Compression of lipid membranes as observed at varying membrane positions

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ABSTRACT We have measured the microscopic isothermal compressibility of dioleoyl- and dimyristyl-phosphatidylcholine multilayers and bilayers as a function of membrane depth by the pressure dependence of the polarization of a series of anthroyloxy fatty acids. In both systems, within experimental error, the compressibility did not change with membrane depth. The magnitudes of the compressibilities matched those of organic solids and those reported for dipalmitoylphosphatidylcholine multilayers from neutron diffraction measurements (Braganza, L. F., and D. L. Worcester. 1986. *Biochemistry.* 25:7484–7488). The bilayer compressibility decreased with temperature and this decrease was similar with membrane depth consistent with the isotropic thermal expansion of membranes previously observed (Scarlata, S. 1989. *Biophys. J.* 55:1215–1223). The vertical compressibility in the *z* direction is much lower than the horizontal (*xy* planes) for probes that lie parallel to the hydrocarbon chains which is consistent with an increase in bilayer thickness. The compressibility for probes that lie perpendicular to the hydrocarbon chains is more isotropic due to their limited spatial access to the *z* plane.

INTRODUCTION

This study focuses on the compressibility of lipid membranes. These systems are highly anisotropic in regards to their fluidity, and due to their quasi-two–dimensional structure, large fluidity gradients exist between the planes parallel and perpendicular to the hydrocarbon chains. Also, the fluidity increases towards the center of membrane as shown by the decrease in fluorescence polarization of probes located at different membrane depths (Thulborn and Sawyer, 1978; Tilley et al., 1979). Because the fluidity is related to the free volume of a system (Batschinski, 1913; Bingham and Kinney, 1940), as is the compressibility, it follows that compressibility gradients may also be present in membranes.

In this work, we examine the effects of high hydrostatic pressure on saturated and unsaturated membranes as a function of membrane position. High pressure has been extensively used to follow changes that occur in membranes as a result of increased lipid packing (for review see MacDonald, 1984). The increase in lipid order with pressure is readily seen by the fact that the application of pressure raises the gel to liquid crystalline phase transition temperature by ~20°C/kbar (where 1 bar = 1.015 atms), (Heremans, 1982; Chong and Weber, 1983). Neutron diffraction studies on saturated phosphatidylcholine multilayers have shown that raising the pressure results in an increase in bilayer thickness (Braganza and Worcester, 1986). Using a free volume model, it was found that at 2,000 bars, the lateral diffusion of a fatty acid in a red blood cell membrane is reduced by a factor of ten (Eisinger and Scarlata, 1987) as compared to the atmospheric value.

The existence of a compressibility gradient may alter the position and location of various membrane components and, indeed, some examples of this have been observed including anesthetics, alcohol, and other small molecules (e.g., Lever et al., 1971; Halsey and Wardley-Smith, 1975; Auger et al., 1988; Chong, 1988; Zakim and Wong, 1990). High hydrostatic pressure may also cause the elimination of some integral membrane proteins from the membrane which is postulated to be due to increased lipid packing (Muller and Shinitzky, 1981; Deckman et al., 1985). To gain insight into the changes in orientation that membrane components may undergo with pressure, we will measure the compressibility of different regions of the bilayer. Previously, we have found that the thermal expansion of dioleoylphosphatidylcholine (DOPC) bilayers is isotropic (Scarlata, 1989). In those studies we measured the temperature dependence of the fluorescence polarization of a series of anthroxyloxy fatty acid probes in which the fluorophore is positioned at varying membrane depths (Thulborn and Sawyer, 1978; Tilley et al., 1979). By relating the polarization to a rotational volume and determining its change with pressure, we will extract the compressibility in different regions in the membrane.

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MATERIALS AND METHODS

The series of anthroyloxy-fatty acids were purchased from Molecular Probes, Inc. (Eugene, OR). Purity was verified by thin layer chromatography in chloroform:ethanol (80:20). Lipids were purchased from Avanti Lipids, Inc. (Birmingham, AL) and used without further purification. Vesicles were prepared by mixing the fluorescent fatty acids with the lipids in chloroform and/or ethanol at a 0.1% mol/mol ratio, drying under nitrogen and then under vacuum, hydrating in 50 mM Hepes-0.16M KCl buffer for multilamellar vesicles and sonicating with a W225 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) for 30 min under nitrogen and spinning at 39,000 rpm for 30 min for small, unilamellar vesicles. Large, unilamellar vesicles were prepared from multilayers by extrusion through a 1-μm filter.

Samples were subjected to high pressure using the apparatus described by Paladini and Weber (1981) with some modifications. All samples returned to their atmospheric value after pressure release, except where indicated. The data presented are the average of three independent runs on different preparations. Polarization values were corrected for pressure-induced window birefringence by the method of Royer and Scarlata (manuscript in preparation) that gave values comparable to those obtained using the method of Paladini and Weber (1981). Fluorescence measurements were carried out on an I.S.S. Greg-PC (I.S.S., Champaign, IL). The anisotropy was measured using an excitation wavelength of either 381 or 316 nm as noted in the text and an emission wavelength of 460 nm. Lifetimes were measured using the 325-nm line from a HeCd laser at the Laboratory of Fluorescence Dynamics at the University of Illinois. Average steady-state lifetimes were found to be 7.9, 11.7, 11.9, and 12.75 for 2-, 6-, 9-, and 12-AS-DOPC multilayers at 22°C. Because lifetimes are proportional to the fluorescence intensities and we were specifically interested in data taken at 316 and 381 nm excitation, in most cases as noted in the text, intensity data were used to assess changes in lifetime. Additionally, the emission spectra of several of the samples were checked and showed no changes under pressure. The limiting anisotropies used here were from previous measurements (Scarlata, 1989): 0.312 for 2-, 6-, 9-, and 12-AS and 0.322 for 11-AU, and 16-AP for 381 nm excitation, and 0.123 for all probes at 316 nm excitation.

Fluorescence anisotropies were scaled to correct for lifetime differences through the Perrin equation:

$$A_0/A - 1 = (1/p - 1/3)/(1/p_0 - 1/3) = RT^*\text{tau}/(V^*\text{eta}), \quad (1)$$

where A_0 and p_0 are the limiting anisotropy and polarization, R is the gas constant, T the absolute temperature, tau the fluorescence lifetime, V the rotational volume assuming a spherical rotor, and eta is the viscosity. The use of this equation is solely to scale the anisotropy and no specific models are assumed.

RESULTS

DOPC studies

DOPC multilamellar vesicles were labeled with 0.1% mol/mol with one of the anthroyloxy probes and the polarization measured as a function of pressure from .001–2 kbar at 22°C. Whereas the polarization increased almost linearly with pressure, the fluorescence lifetimes measured at 325 nm excitation and the fluorescence intensities measured at 381 nm did not change significantly. In Fig. 1 we present the results for 6- and 12-AS under the conditions explored here.

All samples gave reproducible initial polarizations and changes with pressure. Identical polarization data were obtained for small, unilamellar vesicles. The probes in small unilamellar, large unilamellar, and multilamellar vesicles at 22°C and 381 nm excitation show an initial

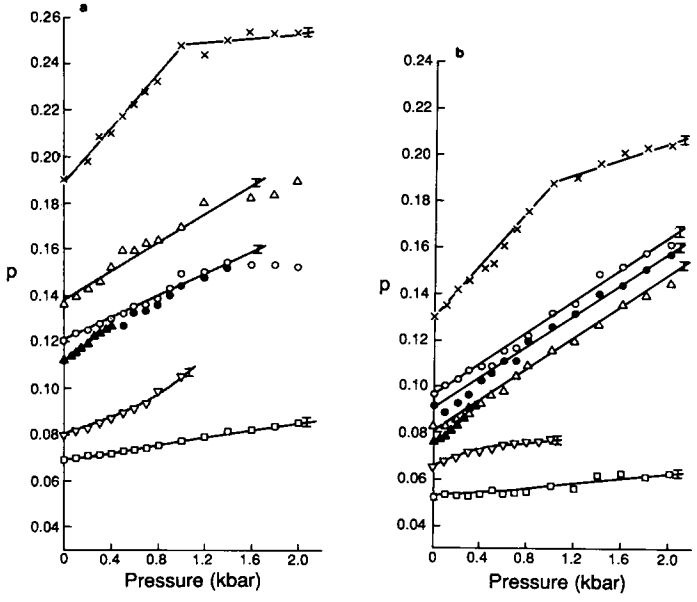

FIGURE 1 The polarization (p) vs. pressure plots of 12-AS (a) and 6-AS (b) in (●) DOPC small unilamellar vesicles, (○) DOPC large unilamellar vesicles, (△) DOPC multilamellar vesicles, (□) DOPC small unilamellar vesicles excited at 316 nm instead of 381 nm, (x) DOPC small unilamellar vesicles at 3°C and (▽) 40°C instead of 22°C, and (▲) DMPC small unilamellar vesicles.

linear increase with the exception of 9-AS which showed a slight curvature at higher pressures and 6-AS where the curvature was much more pronounced and occured at lower pressures (Fig. 1 *b*). While the cause of this curvature is not clear it may possibly be due to the packing of the probes around the double bond, an effect also observed in the pressure dependence of dipyrenylphospholipid probes (Sassaroli and Scarlata, manuscript in preparation). For comparison, the slopes of the linear portion of the polarization versus pressure curve are listed in Table 1. These data do not show any significant difference between multilamellar and unilamellar vesicles indicating that the probe motions observed are local in nature and the overall rotation of the vesicles does not contribute to the depolarization. Similar slopes of the pressure-polarization curves were obtained for all probes with fluorescent groups attached as a side chain of the fatty acid at the indicated positions (2-, 6-, 9-, and 12-AS). In the two samples where the label is on the end of the fatty acid (11-AU and 16-AP), the slopes are comparable to each other but lower than those of the side chain probes.

To determine whether the curvature of the bilayer effects the rotational behavior of the probes under pressure, we tested DOPC-12-AS and DOPC-6-AS in large-unilamellar vesicles. No significant differences were observed.

The above data was obtained using 381 nm excitation where the anisotropy is due to an equal portion of in-plane and out-of-plane probe rotation. When samples are excited at 316 nm, all of the emission depolarization is from out-of-plane motions (Vincent et al., 1982). The data taken at 316 nm also show an apparent linear increase in polarization with pressure (Fig. 1), which is similar for all of the probes. Again, the fluorescence intensities did not change significantly with pressure.

To view the phase transition behavior at different membrane depths, the experiments were repeated at 3°C (Fig. 1). In the fluid phase, the intensity of all the samples remained constant with pressure and the polarization of each increased smoothly. All probes gave identical phase transition pressures of 1.0 ± 0.1 kbar agreeing with previously reported lipid values dT_m/dp of ~20°/kbar (Nagle and Wilkinson, 1978; Heremans, 1982; Chong and Weber, 1983). Upon reaching the gel phase, fusion and subsequent precipitation of the vesicles occurred and the changes were not reversible. We note that Wong and Mantsch (1988) found the main phase transition of DOPC to occur at 5 kbar at 28°C by vibrational spectroscopy. Differences in the apparent dT_m/dp from these results compared to those authors could lie in the large differences in lipid concentration and hydration used in the two techniques. Alternatively, assessment of the phase transition by vibrational measurements entails the measurement of the static concentrations of the *cis* to *trans* isomers on the hydrocarbon chains while our measurements are sensitive to lipid packing. It is possible that the cooperative increase in chain packing occurs previous to complete static alignment of the lipid chains.

The experiments were repeated at 40°C to observe the temperature dependence of the compressibility. However, at this temperature, the samples displayed much higher errors in polarization. 2-AS-DOPC failed to show any change in polarization with pressure, which indicates that the location of the probe changes such that it is no longer sensitive to membrane compression. Data were not taken above 1 kbar due to experimental limitations. 6-AS and 12-AS showed an increase in intensity while the intensity of 9-AS remained constant with pressure.

To determine whether the gel to liquid-crystalline phase transition induced by pressure could be mimicked by reductions in temperature, we measured the polarization of DOPC small unilamellar vesicles labeled with 2-AS, 12-AS, or 16-AP in 55% glycerol-Hepes from 5° down to −25°C. Glycerol was added to preformed bilayers and the resulting solution was sonicated briefly at low power for ~5 min. The addition of glycerol did not affect the fluorescence properties of the vesicles. Unlike pressure, neither the polarization nor the lifetime displayed a break or large change in slope when brought through the phase transition temperature (−18°C). This implies that the combination of decreased volume and decreased kinetic energy caused by lowering the temperature broadens the transition enough to make the rotational motion of the probes similar in the two phases. However, it is possible that the addition of glycerol results in a broadening of the phase transition due to either change in solvent dielectric, bilayer hydration, or osmotic gradients.

TABLE 1 Slopes of the polarization change with pressure

	Probe	MLV	SUV
side probes	2AS	0.039	0.036
	6AS*	0.038	0.037
	9AS	0.039	0.038
	12AS	0.034	0.036
end probes	11AU	0.030	0.028
	16AP	0.028	—

Where MLV-multilamellar vesicles and ULV-unilamellar vesicles. Slopes were calculated from the linear portion of the polarization vs. pressure plots which were from atm to 1.8 to 2.0 kbar except for *(6AS) became nonlinear after 1.4 kbars and are in units of kilobar.

Dimyristoylphosphatidylcholine (DMPC) bilayers

The effect of pressure on the polarization of the anthroyloxystearic acid probes in DMPC small-unilamellar vesicles was also determined (Fig. 1). We note that since the fatty acid probe chains are longer than myristoyl, some interdigitation or looping of the fatty acid may occur, which would most likely broaden the phase transition. All samples showed identical, sharp phase transitions indicating that the bilayers are not greatly perturbed by the presence of probe. Experiments were carried out at 40°C and data in the liquid crystalline phase were collected from atmospheric to 0.4 kbar. All samples showed a smooth increase in polarization while the intensities increased identically by 10%.

DISCUSSION

The behavior of the polarization of the different probes under pressure should allow us to determine the changes in free volume and packing that occur in the membrane as a function of membrane depth. Fluorescence polarization or anisotropy was first described for isotropic rotations and, in a membrane, we expect the probe motions to be anisotropic. We assume here that the probe rotation is fast enough that it fills the space allowed to it by its environment. Kawato et al. (1977) described the anisotropy observed at long times to be due to restricted fluorophore motion in a potential well and introduced a parameter, A_{inf}, that describes this amplitude. A_{inf} has subsequently been related to a lipid order parameter (van Blitterswijk et al., 1981). Thus, under conditions of limited diffusion, depolarization can be described as consisting of a dynamic component due to the rotational rate and a static component resulting from the potential imposed by the surroundings. Correcting the observed anisotropy by A_{inf} will yield the dynamic component of the anisotropy. Our pressure experiments are isothermal and the dynamics of the probes should not change. Thus, the application of pressure will only increase A_{inf} and it is not necessary to extract static and dynamic components from our measurements. To further this point, Weber (1989; Scarlata and Weber, 1990) has shown that fluorescence anisotropy can be described as the result of thermal equilibration of different probe orientations within the fluorescence lifetime in isotropic and anisotropic environments. This description is independent of geometric constraints or use of A_{inf} and allows for detailed analysis of the polarization data without isolation of the dynamic and static components.

The fluorescence polarization or anisotropy (A) of a probe is related to the average cosine square of the angle, θ, swept out by the fluorophore during its lifetime through the following:

$$\langle \cos^2 \theta \rangle = [1 + 2\langle A \rangle / A_0)]/3, \quad (2)$$

where A_0 is the anisotropy in the absence of any rotational motion and corresponds to the angle between the absorption and emission dipoles. Fig. 2 shows the decrease in amplitude with pressure of the different probes exciting at 381 and 316 nm at 22°C. The plots are approximately linear for all the probes.

Although this study focuses primarily on membrane compressibility as a function of depth, comparing the behavior of the probes that are located at the terminus of the fatty acid chain or "end-on" (11-AU and 16-AP) and probes that are labeled on the side of the fatty acid chains "side-on" (the AS series) at two different wavelengths will yield information about how compression is felt in the different membrane planes. Exciting at 316 nm monitors motions from out-of-plane probe rotations while exciting at 381 nm monitors motions due to half in-plane and half out-of-plane rotations (Vincent et al., 1982). For end-on probes, in-plane motion will be in the xz and yz planes of the membrane essentially parallel to the lipid chains while the out-of-plane motion will be in the xy membrane plane perpendicular to the lipid chains. Side-on probes will be positioned 90° with respect to the end-on probes and their in-plane motions will be in the xy and xz membrane planes while the out-of-plane motion will be in the yz membrane plane. We note that the xz and yz planes are degenerate.

Extrapolating the lines in Fig. 2 to the x axis allows us to estimate the pressure at which the probes no longer rotate, assuming the lines do not curve appreciably. Because the side-on probes show larger x-intercepts when exciting at 316 nm (yz membrane plane) as compared to 381 nm (xy and xz membrane planes), this

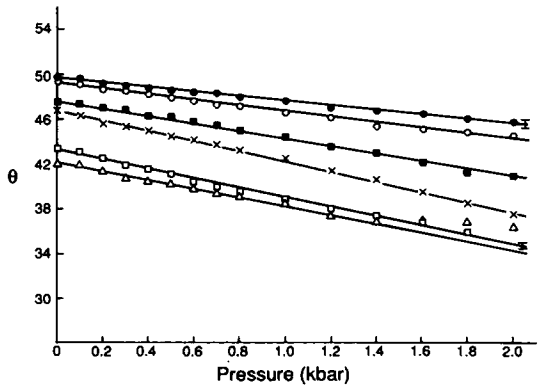

FIGURE 2 The behavior of the angle of rotation, θ, with pressure for 2-AS (x), 6-AS (△), 9-AS (□), 12-AS (■), 11-AU (○) and 16-AP (●).

indicates that higher pressures are needed to stop the motions in the z plane. This result agrees with neutron diffraction studies (Braganza and Worcester, 1986) that show an increase in dipalmitoylphosphatidylcholine (DPPC) bilayer thickness in going from atmospheric to 2 kbar. Unlike the side-chain probes, the end-on probes show similar intercepts in the different membrane planes indicating that pressure is felt differently by the two types of probes. The x intercepts increase with temperature due to membrane expansion.

We can equate the amplitude of the angle swept by the fluorophore during rotation to a volume element and analyze our data by a free volume model in which the increase in polarization with pressure is due to a decrease in the volume in which the probe is allowed to rotate. The isothermal compressibility, K, defined as $-1/V(dV/dp)$, can be determined by the decrease in volume with pressure through:

$$V(p) = V(\text{atm}) \times (1 - Kp), \qquad (3)$$

where $V(p)$ and $V(\text{atm})$ are the volumes at pressure p and atmospheric, respectively, and where the volumes used are the values of $\mathscr{O}$ from Eq. 2. It may be argued that $\mathscr{O}$ is not a true volume element and may not accurately characterize the compressibility. To test whether this is true we took the polarization data at 316 nm excitation where we are viewing emission from dipoles whose motions sweep out a volume similar to a flat-topped cone [$V = pi/3\, l^3 \sin^2(\mathscr{O})$] and calculated the compressibilities using these volume elements. This method resulted in K values identical to, but 20% lower and with more uncertainty, than those obtained just using $\mathscr{O}$. Thus, the direct use of the rotational amplitude allows comparison of the changes in compressibility reported by the different probes. Furthermore, the compressibilities that we calculate for our samples (Table 2, discussed below) are within range of the bilayer compressibilities of -0.01 to -0.06/kbar obtained for oriented DPPC and DPPC/cholesterol multilayers by neutron diffraction (Braganza and Worcester, 1986) and are typical of polymers and organic solids.

We have calculated K for all our samples as a function of pressure (Table 2). The value of K was constant with pressure after initial fluctuation (Fig. 3) and thus a unique value can be reported. These are listed in Table 2. In DOPC at 381 nm excitation, K is relatively constant for the first half of the chain and as the probe is moved towards the center of the membrane, K appears to decrease. However, the small differences in lifetime of the probes may change the apparent values of the compressibilities. Using Eq. 1, we calculated the anisotropies the probes would have if all of the lifetimes were 11.8 ns, and corrected the compressibilities. These data, listed in Table 2 as K', show similar (within error) values

TABLE 2 Compressibility results

	PROBE	K(/kb)	K'(/kb)	x-int (kb)
DOPC	2AS	−0.085 (± 0.005)	−0.070	100.2
22°C, 381 *nm*	6AS	−0.086	−0.086	11.3
	9AS	−0.085	−0.085	10.2
	12AS	−0.070	−0.073	14.1
	11AU	−0.058	−0.059	21.0
	16AP	−0.046	−0.049	24.0
DOPC	2AS	−0.033 (± 0.008)	−0.028	29.1
22°C, 316 *nm*	6AS	−0.044	−0.044	21.7
	9AS	−0.041	−0.041	24.4
	12AS	−0.041	−0.043	25.0
	11AU	−0.034	−0.034	29.1
	16AP	−0.033	−0.035	30.1
DOPC	2AS	−0.121 (± 0.007)	−0.041	8.3
3°C, 381 *nm*	6AS	−0.120	−0.046	9.3
	9AS	−0.120	−0.048	8.2
	12AS	−0.121	−0.052	8.4
DOPC	6AS	−0.041 (± 0.014)	−0.160	24.0
40°C, 381 *nm*	9AS	−0.118	−0.100	22.5
	12AS	−0.025	−0.100	25.0
DMPC	2AS	−0.088 (± 0.009)	−0.034	11.6
40°C, 381 *nm*	6AS	−0.076	−0.034	12.9
	9AS	−0.088	−0.041	11.0
	12AS	−0.078	−0.036	12.2
	11AU	−0.057	−0.018	21.1

Where K is the compressibility, K' is the compressibility from the fluorescence polarization data normalized to a lifetime of 11.8 ns, and x-int is the x-axis intercept of the plot of θ vs. pressure as seen in Fig. 2.

for the side-on probes (2-, 6-, 9-, and 12-AS). The values listed for the two end-on probes (11-AU and 16-AP) are also within error of each other. Thus, within the limits of our measurements, we conclude that the compressibility does not vary significantly with membrane depth.

At the different temperatures, the magnitude of the compressibilities cannot be directly compared because the lifetimes are different. The values (K') listed in Table 2 are the compressibilities that have been corrected for lifetime differences (again normalizing to 11.8

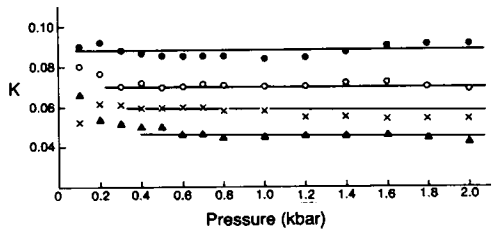

FIGURE 3 The compressibility, K, versus pressure for 2AS (●), (○) 12-AS, (x) 11-AU and (△) 16-AP.

ns) and for any changes in intensity with pressure. These data show that lowering the temperature decreases the compressibility which is a general property of fluids. We note that at 40°C, the compressibility of DOPC begins to approach that of a liquid hydrocarbon (K = 0.1/kbar) corresponding to the higher degree of fluidity at this temperature.

Comparing the compressibilities at 316 excitation to 381 nm, allows us to determine its variation in the different membrane planes assuming the values are linearly related. For the two end-on probes, $K'(xy)$ = 0.0335 and $K'(xz) = K'(yz)$ = 0.0356. Thus, the compression felt by these probes is isotropic. For the four side-on probes, $K'(xy)$ = 0.0765 and $K'(xz) = K'(yz)$ = 0.0395. Therefore, the lateral compression (xy plane) felt by probes that are parallel to the lipid chains is twice as large as in the two other planes and also is twice as large as that felt by probes that are perpendicular to the chains.

The intensities of the DMPC samples all increased by 10% from atmospheric pressure to 0.4 kb. Since the lifetime of the probes is usually proportional to the intensity, any increases would cause the rotational volume to appear greater than what is measured in the absence of intensity change and yield a lower compressibility. The values listed for K' take into account these increases as well as the differences in lifetime between the different probes. Comparing these values to those of DOPC at 40°C, we find that DOPC is approximately twice as compressible, presumably due to the looser packing of unsaturated systems.

Since a lipid bilayer is a quasi-two–dimensional fluid, we also analyzed our data using $A(p) = A(\text{atm}) \times (1 - Kp)^{2/3}$, and the results were identical to the three-dimensional case except that the compressibilities for the end-on and side-on probes were 40 and 50% lower, respectively.

Whereas compressibility is usually described as an overall macroscopic property of a system, in this study we have characterized it on a microscopic scale by the use of fluorescent probes located at different membrane positions. We find that the compressibility does not vary appreciably with membrane depth. We have also found that the compressibility measured for a particular probe will vary depending on the probe's access to the z plane because this plane expands with pressure. Thus, these results imply that if a molecule is positioned completely parallel to the hydrocarbon chains then pressure would not perturb its location but if in other orientations, pressure would force it into more aligned positions.

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