C. This phase was also detected by other investigators (Prasad et al., 1987) using light-scattering techniques at high pressure.

For pure phosphatidylcholine bilayers, the orientation of the headgroup has been well characterized by a variety of physical methods showing that headgroups are aligned approximately parallel to the bilayer surface (Seelig & Seelig, 1980). Studies of the dynamic properties of the headgroups demonstrated that the reorientational motions were reduced by lowering the temperature in the LC state and that a distinct decrease of motions accompanied the phase transition from the LC to the gel phase (Browning, 1981).

Because only one phosphorus ^{31}P with 100% natural abundance is contained in the phospholipid molecule, ^{31}P NMR has become an important tool to study the phospholipid headgroup structure and dynamics. Many ^{31}P NMR experiments have been carried out at ambient pressure (Griffin, 1981; Yeagle, 1990; Seelig, 1978; Seelig et al., 1981; Scherer & Seelig, 1989; Lewis et al., 1984; Ruocco et al., 1985) giving information about the orientation, mobility, and interaction of the phospholipid headgroups.

Boulanger et al. (1980) have reported that the addition of TTC to phosphatidylcholine dispersions in water produced different effects depending on whether the anesthetic was positively charged (pH 5.5) or uncharged (pH 9.0). The changes in bilayer properties were monitored by ^2H and ^{31}P NMR spectroscopy. The fact that the changes observed at low pH in the ^2H and ^{31}P NMR spectra of the phospholipid headgroup in the presence of TTC resemble those produced by ions is most relevant to the present study. These results also suggested that the headgroup undergoes a conformational change upon interaction with the anesthetic. Scherer and Seelig (1989) and Seelig et al. (1987) have carried out thorough and elegant studies on the effects of electric charge on phospholipid headgroups by investigating mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) with charged amphiphiles. Deuterium, ^{31}P, and ^{14}N NMR spectra were recorded for bilayers with varying concentrations of amphiphiles. In agreement with their earlier results (Scherer & Seelig, 1989) on cationic and anionic molecules, they found (Seelig et al., 1987) that the charged amphiphiles have a major effect on the phosphocholine headgroup. The headgroups which are normally aligned parallel to the membrane surface in a pure phospholipid membrane, upon addition of a positively charged amphiphile, change in orientation by a movement of the N^+ end of the dipole toward the water phase. This result was generalized for positively charged anesthetics, charged phospholipids, and peptides.

The results obtained by Scherer and Seelig (1989) in the LC phase were extended by MacDonald et al. (1991) to the gel state of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) using ^2H NMR spectroscopy of specifically choline-deuterated DMPC. McDonald et al. (1991) provided evidence that the choline headgroup of phosphatidylcholine in the gel state also responds to charge changes at the membrane surface; thus, the response of the choline headgroup of phosphatidylcholine to surface charges is qualitatively and quantitatively similar in the LC state and in the gel state of membrane lipids.

In the present study, the ^{31}P NMR lineshapes and ^{31}P spin-lattice relaxation times of pure DPPC bilayers and DPPC bilayers containing charged tetracaine were measured as a function of pressure form 1 bar to 5.1 kbar at 50 °C. The goals of the study were as follows: (1) To determine the behavior of the headgroup in the LC phase and the various gel phases accessible at high pressure; (2) to compare the pressure effects on pure DPPC bilayers with those observed for DPPC/tetracaine bilayers; (3) to determine whether the concept of molecular electrometer as introduced by Seelig (Scherer & Seelig, 1989) is also applicable for gel phases induced at high pressures.

EXPERIMENTAL PROCEDURES

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phospholine (DPPC) and tetracaine hydrochloride (TTC) were purchased from Avanti Polar Lipids (Birmingham, AL) and Sigma Chemical Co. (St. Louis, MO), respectively. They were used without further purification. The DPPC sample purity was checked by thin-layer chromatography (TLC) using chloroform–methanol–water (65:25:4 v/v) as a solvent system before and after the experiments. The results showed a single spot and no decomposition of the sample was found by TLC. 85% H_3PO_4 from Fisher Scientific (Chicago, IL) was used as an external standard in the ^{31}P NMR chemical shift measurements. All analytical grade chemicals required for the buffer solution were purchased from Aldrich Chemical Co., Inc. (St. Louis, MO).

Sample Preparation. For pure phospholipid sample, DPPC was dispersed in deionized and distilled water above the gel–LC phase transition temperature (Cevc & Marsh, 1987) (41 °C). A homogeneous milky dispersion was formed by extensive vortexing, and the sample was transferred directly into the sample cell. The final concentration of the multilamellar DPPC dispersion was 0.2 M. For the sample of phospholipid with tetracaine, the BPC [sodium borate (0.017 N)–sodium phosphate (0.02 N)–sodium citrate (0.02 N)] buffer containing 0.1 M sodium chloride was used. The pH of the solution was adjusted to 5.5 with concentrated HCl. The DPPC was then dispersed in the BPC buffer and TTC was added. The resulting sample was subjected to at least five freeze–thaw–vortex cycles to ensure complete equilibration of TTC between the bilayer phase and aqueous phase. The sample pH was measured and readjusted if necessary. In order to remove possible paramagnetic contaminants, 0.001 M EDTA was added to ensure the accuracy of the phosphorus relaxation time measurements. The final concentrations of DPPC and TTC were 0.2 M and 0.1 M, respectively.

NMR Measurements. ^{31}P NMR measurements were performed on a home-built NMR spectrometer with a wide-bore (130 mm) Oxford 4.2-T superconducting magnet. The spectrometer is interfaced to a GE 293D pulse programmer and a GE/Nicolet 1280 computer system. ^{31}P NMR spectra were obtained at 72.9-MHz spectrometer frequency.

In order to obtain high-sensitivity ^1H decoupled ^{31}P NMR spectra, we designed and constructed a high-pressure probe (1 bar–5 kbar) with a solenoidal sample coil which was doubly tuned for ^{31}P and ^1H. To maximize the filling factor, a free standing coil was used, and the coil was wound directly on the sample cell. The pressure-transmitting fluid CS_2 was used inside the titanium high-pressure vessel, and the sample was separated from the CS_2 fluid by a Teflon piston. The temperature was controlled by a circulating mixture of ethylene glycol and water around the high-pressure vessel and was measured via a copper–constantan thermocouple inside the high-pressure vessel. The temperature was controlled within ±0.2 °C, and the pressure was accurate to ±20 bar. The pressure was measured by a Heise gauge, Model CM-18088

(Newton, Connecticut), with the range 1 bar–7 kbar, minimum scale reading 5 bar. All experiments were carried out at 50.0 °C, while pressure was varied from 1 bar up to 5 kbar. Under these experimental conditions, different phases from the liquid–crystal phase through various gel phases were observed. The equilibration time between measurements was about 1 h within the same phase and at least 2 h between different phases. The probe was retuned for each pressure measurement.

In the ^{31}P NMR experiments, a fully 32-pulse phase cycled Hahn echo sequence (Rance & Byrd, 1983) with inverse gated proton decoupling technique was used. The pulse sequence is shown below:

$$90°_{\alpha}\text{–}t_1\text{–}180°_{\beta}\text{–}t_2\text{–acq}$$

where delay time t_1 or t_2 was chosen according to $t_d < t_1$ $(t_2) \ll T_2$ (t_d is the dead time of the receiver, and T_2 is the transverse relaxation time), the subscripts α and β refer to the phases of the first and second pulses. The Hahn echo sequence was used to overcome the finite recovery time of the receiver and the probe ringdown time. We employed the phase cycling technique to prevent spectra distortion due to the misset timing of the pulse lengths and the proton decoupling technique to avoid spectral distortion due to the ^{31}P–^{1}H dipolar interactions. Data were recorded prior to the echo maximum to avoid missing of the first refocusing point of the echo. After the FID was left-shifted to the echo maximum, the data were transformed starting right on the top of the echo. In order to prevent sample heating from the proton decoupling field, the proton decoupler was gated on during the acquisition and gated off the rest of the time. The decoupling frequency was set at the resonance frequency of the DPPC headgroup protons. The decoupling field strength was 10 kHz compared to the ^{31}P–^{1}H dipolar coupling constants which range from 1 to 6 kHz (Chan et al., 1981) and the broad band heteronuclear decoupling was used to get more efficient proton decoupling. The other experimental conditions were as follows: the 90° pulse length was 7 μs, the pulse spacing was 40–50 μs, the recycle decay was 5–15 s depending on the T_1 values of the various phases. The spectral width was 50 kHz, the experimental line broadening was 100–200 Hz, and the data size of 4K points was used. The data were collected with a quadrature detection, and a total of 3200–7200 scans were taken. The estimated error for the chemical shift anisotropy values was ±1.5 ppm.

In order to measure spin-lattice relaxation times, we have modified the conventional inversion–recovery method by including the Hahn echo and phase-cycling. The pulse sequence is given below:

$$180°_{\text{comp}}\text{–}\tau\text{–}90°_{\alpha}\text{–}t_1\text{–}180°_{\beta}\text{–}t_2\text{–acq}$$

where a composite 180° pulse ($180°_{\text{comp}} = 90°_x 180°_y 90°_x$) was used for inversion pulse to prevent experimental error due to the B_1 field inhomogeneities and resonance offset. The magnetization was sampled at various delay times τ in the range of 0–2T_1, and the partially relaxed spectra were used to monitor the recovery of magnetization. A recycle delay time of at least 5T_1 was chosen to assure the full recovery of the magnetization. The final T_1 values were obtained by a least-squares fit to the amplitude results. The estimated error was about ±8%. The T_1 measurements were carried out under inverse gated proton decoupling conditions.

RESULTS

Using a temperature of 50 °C and the pressure range from 1 bar to 5 kbar in our experiments allows us to determine ^{31}P NMR lineshapes in the liquid–crystalline state and various gel states of DPPC. Figure 1 shows the representative ^{31}P NMR spectra for DPPC multilamellar bilayers at various pressures in the LC phase and the various gel phases induced by high pressure.

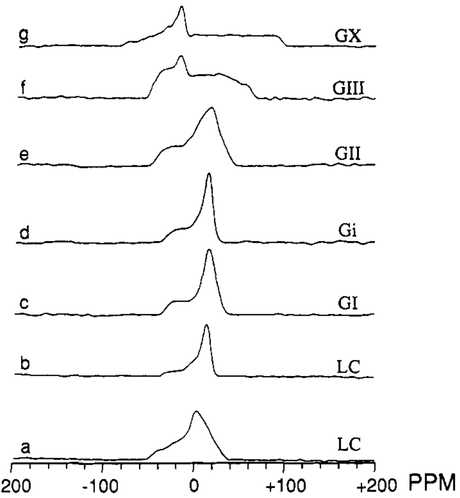

FIGURE 1: ^{31}P NMR spectrum obtained using a single 90° pulse without ^{1}H decoupling in pure DPPC bilayers at 50 °C and 1 bar (a); and ^{31}P NMR spectra obtained using a fully phase-cycled Hahn echo sequence with inversely gated ^{1}H decoupling in pure DPPC bilayers at 50 °C and 1 bar in the LC phase (b); 1 kbar in the gel GI phase (c); 1.75 kbar in the interdigitated Gi gel phase (d); 2.5 kbar in the GII gel phase (e); 3.70 kbar in the GIII gel phase (f); 5.10 kbar in the GX gel phase (g).

As it can be seen from Figure 1, the lineshape distortion caused by proton–phosphorus dipolar interactions was removed by ^{1}H decoupling. The ^{31}P NMR spectra of DPPC in the LC state, gel I, the interdigitated gel Gi phase, and the gel II phase show a typical axially symmetric powder lineshape (Griffin, 1981). In contrast, the lineshape becomes axially asymmetric (Griffin, 1981) in the high-pressure gel III phase. The asymmetry increases further in the last accessible gel phase gel X for which a typical rigid lattice axially asymmetric powder pattern can be observed. The experimental chemical shift anisotropy values, $\Delta\sigma$, were determined using the standard procedures (Seelig & Seelig, 1980; Griffin, 1981). For the axially symmetric lineshapes, the anisotropic part of the time-averaged shielding tensor is defined as

$$\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp} \tag{1}$$

where $\sigma_{11} = \sigma_{22} = \sigma_{\perp}$ and $\sigma_{33} = \sigma_{\parallel}$.

For the axially asymmetric powder pattern the shielding anisotropy was calculated using the following expression:

$$(\Delta\sigma)^2 = 3/2[(\sigma_{11} - \sigma)^2 + (\sigma_{22} - \sigma)^2 + (\sigma_{33} - \sigma)^2] \tag{2}$$

where σ_{11}, σ_{22}, and σ_{33} have their usual meaning and σ denotes the isotropic part of the shielding tensor:

$$\sigma = 1/3(\sigma_{11} + \sigma_{22} + \sigma_{33}) \tag{3}$$

The calculated $\Delta\sigma$ values are given in Table I and plotted as a function of pressure in Figure 3. Only the absolute value of $\Delta\sigma$ can be determined from eq 2, but in view of the fact that $\Delta\sigma$ is negative (Scherer & Seelig, 1989) for the LC phase and the temperature-induced gel phases (gel I and gel II), we expect that $\Delta\sigma$ will be also negative for the high-pressure gel phases of DPPC.

The experimental ^{31}P NMR lineshapes for DPPC bilayers containing tetracaine are shown in Figure 2, and the calculated $\Delta\sigma$ values are given in Table I and also plotted as a function of pressure in Figure 3, in order to permit a direct comparison between the $\Delta\sigma$ values obtained for pure DPPC and

Table I: Experimental ^{31}P Chemical Shift Anisotropy ($\Delta\sigma$) and ^{31}P Spin–Lattice Relaxation Times (T_1) for DPPC and DPPC/TTC Multilamellar Bilayers at 50.0 °C

DPPC			DPPC/TTC		
P(kbar)	$\Delta\sigma$ (ppm)	T_1 (s)	*P*(kbar)	$\Delta\sigma$ (ppm)	T_1 (s)
LC phase			LC phase		
0.001	−46	3.5	0.001	−59	3.0
0.125	−47	3.2	0.125	−60	2.8
0.250	−49	3.0	0.250	−62	2.5
0.375	−50	2.8	0.375	−64	2.2
0.500	−52	2.5	0.500	−65	2.0
gel I phase			0.625	−67	1.7
0.750	−59	1.6	0.750	−68	1.5
1.000	−66	1.8	gel INT phase		
1.300	−72	2.0	1.000	−58	1.6
gel INT phase			1.300	−59	1.8
1.500	−56	1.6	1.500	−60	1.9
1.750	−58	1.8	1.750	−62	2.0
2.000	−60	1.9	2.000	−64	2.1
gel II phase			2.250	−67	2.3
2.250	−76	2.4	2.500	−70	2.4
2.500	−79	2.6	2.750	−75	2.6
2.750	−83	2.8	3.000	−83	2.8
gel III phase			high-pressure gel phases		
3.250	−97	3.0	3.250	−101	3.1
3.700	−108	3.2	3.700	−115	3.4
4.200	−119	3.5	4.200	−129	3.8
gel X phase			4.500	−140	4.1
4.500	−125	3.7	4.800	−153	4.3
4.800	−135	4.0	5.100	−172	4.6
5.100	−152	4.2			

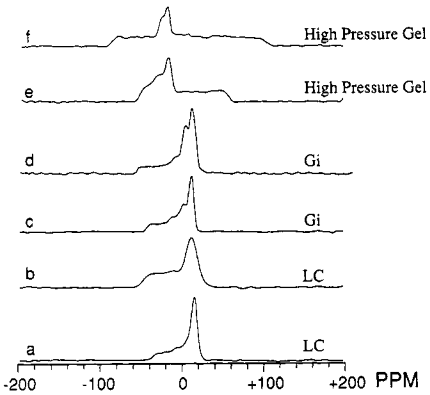

FIGURE 2: ^{31}P NMR spectra obtained using a fully phase-cycled Hahn echo sequence with inversely gated ^1H decoupling in DPPC/TTC bilayers at 50 °C and 1 bar in the LC phase (a); 750 bar in the LC phase (b); 1 kbar in the interdigitated Gi gel phase (c); 2.75 kbar in the interdigitated Gi gel phase (d); 3.25 kbar in the high pressure gel phase (e); 5.1 kbar in the high-pressure gel phase (f) (for details, see the text).

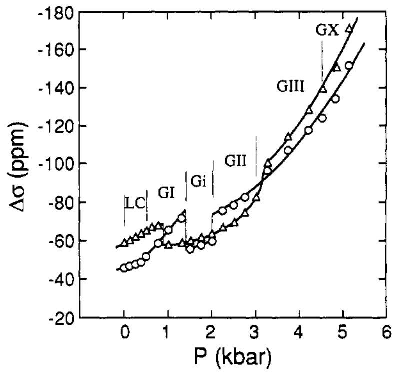

FIGURE 3: Pressure dependence of ^{31}P chemical shift anisotropy values for DPPC bilayers (○) and DPPC/TTC bilayers (△) at 50 °C. The pressure ranges for different phases of pure DPPC are indicated (for detailed discussion, see the text).

DPPC/TTC bilayers. In Figure 2, the small peak near the main peak in the interdigitated gel phase (Figure 2c,d) arises from the buffer phosphate ion. According to Brown and Seelig (1977), no spectral changes in the ^{31}P lineshapes of DPPC are due to general ionic strength effects, and consequently, the addition of the buffer used in our DPPC/TTC experiment has no effect on the observed ^{31}P lineshapes. Similarly, as for pure DPPC bilayers, the ^{31}P lineshape shows the characteristic axially symmetric powder patterns for the LC phase and the interdigitated Gi gel phase but changes to an axially asymmetric powder pattern in the high-pressure gel phases.

The effect of pressure on the measured chemical shift anisotropy values, $\Delta\sigma$, is shown graphically in Figure 3 for both pure DPPC and DPPC/TTC bilayers. For pure DPPC, the absolute value of $|\Delta\sigma|$ increases slightly with pressure in the LC phase in the pressure range from 1 bar to 500 bar. In the gel I phase, the $|\Delta\sigma|$ value continues to increase with pressure until the phase transition gel I/Gi is reached. As expected, the $|\Delta\sigma|$ values for the interdigitated gel phase of DPPC are smaller than in the other gel phases. An abrupt change to higher $|\Delta\sigma|$ value occurs at the Gi/gel II phase transition pressure. In the gel phases gel II, gel III, and gel X, the $|\Delta\sigma|$ values increase continuously with increasing pressure, and it is evident that there are no distinct changes in the $\Delta\sigma$ vs pressure plots associated with the expected gel II/gel III and gel III/gel X phase transitions.

In agreement with literature (Scherer & Seelig, 1989; Boulanger et al., 1980), the $|\Delta\sigma|$ value increases from −46 to −59 ppm when charged tetracaine is added to DPPC bilayers in the LC state at 1 bar and 50 °C. Again, we find that $|\Delta\sigma|$ increases with pressure up to 0.8 kbar, but then, the $|\Delta\sigma|$ value changes abruptly to a lower value indicating a direct transition to the interdigitated gel phase (Prasad et al., 1987). As pressure increases, the $|\Delta\sigma|$ value gradually increases in the region between 1 kbar and 3.1 kbar and finally approaches the value of $\Delta\sigma$ found for pure DPPC bilayers at about 3.1 kbar. It can be noticed that the $\Delta\sigma$ values in this pressure range are lower than those of pure DPPC in gel I and gel II phases but greater than those in the interdigitated gel Gi phase

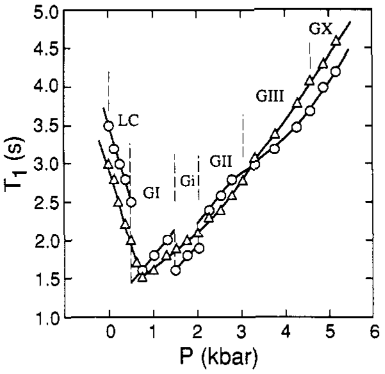

FIGURE 4: ^{31}P spin-lattice relaxation times (T_1) as a function of pressure for pure DPPC bilayers (○) and DPPC/TTC bilayers (△) at 50 °C. The pressure ranges of differential phases of pure DPPC are indicated (for detailed discussion, see the text).

of pure DPPC. Finally, in the region between 3.1 and 5.1 kbar, the $|\Delta\sigma|$ values increase monotonically and are greater than those of pure DPPC.

In order to obtain more information on the dynamics of the phospholipid headgroups, we have also measured the pressure dependence of the ^{31}P spin-lattice relaxation times, T_1, in bilayers of pure DPPC and DPPC with tetracaine. The experimental T_1 values are listed in Table I, and the pressure dependence of T_1 for both DPPC and DPPC/TTC is shown in Figure 4. As it can be seen from Figure 4, there are three distinct breaks in the T_1 vs pressure dependence observed for DPPC bilayers, corresponding to the phase transitions LC/gel I, gel I/interdigitated gel Gi, and Gi/gel III, respectively. In the LC phase, the T_1 value decreases nearly linearly with increasing pressure. At the LC/gel I phase transition pressure, the T_1 value drops from 2.5 to 1.5 s. In the gel I phase, the T_1 value increases with increasing pressure indicating a transition from the fast correlation time regime ($\omega_0\tau_c \ll 1$) to the slow correlation time regime ($\omega_0\tau_c \gg 1$). It is interesting to observe in Figure 4 the clear demarcation of the interdigitated gel phase Gi, as the T_1 value drops at about 1.4 kbar and returns to a higher value at about 2 kbar. In the subsequent gel II, gel III, and gel X phases, the T_1 values increase continuously with pressure, and no major sudden changes in the T_1 value are observed at the expected phase transition pressures corresponding to gel II/gel III and gel III/gel X phase transitions.

DISCUSSION

The structural information on the effects of pressure on DPPC bilayers is relatively limited (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989; Winter et al., 1991). Neutron scattering experiments (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989) on DPPC bilayers under high pressure (maximum pressure 2–3 kbar) revealed the following features. In the LC phase, the *d*-spacing, which characterizes the bilayer thickness, including the water layer in the headgroup region, increased with increasing pressure at 63 °C from 66 Å at 1 bar to 67.5 Å at 861 bar. In the gel phases, however, the *d*-spacing decreased as a function of pressure. Small-angle neutron scattering studies (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989) reported the existence of a pressure-induced interdigitated phase Gi in DPPC bilayers at pressures above 1 kbar and temperatures above 40 °C. This interdigitated phase was also seen by other investigators using light scattering techniques at high pressure (Prasad et al., 1987). The general pressure–temperature phase diagram for DPPC-d_{62} was described in detail in our earlier study (Driscoll et al., 1991b), which also provided the notation for designating the various gel phases. In this high-pressure deuterium NMR study of DPPC-d_{62}, we found that the phase change GIII/GX does not correspond to the GIII/GIV phase transition described by Wong et al. (1988). At the present time, we have no detailed structural information on the high-pressure gel GX phase.

The ^{31}P lineshapes, as shown in Figure 1 at different pressures, agree well with those for the corresponding temperature-induced phases (P_{β}' abnd L_{β}') at ambient pressure with the exception of the interdigitated gel phase, Gi, which cannot be attained by varying temperature only. The observed $\Delta\sigma = -46$ ppm at 1 bar and 50 °C in the LC phase is in good agreement with that of −47 ppm reported previously by others (Scherer & Seelig, 1989). The transition from the LC phase to the gel I phase is accompanied by a reduction of gauche isomers in the acyl chains (Wong et al., 1988) affecting the available space for the headgroup motions and resulting in an increase of the absolute values of $|\Delta\sigma|$. The abrupt decrease of $|\Delta\sigma|$ that accompanies the gel I/Gi phase transition provides supporting evidence for the existence of the interdigitated phase, Gi, in which the phospholipid headgroup has more available space and is thus less restricted. In this phase, the faster motion of the headgroup averages the chemical shift anisotrpy leading to lower $|\Delta\sigma|$ values. In contrast, in the gel II phase, there is a further reduction of gauche conformers (Wong et al., 1988), and the two acyl chains are almost extended which makes the chains tilt with respect to the bilayer normal. As a result, the available headgroup space decreases again, and the $|\Delta\sigma|$ value returns to higher values. In the gel III phase, the headgroup motion is further restricted, and the $|\Delta\sigma|$ value continues to increase with pressure. In agreement with our results (Driscoll et al., 1991b) for DPPC-d_{62}, the phase change from gel III to gel X phase corresponds to a transformation to a rigid lattice spectrum, which shows a typical ^{31}P axially asymmetric powder pattern. However, in contrast to the deuterium lineshapes, there is no discontinuity in $\Delta\sigma$ at the phase transition pressure corresponding to gel II/gel III and gel III/gel X phase transitions. This may be due to the fact that the phase transitions are driven by the cooperative interactions between the DPPC fatty acid chain segments.

The increase in pressure in all phases is accompanied by an increase in the ^{31}P chemical shift anisotropy $|\Delta\sigma|$, as headgroup motions are slowed down, and the motional averaging is reduced. However, in view of the results obtained by neutron scattering (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989) and X-ray diffraction (Stamatoff et al., 1978), an alternative explanation should be considered for the $|\Delta\sigma|$ behavior in the LC phase. Increasing pressure causes a decrease in overall volume available for the DPPC molecules by "squeezing" them together, with an increase in the overall interactions. 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 per headgroup thus decreases. With pressure, the headgroup which is normally oriented parallel to the bilayer surface may change its orientation, thus contributing to the measured increase in bilayer thickness and leading to higher $|\Delta\sigma|$ values. In the gel phases, this explanation is not applicable, as the bilayer thickness decreases with increasing pressure (Braganza & Worcester, 1986a,b; Winter & Pilgrim, 1989). As is well documented in the literature (Griffin, 1981; Seelig, 1978; Skarjune & Oldfield, 1991), the knowledge of the ^{31}P $\Delta\sigma$ value alone is not sufficient to determine the average orientation of the headgroup; therefore, we attribute the increase in $|\Delta\sigma|$ due to pressure in the various phases of DPPC as a consequence of slower motions.

For the DPPC/TTC bilayers, the dependence of $\Delta\sigma$ upon pressure, as shown in Figure 3, can be divided into three regions. In the first region, between 1 bar and 0.8 kbar, which corresponds to the LC phase, the $|\Delta\sigma|$ values for the DPPC/TTC bilayers are greater than those for pure DPPC bilayers, as expected (Scherer & Seelig, 1989; Boulanger et al., 1980). The $\Delta\sigma$ change due to the charged form of tetracaine will be discussed in more detail later. The distinct break which occurs at about 0.8 kbar represents the phase transition which is different from results for pure DPPC in three respects. First of all, the critical pressure of the main phase transition is shifted from ~0.5 kbar to 0.8 kbar. This increase in critical pressure upon addition of anesthetics has also been observed in the DPPC/TTC system by differential scanning calorimetry and small-angle neutron scattering (Winter & Pilgrim, 1989). Moreover, it is consistent with the depression of the main phase transition temperature at ambient pressure (Macdonald, 1978; Ueda et al., 1977; Frenzel et al., 1978; Trudell et al., 1975). Second, the phase transition is accompanied by an abrupt change in the $\Delta\sigma$ value which is not observed in the pure DPPC bilayers. Finally, instead of the observed $|\Delta\sigma|$ increase for pure DPPC, the $|\Delta\sigma|$ value decreased significantly at the phase transition pressure. Structural studies of the DPPC/TTC system by X-ray diffraction showed that under certain conditions incorporation of TTC caused the hydrocarbon chains to interdigitate (McIntosh et al., 1983). In addition, it was found by DSC experiments (Kamaya et al., 1981) that the pretransition peak disappeared upon the addition of anesthetics. In the second region, from ~0.8 kbar to 3 kbar, the $|\Delta\sigma|$ values are significantly lower than those of pure DPPC bilayers in the gel I and gel II phases but are slightly higher than those in the interdigitated Gi gel phase. Thus, our results indicate that the addition of the charged form of TTC leads to the formation of the interdigitated gel Gi phase directly from the LC phase. In the Gi phase the $|\Delta\sigma|$ values increase with pressure and gradually approach the $\Delta\sigma$ values of pure DPPC in gel II and gel III phases. In the third region, from about 3.1 kbar to 5.1 kbar, the $|\Delta\sigma|$ values are greater than those observed for pure DPPC bilayers, similar to the situation for the LC phase. The question of the possible expulsion of anesthetic from the bilayer by high pressure cannot be answered by this study, but the fact remains that the $|\Delta\sigma|$ for the DPPC/TTC system is higher than the corresponding $\Delta\sigma$ values observed for the pure DPPC bilayers, even in the pressure range from 3.1 kbar to 5.1 kbar.

As we indicated in the introduction, one of the objectives of this study was to examine our data in light of the results obtained by Scherer and Seelig (1989), Seelig et al. (1987), and MacDonald et al. (1991), who investigated the influence of membrane surface charges upon the conformation of the choline headgroup in various phosphatidylcholines.

Boulanger et al. (1980) reported that at low pH the changes in the ^{2}H and ^{31}P NMR spectra of the phospholipid headgroup in the presence of tetracaine resemble those produced by the presence of ions and suggested that the headgroup undergoes conformational changes upon interaction with the anesthetic. In their detailed study of charge effects on the phospholipid headgroup in phosphatidylcholine bilayers, Scherer and Seelig (1989) used various charged amphiphiles and extended their observations also to charged local anesthetics. It is well established that the P–N dipole of the headgroup is approximately parallel to the membrane surface in pure phospholipid membranes, but the addition of charged ions, positively charged amphiphiles, and positively charged local anesthetic moves the N$^+$ end of the dipole toward the water phase and changes the headgroup orientation.

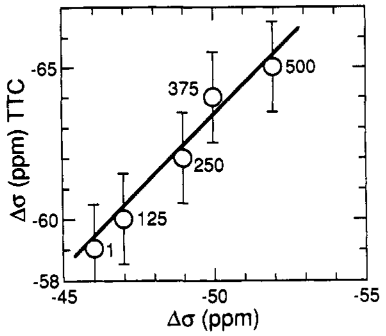

FIGURE 5: ^{31}P chemical shift anisotropy $\Delta\sigma$ for DPPC/TTC as a function of $\Delta\sigma$ for pure DPPC in the liquid–crystalline phase at 50 °C. The numbers next to the experimental points denote pressure in bars.

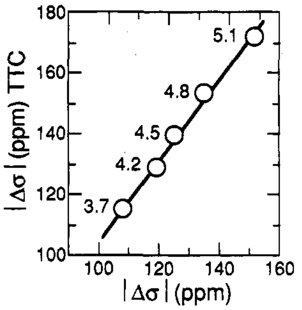

FIGURE 6: ^{31}P chemical shift anisotropy $\Delta\sigma$ for DPPC/TTC as a function of $\Delta\sigma$ for pure DPPC in the high-pressure gel phases at 50 °C. The numbers next to experimental points denote pressure in kilobars.

Scherer and Seelig (1989) introduced the following expression to calculate $\Delta\sigma$ after the addition of a positively charged amphiphile which leads to a decrease in the ^{31}P $\Delta\sigma$ value:

$$\Delta\sigma = -47.6 - 46.2X_B \qquad (4)$$

where X_B is the molar ratio of the positively charged amphiphile. It is of interest to use this expression, derived for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) to calculate the $\Delta\sigma$ change for our system. Using this expression to calculate $\Delta\sigma$ at 1 bar and 50 °C for DPPC/TTC (X_B = 0.33), we obtain $\Delta\sigma = -62.9$ ppm, which compares favorably with the experimental value $\Delta\sigma = -59$ ppm. This encouraging result led us to examine in more detail experimental $\Delta\sigma$ values for the LC phase. Since the above expression was derived for POPC and a different temperature, we modified this expression for our experiments on DPPC to the following form:

$$\Delta\sigma = -46 - 39.4X_B \qquad (5)$$

From Figure 5 we can readily see that within the LC phase the $\Delta\sigma$ values for DPPC/TTC parallel those for the pure DPPC bilayers independently of the pressure. It is interesting to note that eq 5 describes quantitatively the behavior of $\Delta\sigma$ for our system. What it suggests is that the orientation of the headgroup changes upon the addition of the charged anesthetic and that the difference in $\Delta\sigma$ for the pure DPPC system and DPPC/TTC is not affected by the increasing pressure in the LC phase.

In view of the results obtained by McDonald et al. (1991), we turn our attention to the high-pressure gel phases, i.e., to the pressure region above ~3.5 kbar. Figure 6 shows the dependence of $\Delta\sigma$ for DPPC/TTC upon $\Delta\sigma$ for pure DPPC, but in this case there is a small increase in the differences in the $\Delta\sigma$ values with increasing pressure. Nevertheless, the average $\Delta\Delta\sigma$, defined as the difference between $\Delta\sigma$ for DPPC/TTC and that for pure DPPC is −12.4 ppm, while eq 5 predicted a $\Delta\Delta\sigma = -13$ ppm. This result provides supporting evidence for the proposal of McDonald et al. (1991) that the choline headgroup of phosphatidylcholine responds to the surface charge effects even in gel-state lipid membranes.

The final topic of discussion deals with the pressure dependence of the measured ^{31}P spin-lattice relaxation times, as listed in Table I and depicted in Figure 4. The spin-lattice relaxation time (T_1) experiments clearly show a minimum of T_1 versus pressure as it can be seen in Figure 4. In the LC phase, the T_1 values decreased with increasing pressure in agreement with variable temperature studies at ambient pressure. Our T_1 value of 3.0 s at 1 bar and 50 °C is comparable with the literature value of 3.05 s at 1 bar and 47 °C measured at 36.4 MHz (Browning, 1981). However, the T_1 values increased considerably with pressure in the various gel phases, an observation which is clearly different from the results of the variable temperature studies where the T_1 values were only slightly temperature dependent (Browning, 1981). One explanation for this difference is that the T_1 values in the variable temperature studies were performed at 36 MHz, whereas our frequency was 73 MHz. According to previous studies (Yeagle et al., 1975, 1977), the T_1 values measured at 36 MHz are dominated by phosphorus–proton dipolar interactions. The differences of the T_1 values measured at 36 MHz and 73 MHz suggest that there is an additional contribution from the relaxation via chemical shift anisotropy. This disprepancy has also been reported for DOPC multilamellar bilayers (Seelig et al., 1981).

In the ^{31}P NMR T_1 measurements, the observed spin lattice relaxation time of the phosphorus can be expressed as follows (Seelig et al., 1981):

$$\frac{1}{T_1^{\mathrm{OBS}}} = \frac{1}{10}K^2\chi + \frac{3}{20}\omega_{\mathrm{P}}^2\Delta\sigma^2\frac{2\tau_{\mathrm{c}}}{1+\omega_{\mathrm{P}}^2\tau_{\mathrm{c}}^2} \tag{6}$$

$$K = \hbar\gamma_{\mathrm{H}}\gamma_{\mathrm{P}}\sum_i \langle r_i\rangle^{-3} \tag{7}$$

$$\chi = \frac{\tau_{\mathrm{c}}}{1+(\omega_{\mathrm{H}}-\omega_{\mathrm{P}})^2\tau_{\mathrm{c}}^2} + \frac{3\tau_{\mathrm{c}}}{1+\omega_{\mathrm{P}}^2\tau_{\mathrm{c}}^2} + \frac{6\tau_{\mathrm{c}}}{1+(\omega_{\mathrm{H}}+\omega_{\mathrm{P}})^2\tau_{\mathrm{c}}^2} \tag{8}$$

γ and ω refer to the gyromagnetic ratio and the resonance frequency of the corresponding nucleus, τ_c is the correlation time of the motion, assuming isotropic reorientation, and $\langle r_i\rangle$ is the average distance between phosphorus and surrounding protons.

It is evident that the term K is difficult to evaluate as both pressure and the addition of TTC may change the arrangement of protons in the immediate environment of the phosphate group and may also change the relative intra- and intermolecular contributions to relaxation. Therefore, we only take advantage of the fact that there is a minimum in the plot of T_1 vs pressure to calculate τ_c from $\omega_P\tau_c \sim 1$. By substituting τ_c into eq 6, we can calculate $K = 3.1 \times 10^4$ s^{-1} subject to the uncertainty expressed above. The calculated τ_c values range from 0.3 ns (1 bar, LC phase DPPC) to ~37 ns for the high-pressure gel X phase at 5.1 kbar.

Furthermore, the τ_c value in the gel II phase at about 2 kbar is 5 ns, which is in good agreement with 4 ns found from dielectric measurements (Shepherd & Buldt, 1978) for DPPC dispersions in the L_{β}' phase at 30 °C. However, in view of the uncertainty of the K term in eq 6, we do not consider it appropriate to discuss in detail the calculated τ_c values. The only additional comment is that we find that τ_c is in the order of 10^{-9} s for the motion of the whole headgroup, whereas the correlation times (Gally et al., 1975; Akutsu & Seelig, 1981) for the ethylene and methyl groups are much faster, in the order of 10^{-11} s. In general, the τ_c values for DPPC/TTC are higher than the corresponding τ_c's for the pure DPPC bilayers over the whole range of pressures studied.

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Barnase Has Subsites That Give Rise to Large Rate Enhancements[†]

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ABSTRACT: Barnase is found to have a series of subsites for binding its substrates that confers large rate enhancements. Ribonucleotide substrates of the type $Zp_0Gp_1Xp_2Y$ have been synthesized, where p is phosphate, X, Y, and Z are nucleosides, and G is guanosine. G occupies the primary specificity site. The most important subsite is for p_2, followed by that for Y. There appears to be no subsite for the Z or p_0 positions. Occupation of the subsite for p_2 gives rise to a 1000-fold increase in k_{cat}/K_m, composed of a 100-fold increase in k_{cat} and a 10-fold decrease in K_m. The Y subsite gives rise to further 20-fold increase in k_{cat}/K_m. Rates approaching diffusion control for k_{cat}/K_m are observed. k_{cat} for the dinucleotide monophosphate GpU $= 0.55\ s^{-1}$, and $K_m = 240\ \mu M$; this compares with $53\ s^{-1}$ and $20\ \mu M$ for GpUp, and $3.3 \times 10^3\ s^{-1}$ and $17\ \mu M$ for GpApA (the best substrate tested). Cleavage occurs at the 3′-phosphate of guanosine in all cases. There are differences in base specificity at the two subsites for X and Y downstream of the scissile bond. The binding energies of different substrates have been analyzed using thermodynamic cycles. These show that the contributions of the X and Y sites are nonadditive.

Barnase is a small extracellular ribonuclease from *Bacillus amyloliquefaciens* and is a member of a family of homologous microbial nucleases (Hill et al., 1983), of which the best characterized is RNase[1] T1. Barnase consists of a single polypeptide chain of 110 amino acids, $M_r = 12\,382$, with no disulfide bridges (Hartley & Baker, 1972). The small size has facilitated structural and physical studies. The crystal structure has been solved at 2-Å resolution (Mauguen et al., 1982). More recently, a complex with the deoxydinucleotide d(GpC) at 1.9-Å resolution has also been solved (Baudet & Janin, 1991), but the binding of the dinucleotide is a nonproductive mode for a substrate. The sequence-specific 2D ^{1}H NMR spectrum of barnase has been assigned (Bycroft et al., 1990) and the solution structure determined (Bycroft et al., 1991). Barnase has also been extensively studied as a paradigm for protein folding and stability [e.g., Matouschek et al. (1990) and Serrano et al. (1991)].

The mechanism of action of barnase is similar to that of pancreatic ribonuclease in that a 2′,3′-cyclic nucleotide intermediate is formed in the first step (i.e., transesterification) of the reaction, followed by a much slower hydrolysis of this intermediate to give a 3′ nucleotide (Figure 1). On the basis of chemical modification, site-directed mutagenesis, and primary sequence homologies between members of the family of microbial ribonucleases, three important catalytic residues have been identified in barnase: His-102 (putative general acid), Glu-73 (putative general base), and Arg-87 (Arata et

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[1] Abbreviations: RNase, ribonuclease; d(GpC), 2′-deoxyguanylyl-(3′→5′)-2′-deoxycytidine; 2′,3′-cGMP, guanosine 2′,3′-cyclic monophosphate; 2′,3′-cAMP, adenosine 2′,3′-cyclic monophosphate; CpGc-2′,3′-p, cytosyl(3′→5′)guanosine 2′,3′-cyclic monophosphate; N, any nucleoside. For other nucleoside and nucleotide abbreviations, see IUPAC–IUB Commission on Biochemical Nomenclature (1970), for example, GpUp, guanylyl(3′→5′)uridine 3′-monophosphate.

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