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High-Pressure 2D NOESY Experiments on Phospholipid Vesicles

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Two-dimensional NOESY spectra obtained at high pressure are reported for sonicated vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) model membrane systems. The 2D NOESY spectra were measured in the liquid-crystalline state as a function of pressure at temperatures of 28°C for POPC and 64°C for DMPC. A phenomenological discussion of the strong effect of pressure on the build-up rates of cross peaks in the two-dimensional spectra is presented.

The great interest in understanding biomembrane structure and function has stimulated intensive investigations of model membrane systems, in particular, the phosphatidylcholine systems (*1, 2*). A common feature of these membrane phospholipids is the existence of a reversible gel to liquid-crystalline (LC) phase transition. Below a certain temperature T_m, the lipid bilayers are in an ordered gel state ($L_{\beta'}$), characterized by a relatively rigid packing of their hydrocarbon chains. Above T_m, the lipid bilayers are in a fluid-like state (L_α) with large conformational disorder along the hydrocarbon chains.

As is well known, NMR spectroscopy is a very powerful technique for studying structure and dynamics of biomembrane systems. Recently, we developed a high-resolution probe for high-pressure NMR measurements on biological systems (*3*), which enables us to study the structure and dynamical properties of model membranes at high pressure by a variety of NMR techniques. We have already shown that proton-decoupled natural abundance ^{13}C NMR spectroscopy is a promising tool for studying the phase transition behavior of these systems at high pressure (*3*).

Two-dimensional nuclear Overhauser effect spectroscopy has been proven to be a valuable technique (*4*) which provides both structural and dynamical information. Dipolar cross-relaxation, responsible for the NOE, depends on both spatial proximity and relative motion of the interacting nuclei. Recently, NOESY has been

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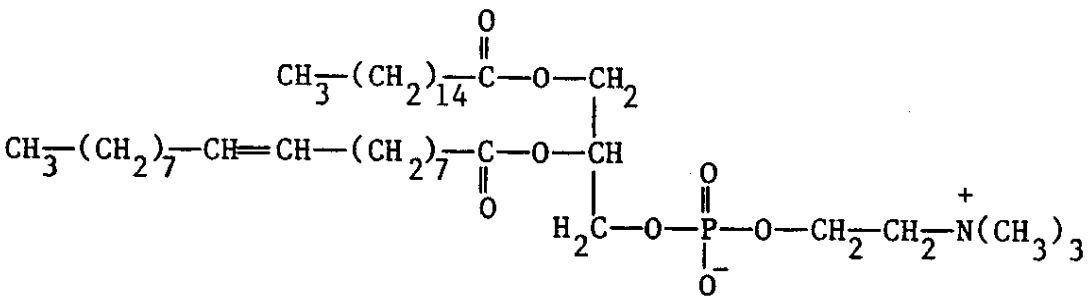
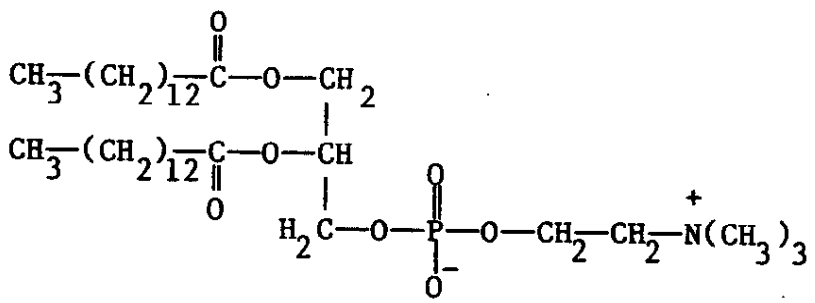
successfully applied to study membrane systems (*5–8*) and their interactions with probe molecules at ambient conditions.

In order to test the performance of the high-pressure NMR systems we have decided to continue our studies of bilayer membrane systems using the phospholipid POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine:



POPC is not a highly studied phospholipid, and yet is a very important component of animal cell membranes. The double bond on the *sn*-2 chain of this lipid gives POPC the property of being highly fluid at physiological temperature and also makes it an ideal system for studies at increased pressure near ambient temperature.

During the course of our experiments on POPC we observed a strong effect of increased pressure on the intensity of the NOESY cross peaks in the POPC, and this led to experiments on DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine):



This molecule represents a simpler system with a wealth of experimental NMR data available at ambient pressure and different temperatures. A phenomenological discussion of the pressure effects on the NOE build-up curves is given.

EXPERIMENTAL

Materials. Small vesicles of POPC and DMPC (Sigma) were prepared by ultrasonic irradiation of the lipid dispersed in 99.8% D_2O (Aldrich Chemical Co.). The sonication was performed by using a Heat System/Ultrasonics (Plainview, New York) sonicator for a total time of about 15 min. During that period the sample tube was cooled in a water bath to prevent overheating; however, the temperature of the

sample was kept above 24°C, i.e., above the LC–gel phase transition temperature. The resulting translucent solution was centrifuges for 15 min at 10,000 cps to remove any larger particles and titanium dust from the tip of the sonicator. The final concentration of the lipid used was 0.09 *M* POPC or DMPC in all the experiments. For the high-pressue experiments, the samples were placed in glass cells with Teflon bellows.

NMR measurements. The proton spectra were recorded at 180 MHz on a home-built NMR spectrometer (with a wide-bore (130 mm) Oxford 4.2 T superconducting magnet) interfaced to a General Electric 293D pulse programmer and a 1280 data system. The high-resolution, high-pressure probe was a modification of a probe described earlier (*9*) and will be described elsewhere in detail (*10*). The high-pressure vessel was fabricated from titanium alloy. The resolution of the nonspinning samples in this high-pressure setup is 2 Hz for protons. Pressures (up to 3500 bar) were generated with a piston screw pump. The pressures, accurate to ±50 bar, were measured with a digital pressure gauge. The pressure-transmitting fluid (CS_2) was separated from the sample using Teflon bellows. The temperature was controlled to within ±0.5°C by circulating an ethylene glycol/water mixture from a circulator through a thermostating jacket around the high-pressure vessel and was measured using a copper–constantan thermocouple inside the high-pressure vessel.

GEM software was used to collect and process the two-dimensional data sets. The 2D phase-sensitive NOESY spectra were obtained using the method of States *et al.* (*11*). An incremented π pulse was used during the mixing period to suppress *J* cross peaks. The spectra were obtained with a spectral width of 600 Hz and a 3 s delay. The spectra were zero-filled, multiplied by a Gaussian filter (10 Hz), and Fourier-transformed in both dimensions, followed by symmetrization. The final data size of the absorptive part of the spectrum was 256 × 256 points and 64 acquisitions were taken for each data block. 2D NOESY spectra were taken at mixing times t_m from 50 to 1200 ms. A typical experiment required about eight hours of spectrometer time for one mixing time. Diagonal and cross peaks were compared by normalizing the 2D spectra to the shortest mixing time. The T_1 relaxation times were obtained by the inversion-recovery method with a composite π pulse to compensate for any pulse imperfection. The estimated error in T_1 is approximately 10%.

RESULTS AND DISCUSSION

The chemical-shift assignments for POPC as reported by Neumann *et al.* (*12*) are given in Table 1 and Fig. 1 and show the 1D proton spectrum of sonicated POPC vesicles at 0.09 *M* concentration in D_2O and 28°C together with the 2D NOESY spectrum of POPC under the same experimental conditions for the mixing time τ_M = 250 ms. It should be pointed out that all experiments carried out in this study are for the liquid-crystalline fluid phase of the phospholipid. One can clearly see the presence of many intense cross peaks between the individual resonances. To illustrate the performance of the high-pressure high-resolution probe, we also give Fig. 2 which shows the 2D NOESY spectrum of POPC at 800 bar for the same mixing time τ_M = 250 ms. A qualitative comparison of the 1 and 800 bar spectra demonstrates that the quality of the spectra does not deteriorate at high pressures.

TABLE 1

Chemical-Shift Assignments for POPC (*12*)

Peak	Chemical shift (ppm from TMS)	Group assignment	No. of hydrogens
A	5.25	$-C\underline{H}=C\underline{H}-$	2
B	4.68	HDO	1
C	4.25	$-C\underline{H}_2-CH_2-N^+(CH_3)_3$	2
D	3.65	$-C\underline{H}_2-N^+(CH_3)_3$	2
E	3.18	$CH_2-N^+(C\underline{H}_3)_3$	9
F	2.63[a]	—	
G	2.24	$C-C\underline{H}_2-$	4
H	1.95	$-C\underline{H}_2-CH=$	4
I	1.17	$-C\underline{H}_2-$ (bulk methylene)	48
J	0.80	$-CH_2-C\underline{H}_3$	6

[a] Low level of impurity seen as a cross peak.

During our experiments we observed a drastic pressure effect on the intensity of the cross peaks in POPC even for a short mixing time of τ_M = 75 ms. Figures 3a, 3b, and 3c show convincingly the increase of intensity and also the number of detectable cross peaks accompanying the change of pressure from 800 to 1400 bar at τ_M = 75 ms. An evaluation of the NOE build-up curves revealed the increase in cross-peak intensities with increasing pressure for all cross peaks observed for the sonicated POPC vesicle system.

In order to provide more quantitative information on the effects of pressure on cross-peak intensities, we decided to carry out 2D NOESY experiments on DMPC which represents a simpler phospholipid system. Figures 4–6 show the evolution of the cross-peak intensities for the $(CH_2)_n/CH_3$, $(CH_2)_n/NMe_3$, and CH_3/NMe_3 resonances for DMPC vesicles as a function of mixing time τ_M in the pressure range from 1 to 2000 bar at T = 64°C, i.e., in the liquid-crystalline phase.

The intensities of the cross peaks are normalized to those of the corresponding diagonal peaks at the shortest mixing time used (τ_M = 50 ms). The characteristic NOE build-up curves (*4, 5*) as given in Figs. 4–6 show a drastic dependence upon pressure as the cross-peak intensities grow stronger with increased pressure. This is more clearly illustrated by Fig. 7 where the pressure dependence of the different cross peaks is plotted for the mixing time of 200 ms.

In view of the complexity of the cross-relaxation phenomena in membranes, a rigorous theoretical description of the results of our high-pressure NOESY experiments is not possible at the present time, and therefore one must resort to a qualitative, phenomenological interpretation. In a general way we can write the following expression for the pressure dependence of the cross-relaxation rate σ_{ij} between spins i and j,

$$\sigma_{ij} \propto [r_{ij}; S_{HH}(P); \tau_\perp(P); \tau_\parallel(P); \tau_J(P); SD(P)], \qquad [1]$$

where r_{ij} is the distance between spins i and j, $S_{HH}(P)$ is the pressure-dependent order

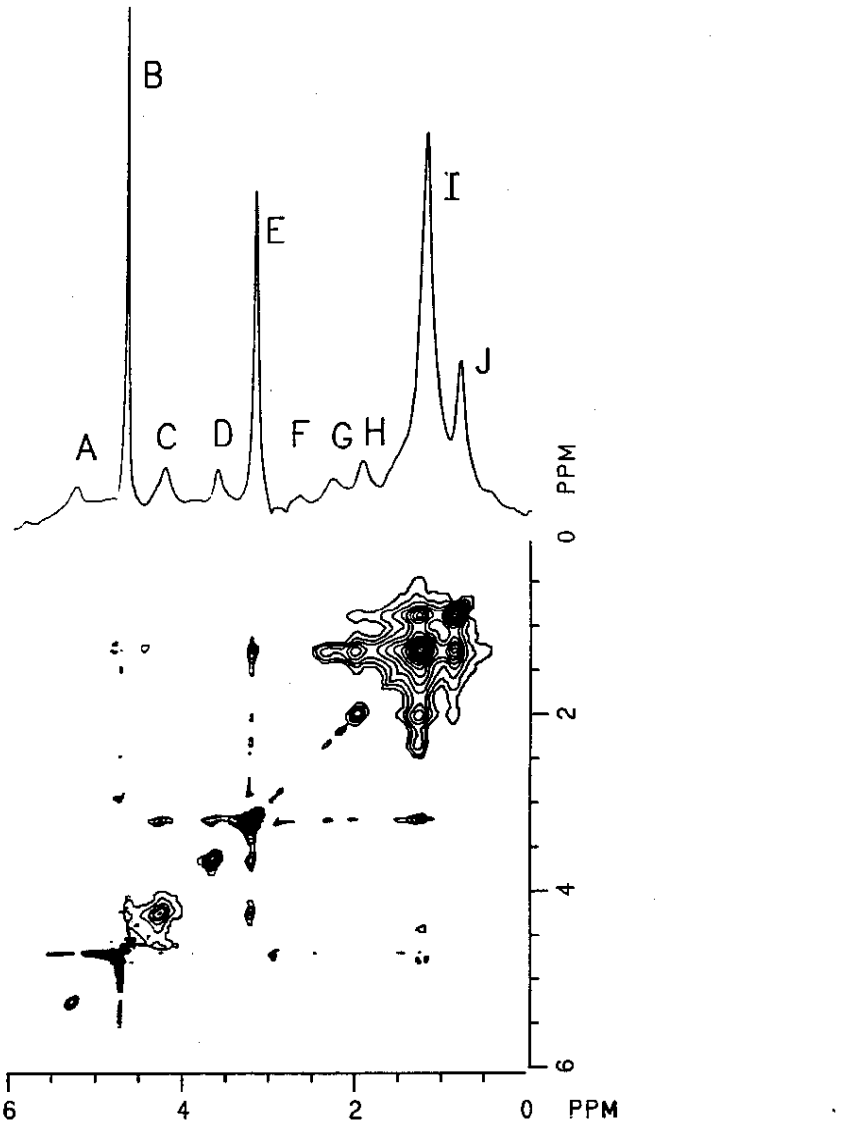


FIG. 1. 2D NOE spectrum for sonicated POPC vesicles (0.09 *M*) at 28°C and 1 bar. The 1D spectrum included was taken with 16 acquisitions and a data size of 4K. The 2D phase-sensitive NOESY spectrum for $\tau_M = 240$ ms; 64 acquisitions; data size 1024 × 1024.

parameter, $\tau_i(P)$ are the various correlation times as discussed by Mayer *et al.* (*13*) in their NMR study of DMPC, and SD(P) denotes the spin diffusion contribution to cross-relaxtion.

We emphasize that this general expression is used only to help us to understand the origin of the strong pressure dependence of σ_{ij} and also to indicate the complexity of the problem. Therefore, we did not attempt to specify the functional form of the σ_{ij} dependence upon the individual terms given in Eq. [1], and we also ignored any possible interdependence of these terms. In all of our discussion of the pressure effects on the NOE build-up curves, we take r_{ij} independent of pressure as we assume that the main pressure effect comes through changes in the correlation times, in the order

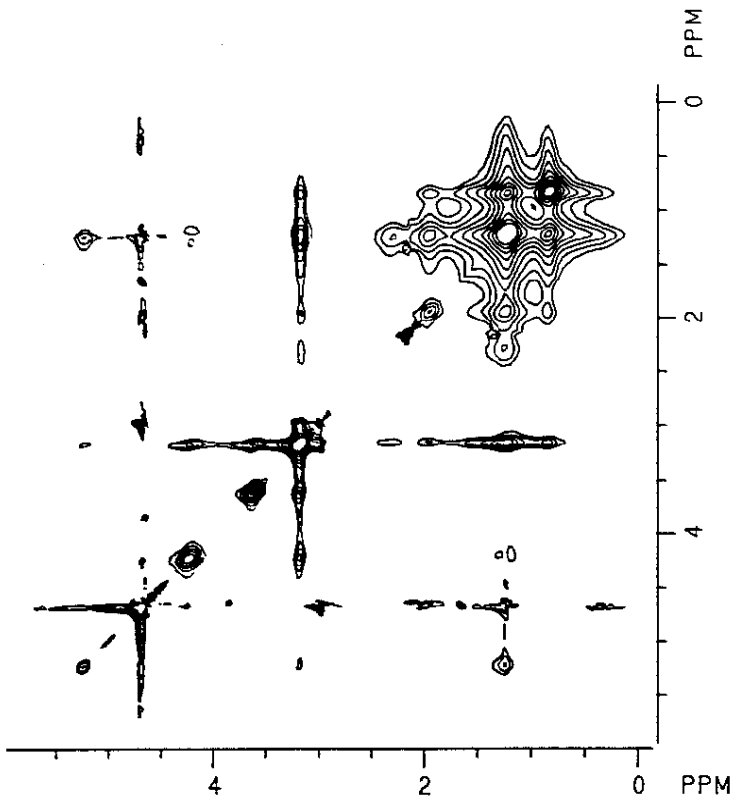


FIG. 2. 2D NOE spectrum for sonicated POPC vesicles (0.09 *M*) at 28°C and 800 bar for τ_M = 250 ms.

parameter, and even in the change of efficiency in spin diffusion. It is well known that pressures up to 15 kbar do not change bond angles or bond distances, but of course, they can change conformations. Since the functional form of the pressure dependence of the individual terms in Eq. [1] is not known, we use the following approach to discuss the strong pressure effects on the NOESY build-up curves. The cross-relaxation rate σ_{ij} between spins i and j is given by (*14*)

$$\sigma_{ij} = \frac{\hbar^2\gamma^4}{10}\frac{1}{r_{ij}^6}[3J(2\omega) - 0.5J(0)], \qquad [2]$$

where r_{ij} is the distance between spins i and j, γ is the magnetogyric ratio, and $J(2\omega)$ and $J(0)$ are the spectral density functions. Adopting the treatment used recently by Cafiso *et al.* (*7*), we can also write

$$J(\omega) = (1 - S_{HH}^2)J^f(\omega) + S_{HH}^2 J^s(\omega) \qquad [3]$$

$$J^{f,s}(\omega) = 2\tau_c^{f,s}/(1 + \omega\tau_c^{f,s}), \qquad [4]$$

where τ_c is the correlation time. Equation [4] represents a simple approximation of the correlation function taking into account the fast (f) and slow (s) motions.

In order to estimate the pressure effects on σ_{ij}, let us first assume that the main effect of pressure is to change the various correlation times characteristic for the dynamic behavior of DMPC under conditions studied (T = 64°C; P = 1 to 2000 bar). The

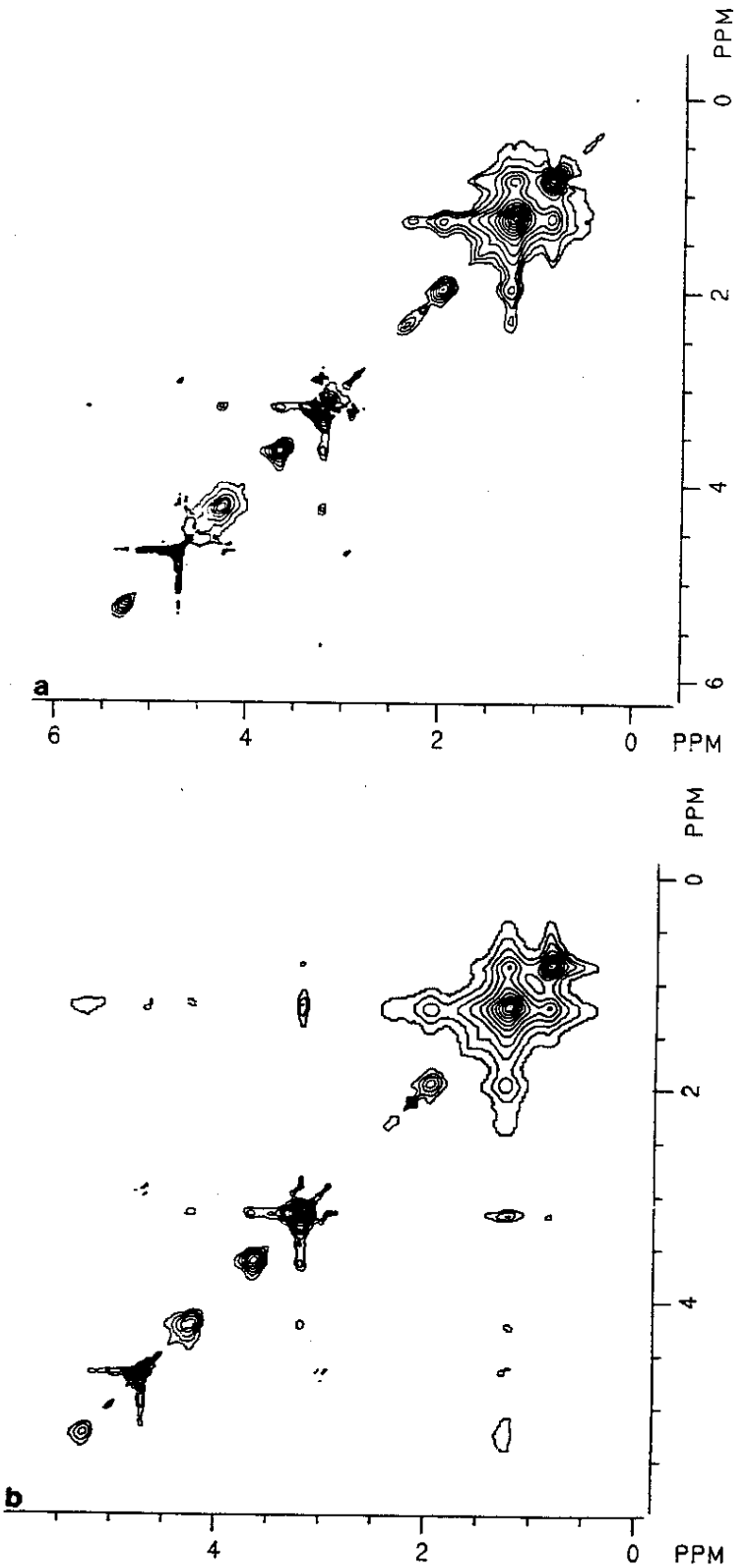


FIG. 3. 2D NOE spectra for sonicated POPC vesicles (0.09 *M*) at 28°C and (a) 1 bar, (b) 800 bar, and (c) 1400 bar; for τ_M = 75 ms and 64 acquisitions.

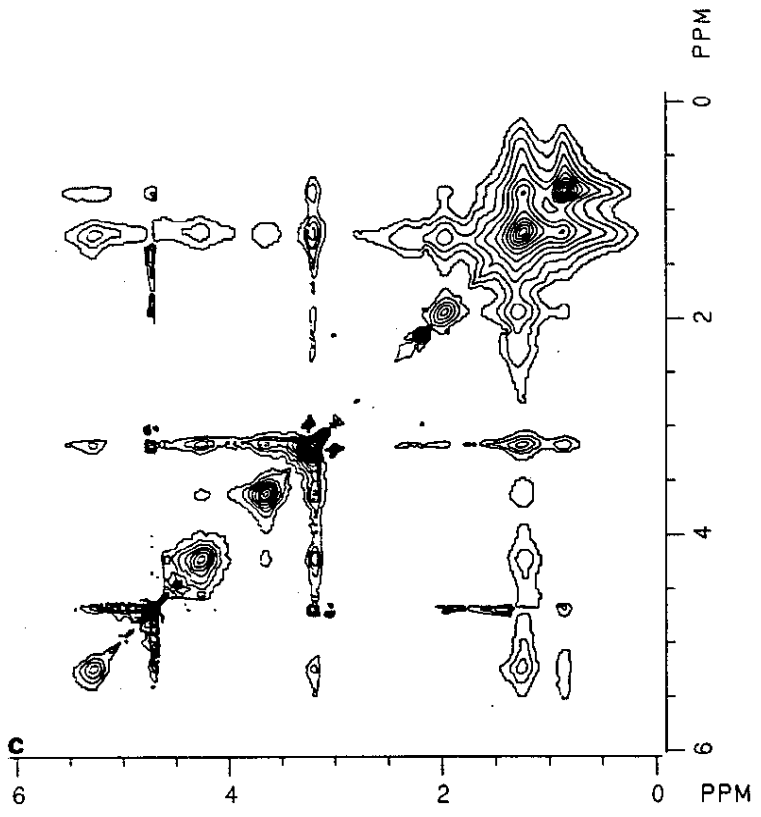


FIG. 3—*Continued*

Debye equation provides the relationship between the reorientational correlation time and shear viscosity. In our earlier study (*15*) we measured viscosity of D_2O as a function of pressure, and the increase in pressure from 1 to 2000 bar produced a 10% change in shear viscosity. Of course, we are aware of the inherent approximation by expecting that the various correlation times will all follow the change in viscosity.

In a recent deuteron NMR relaxation study of DMPC, Mayer *et al.* (*13*) reported the values of the various correlation times at the temperature used in our experiments ($T = 64°C$). Using the results of these experiments (*13*), we take $\tau_s = 10^{-8}$ s and $\tau_f = 10^{-11}$ s and $S_{HH} = 0.36$ for DMPC at 64°C and 1 bar. We assume that τ_f and τ_c follow the pressure change in shear viscosity and that the order parameter S_{HH} does not change with pressure. Using this approach, one calculates a very small relative change in σ_{ij} when going from 1 to 2000 bar. In order to reproduce the large pressure changes in σ_{ij} as seen in Figs. 4–6, one must allow the order parameter to increase with pressure from 0.36 at 1 bar to 0.6 at 2000 bar. This is not surprising as it is expected that the order parameter in membranes is strongly temperature dependent and increases with decreasing temperature, a trend which parallels the increase in pressure. The results of recent high-pressure neutron scattering experiments (*16*) show that the order parameter increases with the increasing pressure in the liquid-crystalline phase of DMPC.

So far we have not considered the term $SD(P)$ giving the possible contribution of spin diffusion to cross-relaxation. It should be mentioned that the role of spin diffu-

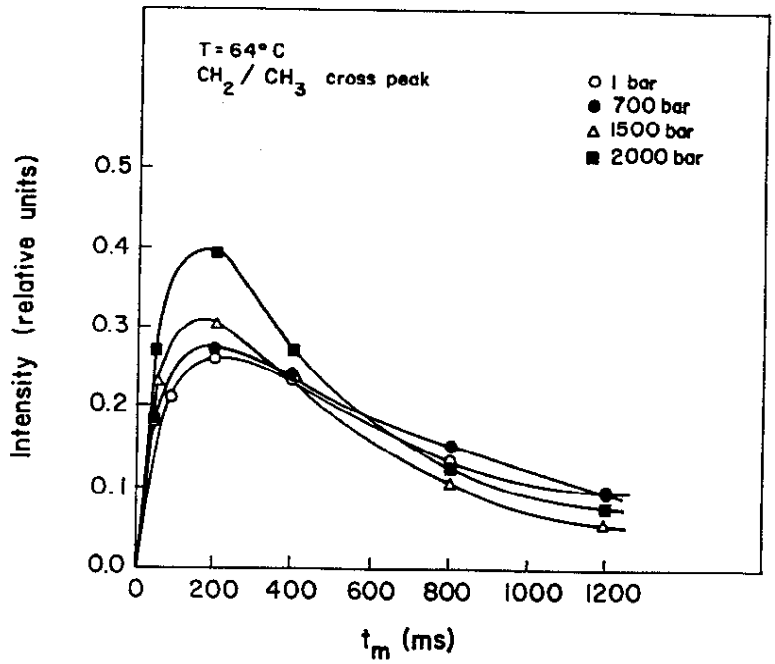


FIG. 4. The intensity of the $(CH_2)_n/CH_3$ cross peak in sonicated DMPC vesicles (0.09 M) at different pressures (from 1 bar to 2 kbar) as a function of the mixing time τ_M ($T = 64°C$).

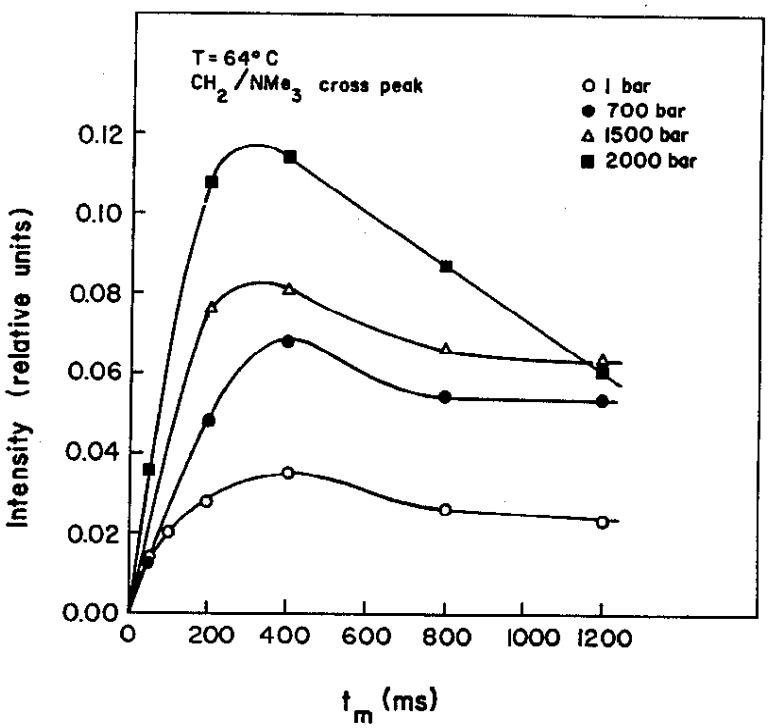


FIG. 5. The intensity of the $(CH_2)_n/NMe_3$ cross peak in sonicated DMPC vesicles (0.09 M) at different pressures from 1 bar to 2 kbar as a function of the mixing time τ_M ($T = 64°C$).

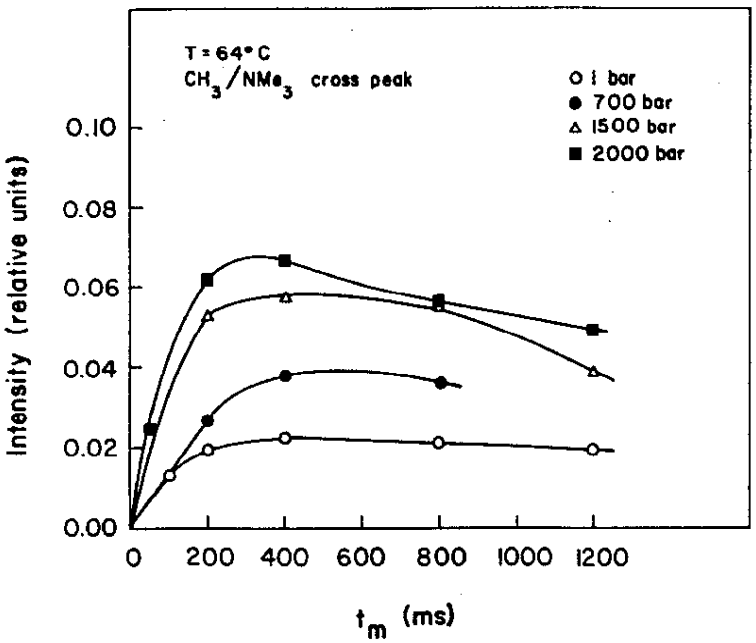


FIG. 6. The intensity of the CH_3/NMe_3 cross peak in sonicated DMPC vesicles (0.09 *M*) for different pressures from 1 bar to 2 kbar as a function of the mixing time τ_M (T = 64°C).

sion as a source of cross-relaxation in membrane systems has been a subject of considerable discussion (*6, 8, 16, 17*). It would appear that the current view is that spin diffusion contributes to cross-relaxation of model membranes, but that it does not represent the dominant contribution. In an attempt to provide some additional information on the role of spin diffusion in the DMPC vesicles studies, we measured the temperature and pressure dependence of the proton spin–lattice relaxation time, T_1, for the choline group, protons, methylene protons, and the methyl protons. The results can be summarized as follows. First of all, in agreement with the results of other workers (*17, 18*), we obtained different T_1 values for the individual groups of protons which suggests that spin diffusion cannot be very strong as it would result in identical T_1 values for all protons. Second, the T_1 values increase with increasing temperature and decrease with increasing pressure, a behavior typical for the motionally narrowed regime. Again, this observation would suggest that spin diffusion is weak. Third, even if one considers a weak spin diffusion, the pressure increase in the order parameter may result in a more efficient spin diffusion. For the pressure increase from 1 to 2000 bar at 64°C, the T_1 values changed on the average by a factor of 1.5 for the three groups of protons of DMPC, i.e., exhibiting a larger change than one would predict if the increase in shear viscosity was solely responsible for the T_1 change. In the earlier discussion we mentioned that the D_2O viscosity increases by a factor of 1.1 at 64°C for the pressure change from 1 to 2000 bar.

From the above phenomenological discussion, it is clear that quantitative interpretation of the observed pressure effects on σ_{ij} cannot be carried out solely on the basis of our preliminary experiments reported in this study, and therefore systematic high-pressure NMR studies of selectively deuterated phospholipids are needed to deter-

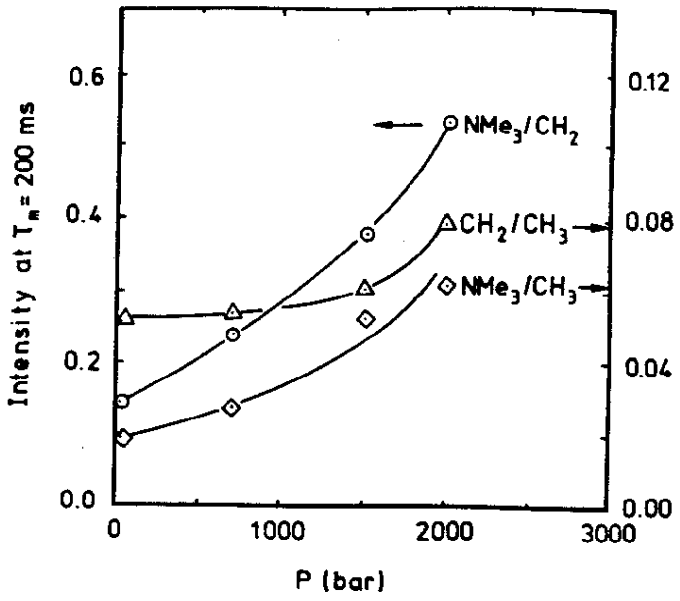


FIG. 7. The intensity of the $(CH_2)_n/CH_3$, $(CH_2)_n/NMe_3$, and CH_3/NMe_3 cross peaks at the mixing time τ_M = 200 ms in sonicated vesicles of DMPC at 64°C as a function of pressure. The estimated error of the intensity is ±10%.

mine the pressure/volume effects on the various correlation times and order parameters in POPC and DMPC. These experiments are in progress in our laboratory.

In our experiments we demonstrated for the first time the feasibility of performing the 2D NMR experiments on biochemical systems at high pressure. In view of the current high interest in volume effects on biopolymers (*18, 19*), it is important to take advantage of the high information content of the 2D NMR experiments performed at high pressure.

ACKNOWLEDGMENTS

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