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DIFFERENTIAL POLARIZED PHASE FLUOROMETRIC STUDIES OF PHOSPHOLIPID BILAYERS UNDER HIGH HYDROSTATIC PRESSURE

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Differential polarized phase fluorometry was used to quantify the rotational rate (R) and limiting anisotropy (r_∞) of the membrane probe diphenylhexatriene (DPH) in solvents and lipid vesicles exposed to hydrostatic pressures ranging from 1 bar to 2 kbar. These measurements reveal the effect of pressure on the phase-transition temperatures of the phosphatidylcholine vesicles, and the effects of pressure on order parameter of the acyl side-chain region of the membranes, the latter as indicated by r_∞. In addition to the well-known elevation of the transition temperature (T_c) with pressure, our results demonstrate that increased pressure restores the order of the bilayers to that representative of temperatures below the transition temperature. We also found that solvents which allowed free isotropic rotation of DPH at 1 bar no longer allowed free rotation when sufficiently compressed; moreover, the apparent DPH rotational rate increased with r_∞. Pressure studies using both DPH and the charged DPH analogue, trimethylammonium DPH (TMA-DPH) indicated that the T_c of dipalmitoylphosphatidylcholine vesicles increased 23 K/kbar and an apparent volume change of 0.036 ml/mol lipid at the phase transition. Assuming, as has been proposed, that TMA-DPH is localized near the glycerol backbone region of the bilayers, these results indicate a similar temperature- and pressure-dependent phase transition in this region and the acyl side-chain region of the membrane.

Introduction

A substantial body of work has emerged wherein the structure and function of cell membranes and their constituents have been examined using fluorescent probe molecules. Among the most widely used methods have been those employing fluorescence anisotropy measurements of polyenes, such as diphenylhexatriene. As a first approximation, such measurements may be interpreted in terms of 'microviscosity' within the bilayer, which has been correlated with many aspects of membrane structure and function, including the gel-liquid crystalline phase transition [1,2]. We and others [3–7] have extended the usefulness of such probes by use of differential polarized phase fluorometry. This technique is comparable to the more familiar measurements of time-resolved decays of fluorescence anisotropy [8], except that the incident light is modulated rather than pulsed. Differential-phase measurements of DPH in lipid bilayers demonstrated that the rotational motion of the probe molecule was hindered in the bilayer [9]. The motions of the probe were further resolved in subsequent studies [10,11] which revealed both the

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-(trimethylamino)phenyl)-6-phenylhexyl-1,3,5-triene; DOPC, DMPC and DPPC, dioleoyl-, dimyristoyl- and dipalmitoyl-L-α-phosphatidylcholine, respectively; τ_p and τ_m, fluorescence lifetime by the phase shift and demodulation methods, respectively.

rotational rate (R) of the probe in the bilayer and the degree to which these motions were hindered. The latter is determined from the limiting anisotropy (r_∞), which reflects the average order parameter of the membranes [12,13]. Investigators using time-resolved methods have arrived at similar conclusions, but within a somewhat different theoretical framework [14,15]. From previous investigations [11] we recognized that differential phase fluorometry provided a relatively straightforward and dependable measurement of the diffusive motions of DPH in solvents and in membranes. We extended our differential-phase studies of lipid bilayers into the realm of high pressure.

The response of many biological systems to high hydrostatic pressure has been examined since Bridgman's [16] pioneering study of serum albumin. Proteins [17–19], nucleic acids [20], and membranes [21] have been examined, along with a large array of in vivo systems [22,23]. Although the above studies were undertaken for a variety of reasons, including the need to understand the response of organisms to pressure, the advantages of pressure as a perturbing agent for biophysical research should be stressed. The primary virtue of perturbing biochemical systems with pressure is that the thermodynamic analysis of the response is usually straightforward. This is in contrast to variables, such as temperature, which cannot be varied over a wide range with biological samples. A second advantage is that within the pressure range of interest (under 10 kbars, where water freezes at 20°C), the pressure is insufficient to distort either bond lengths or bond angles. The molecules in a system respond to pressure by packing themselves more efficiently and/or undergoing conformational changes via rotations around single bonds. It is important to note that these changes occur to reduce the volume of the entire system, and not just the separate components. The inter- and intramolecular interactions which may be perturbed by pressure of less than 10 kbars include the weak interactions, such as electrostatic interactions, hydrophobic interactions and hydrogen bonds, all of which play an important role in the structure and function of most biological molecules.

Despite the difficulties of pressure studies, the most serious of which is the necessity for isolation the sample, the pressure response of cell membranes and related model systems has been studied using several techniques, including dilatometry [24], light scattering [25], electron spin resonance [26] and X-ray diffraction [27]. Among the interesting results was the finding that the effects of pressure and temperature on the gel-liquid crystalline phase transition were reciprocal, i.e., the phase transitions obeyed the Clausius-Clapeyron equation:

$$\frac{dT_m}{dP} = \frac{T_m \Delta V}{\Delta H} \frac{1}{C} \quad (1)$$

where dT/dP is the change in the transition temperature (T_m) with pressure, ΔV is the volume change which occurs at the transition, ΔH the enthalpy change and C is a constant and is equal to $41.3 \cdot 10^3$ [24]. In this report we examine further the effects of pressure on membranes. In particular, we examined the effects of pressure on the order parameters of the membranes, and on the transition temperatures as observed by probes which localize either in the acyl side-chain or glycerol backbone region of the membranes.

Materials and Methods

The experiments were performed essentially as described by Lakowicz and co-workers [9,11], using reagents from the same sources, except as described below. Lipids were dispersed in 0.01 M Tris buffer, containing 0.05 M KCl, pH 7.5. The pK_a of Tris is essentially invariant with pressure. The probes were not mixed with the phosphatidylcholines before sonication. Instead, unilamellar phosphatidylcholine vesicles were labeled by vigorous mixing of the vesicles with small aliquots of millimolar solutions of DPH in tetrahydrofuran, or TMA-DPH in dimethylsulfoxide, until a suitable absorbance at 360 nm was achieved. Phase and modulation lifetimes were measured by comparison with an external reference solution of dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) in ethanol [28], using a reference lifetime of 1.45 ns. The modulation frequency was 30 MHz.

Fluorescence measurements under pressure

The pressure vessel used in this study was es-

sentially identical to that used by Paladini and Weber [29]. For improved temperature control, our thermal jacked surrounded the sides of the pressure vessel and was also in contact with the bottom of this vessel. Furthermore, the internal sample holder was a small cage-like device which was fixed to the base of the sample chamber and prevented motion of the cylindrical sample vial. The use of a high-pressure vessel introduces two types of errors in the observed fluorescence parameters. First, pressure induces birefringence in the quartz windows. This depolarizes the excitation and the fluorescence, resulting in decreased values of the apparent steady-state anisotropies (r) and the differential tangents (tan Δ). Secondly, the use of a pressure vessel requires that lifetime measurements be performed using an alternate light path [30], which in turn results in errors in the measured phase and modulation values.

The pressure-induced birefringence of the windows decreases the apparent polarization by over 20% at 2000 bar. Paladini and Weber [29] were able to measure the depolarization introduced by each window by measuring the polarization of fluorescein in vitrified glycerol solution as a function of pressure. We use an essentially identical procedure, except that we simply multiplied our observed anisotropies by a correction factor derived for DPH in propylene glycol at $-60°C$. This factor is given by r_0/r_{obs}, where r_0 is the anisotropy observed for DPH in the absence of both rotational diffusion and pressure-induced depolarization, and r_{obs} is the anisotropy observed for the same solution, but now depolarized by the pressure-induced birefringence. The correction factors seem to change little over a period of months. In addition, the bomb does not transmit horizontally and vertically polarized light equally well. This effect is corrected for using the usual G-factor employed in steady-state anisotropy measurements [31].

It is also necessary to correct the differential tangents for the birefringence of the quartz windows. This was accomplished using the procedure described by G. Weber (personal communication). The corrected value of tan Δ is given by

$$\tan \Delta = \tan \Delta_{obs}/(1-2\alpha) \tag{2}$$

where tan Δ_{obs} is the measured differential tangent and α is the correction factor described by Palidini and Weber [29], but derived in our case using DPH in propylene glycol at $-60°C$. Differential polarized lifetimes were determined by serial measurements in an L-format, rather than by the simultaneous T-format method used previously [11].

Lifetime measurements on samples contained within the high-pressure cell require the use of a separate light path for determination of the phase and modulation of the incident light. In our instrument about 5% of the modulated exciting light was diverted by a beam splitter to excite the reference fluorophore. Emission from the reference is reflected to the photomultiplier tube by means of a sliding mirror [30]. The phase and modulation of the reference and the sample are compared by sliding the mirror in and out of position.

The different geometries of the sample and reference light paths introduced small artifacts into these measurements. This was apparent when the lifetime of the reference fluorophore was measured in the pressure vessel. The values of the artifacts were small and usually stable. Typically, the emission from the reference cuvette in the alternate light path appears to have 5% less modulation and to be phase-shifted $-2.5°$ at 30 MHz, in comparison with the same compound in the sample cell in the bomb. In practice, we make this comparison before each pressure run. The corrected phase angle (ϕ) and demodulation factor (m) are obtained using

$$\phi = \phi_{obs} + \phi_c \tag{3}$$

$$m = m_{obs}/m_c \tag{4}$$

where obs indicates the observed values for the sample, and ϕ_c and m_c are the values observed when the bomb contains a sample identical to the reference fluorophore. This correction is invariant with pressure and temperature within the ranges described below; however, alignment of the system seems to be crucial in applying this procedure successfully. Particular care is taken in maintaining the orientation of the bomb around its vertical axis in this (and all other) measurements.

The corrected anisotropies, phase lifetimes and

differential tangents were used to calculate the rotational rate (R) of the probe and its limiting anisotropy (r_∞) using the equations described previously [11].

Results

Measurements in homogeneous solution

Two solvents, propylene glycol and mineral oil, were chosen for pressure studies of DPH in homogeneous solution to serve as a basis for comparison with the vesicle preparations. These solvents were used because the DPH lifetimes, anisotropies and differential tangents in each were known over a range of temperatures from previous work [11], and because DPH behaved as a free isotropic rotor in both solvents. Fig. 1 depicts the corrected phase (τ_p) and modulation (τ_m) lifetimes of DPH in propylene glycol at 0, 18 and 50°C, and in mineral oil at 40°C. Overall, the agreement between the phase and modulation lifetime was ± 0.5 ns, indicating that the decay of DPH intensity was dominated by a single exponential over these pressures and temperatures. Reliable determination of phase and modulation lifetimes requires careful measurement of the correction factors (Eqns. 3 and 4) and accurate positioning of the bomb within the instrument. The modest pressure-dependent decreases in lifetime seen for DPH in propylene glycol and in mineral oil are comparable to those seen by Brey et al. [32] for DPH in toluene and methylcyclohexane. Since we did not measure quantum yields we cannot conclude that the same mechanism of quenching via enhanced intersystem crossing suggested by those investigators is operating here. The fact that the apparent lifetimes in propylene glycol remain mostly constant with pressure argues that different mechanisms are operating in the two solvents. This differing response to pressure is not unexpected given the sensitivity of the radiative rates of diphenyl polyenes to variations in temperature [33], viscosity [34] and solvent polarizability [35].

The steady-state anisotropies observed for DPH in the two solvents are depicted in Fig. 2. The observed values have been corrected for pressure-induced birefringence of the windows as described in Materials and Methods. In both solvents the steady-state anisotropy of DPH increases with increasing pressure. The anisotropy of DPH in mineral oil increases more rapidly than that of DPH in propylene glycol. This difference is possibly a result of the higher compressibility of the aliphatic solvent. Alternatively, this difference may be a result of a pressure-induced ordering of the mineral oil (see Fig. 4 and the related discussion).

The tan Δ values measured at 30 MHz for DPH in the two solvents are depicted in Fig. 3. These differential measurements are performed without using the alternate light path, and they are not subject to the phase artifact introduced by this procedure. However, these values were corrected

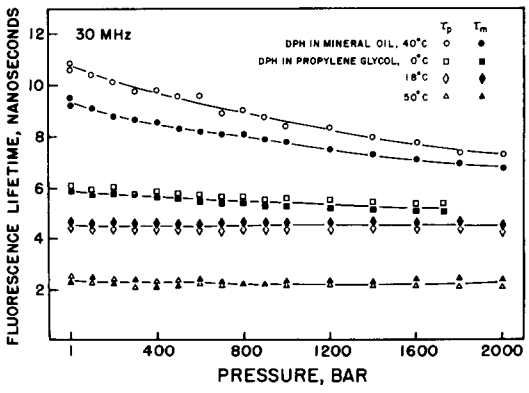

Fig. 1. Pressure dependence of the phase modulation lifetimes of DPH in propylene glycol and mineral oil. The lifetimes were corrected for the optical effects of the pressure bomb and the alternative light path, as described in Materials and Methods.

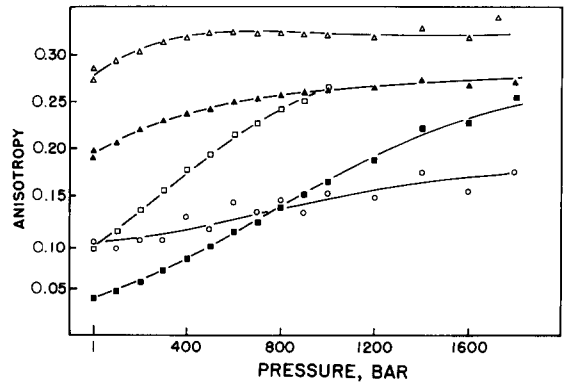

Fig. 2. Pressure-dependent steady-state anisotropies of DPH in mineral oil (□, 20°C; ■, 40°C) and propylene glycol (△, 0°C; ▲, 18°C; ○, 50°C). These values were corrected for the pressure-induced birefringence of the bomb windows, as described in Materials and Methods.

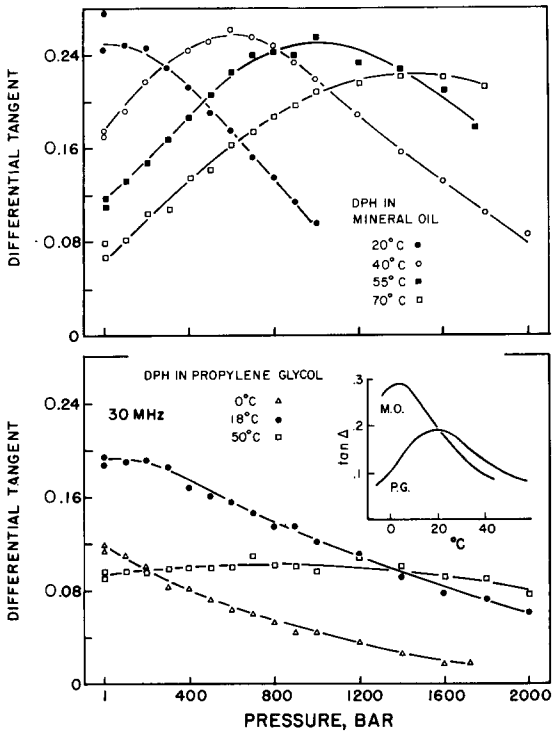

Fig. 3. Differential tangents for DPH in propylene glycol (P.G.) and mineral oil (M.O.). These were corrected for the birefringence, as described in Materials and Methods (equation 2). The inset shows the temperature dependence of tan Δ in these solvents (from Ref. 11).

for the pressure-induced birefringence of the pressure cell windows (Eqn. 2). We judge their accuracy to be ± 0.01, or 0.05 ns. In mineral oil, the pressure response of tan Δ reflects the behavior of an isotropic rotor in a solvent whose viscosity is increasing with pressure. For reference the temperature-dependent tan Δ values at one atmosphere of pressure are shown as an insert. The position of the tan Δ_{max} on the insert may be thought of as a temperature (and therefore a viscosity) where the rotational rate of DPH and its decay rate are comparable in magnitude. At temperatures above 5°C in mineral oil we expected that by increasing the viscosity with pressure, the values of tan Δ would increase to tan Δ_{max} and afterwards decrease; that is, the data would 'backtrack' to lower temperature along the curve shown in the inset. For DPH in mineral oil, the form of the data was approximately as expected. Upon increasing the pressure we observed maximum values of tan Δ at higher temperatures; that is, higher pressures were required to achive tan Δ_{max}. The modest decrease in tan Δ_{max} seen at higher temperature and pressures is due both to the pressure-dependent decrease in the DPH lifetime and to the appearance of partially hindered rotations at these higher pressures.

The differential tangents of DPH in propylene glycol are also shown in Fig. 3. From the inset one notices that tan Δ_{max} is found near 20°C. Hence, at temperatures above 20°C we expect tan Δ to increase with pressure, and at temperatures below 20°C one expects tan Δ to decrease with increasing pressure. As expected, at 0 and 18°C, the values of tan Δ were found to decrease with increasing pressure. Also as expected, the tan Δ values at 50°C increased with increasing pressure. However, the extent of the increase is small. This is probably because the fluorescence lifetime (τ) of DPH is shorter at this temperature (Fig. 1 and Ref. 11).

The data in Figs. 1–3 were used to calculate the effects of pressure on the rotational rate (R) and limiting anisotropy (r_∞) of DPH in these solvents.

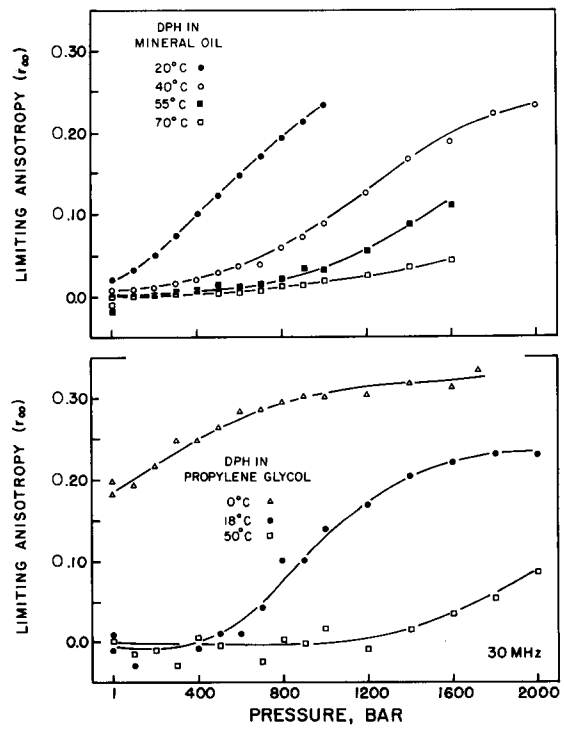

Fig. 4. Pressure-dependent limiting anisotropies (r_∞) for DPH in mineral oil and propylene glycol.

Increased pressures result in hindered rotational motions for DPH (Fig. 4). Surprisingly, the increase is r_∞ is substantial. In both solvents, the rotational motions are hindered at lower pressures when the temperature is also lower. In the case of mineral oil it seems likely that pressure induces partial freezing of this heterogeneous solvent, and that the diffusive motions of DPH within these clusters is hindered. This explanation seems less likely for propylene glycol, which forms a glass rather than crystallizing. The derived pressure-dependent rotational rates are also of interest. In mineral oil these values decrease with pressure. This is the expected result since the viscosities of aliphatic solvents invariably increase with increasing pressure [36,37]. More complex behavior is seen for the rotational rates of DPH in propylene glycol. In this solvent the rotational rates are constant or increase with increasing pressure. This is surprising because the viscosity of a similar solvent, glycerol, is known to increase with pressure [38,61]. However, the viscosity of water is known to decrease initially [39,61] and its rate of rotational diffusion [40] is known to increase with increasing pressure. These unusual changes are thought to result from disruption of the hydrogen bonded structure of water with increasing pressure. It seems likely that similar effects could be present in propylene glycol.

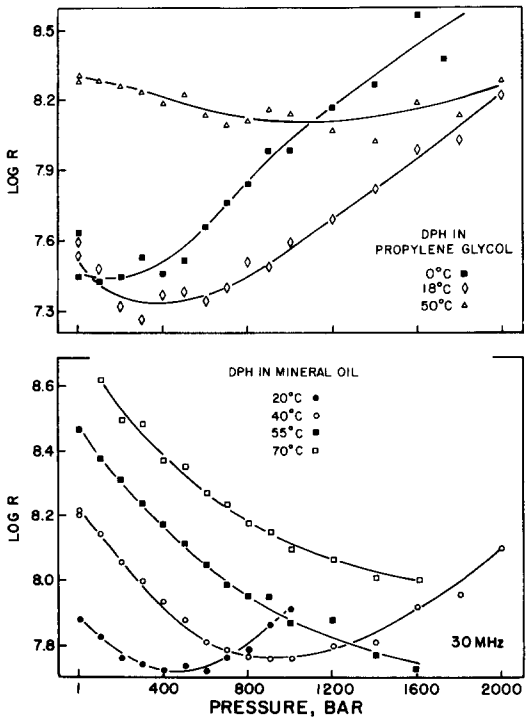

Fig. 5. Pressure-dependent rotational rates for DPH in mineral oil and propylene glycol.

Measurements in saturated phosphatidylcholine vesicles

We used measurements of the lifetimes, anisotropies and differential tangents of DPH-labeled vesicles to determine the effects of pressure on the phase-transition temperatures and on the order parameters of bilayers. Fluorescence lifetimes of DPH in DMPC and DPPC vesicles are shown in Figs. 6 and 7. We note that the samples had similar apparent turbidity before and after raising pressure, suggesting that the state of the phospholipids as unilamellar vesicles did not change during the experiments. Phase and modulation lifetimes generally agreed as closely as in homogeneous solution, and indicate that the decay of fluorescence intensity is mostly a single exponential. We do not feel that phase and modulation data are accurate enough to allow interpretation of these data in terms of a multi-exponential decay of intensity. When the phase and modulation lifetimes are not in agreement we believe this is due primarily to errors in determining the phase and/or modulation correction factors for the experiment (Eqns. 3 and 4). Except for DPPC at 18°C, τ_p is generally slightly longer than τ_m, which is normally

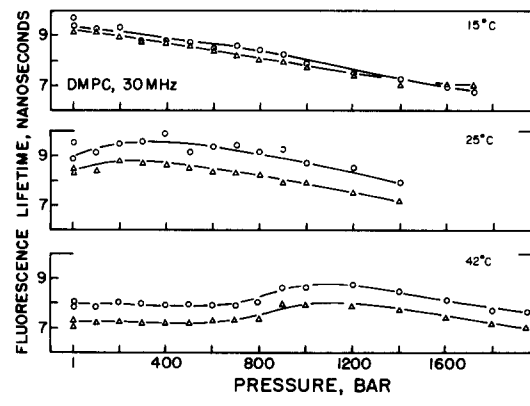

Fig. 6. Pressure-dependent fluorescence lifetimes of DPH in DMPC vesicles. ○, τ_p; △, τ_m.

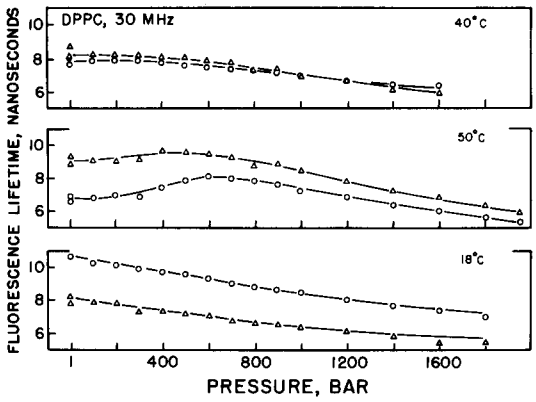

Fig. 7. Pressure-dependent fluorescence lifetimes of DPH in DPPC vesicles. ○, τ_p; △, τ_m.

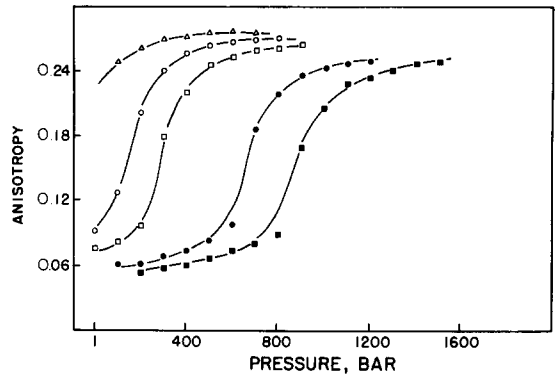

Fig. 9. Pressure-dependent anisotropies of DPH in DPPC vesicles. △, 37°C; ○, 43°C; □, 46.5°C; ●, 54.5°C; ■, 60°C.

the case for a fluorophore undergoing an excited-state reaction [41]. Inasmuch as DPH is not known to undergo any spectrally observable excited state reaction, we believe that this is due to errors in measuring the correction factors. The small differences seen in the phase and modulation lifetimes do not significantly affect the interpretation of our results. The lifetimes showed broadly similar behavior in both phospholipids. Increasing pressures resulted in shorter lifetimes, except at the pressure-induced phase transition, where a slight increase in lifetime was observed.

The steady-state anisotropies of DPH-labeled DMPC and DPPC vesicles are shown in Figs. 8 and 9. These pressure profiles are essentially mirror images of the temperature profiles of DPH anisotropy for these lipids [11,42]. Indeed, the essential reciprocity of pressure and temperature in their effects on the lipid bilayer phase transition has long been recognized for lipid dispersions [24–27]. Quantitatively, the midpoint pressure of the phase transition has been found to obey the Clausius-Clapeyron equation. The pressure midpoint of the phase transition (judged as being the steepest part of each curve) is plotted as a function of temperature in Fig. 10 for DMPC and DPPC. Evidently, our results for DPPC echo those previously mentioned, in that dT/dP is invariant with pressure and equals 22.5 degrees/kbar which corresponds to a volume change of 0.0356 ml/g for the phase transition, with $\Delta H = 8.7$ kcal/mol [43]. This is in excellent agreement with the previously

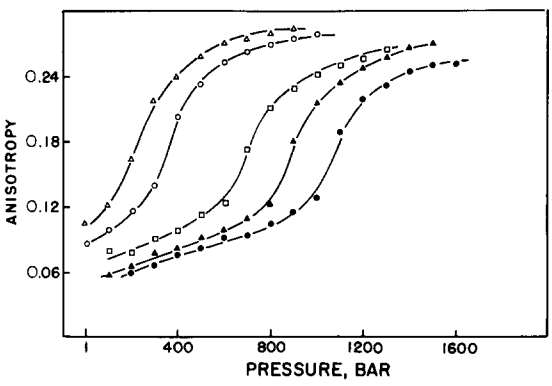

Fig. 8. Pressure-dependent anisotropies of DPH in DMPC vesicles. △, 28°C; ○, 31°C; □, 38°C; ▲, 42°C; ●, 45°C.

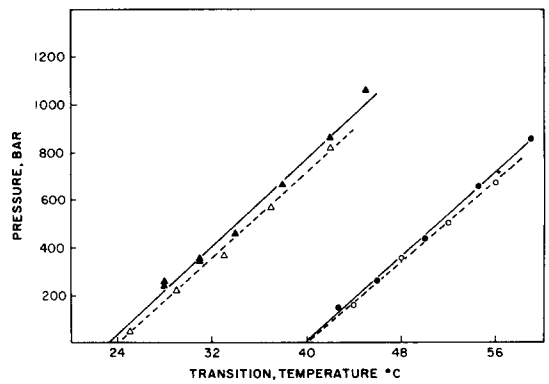

Fig. 10. Effect of pressure on the phase-transition temperature of DMPC (triangles) and DPPC (circles) vesicles. The transition temperatures were estimated from the steady-state anisotropies of DPH (closed symbols) and TMA-DPH (open symbols).

TABLE I

VOLUME CHANGES AND THE PRESSURE DEPENDENCE OF THE PHASE TRANSITION OF DMPC AND DPPC VESICLES

ΔV was calculated using Eqn. 1 and ΔH = 5.4 and 8.7 kcal/mol for DMPC and DPPC, respectively [43].

Lipid/probe	dT_m/dP (K/kbar)	ΔV (ml/g)
DPPC/DPH	22.5	0.0356
DMPC/DPH	21.5	0.0241
DPPC/TMA-DPH	23.4	0.0371
DMPC/TMA-DPH	22.2	0.0249

reported values 0.033 ml/g [24,44]. Also, our results for the DMPC vesicles, $dT_m/dP = 21.5$ degrees/kbar and the calculated value of $\Delta V = 0.024$ ml/g (Table I) are in good agreement with earlier results [43,45,64]. Comparable results for the pressure dependence of T_c, as estimated from the steady-state anisotropies, were obtained by Weber and co-workers [46].

Also summarized in Fig. 10 is the effect of pressure on the phase transitions of DPPC vesicles as observed from the steady-state anisotropies of TMA-DPH. This charged analogue of DPH is thought to localize at the lipid/water interface of vesicles [47]. Clearly, the transition temperatures and the pressure dependence of these values are essentially identical in both cases. We find this result surprising because the phase transition, which is so apparent using measurements sensitive to acyl side-chain motion, is not detected by the probe 2-*p*-toluidinylnaphthalene-6-sulfonic acid which is localized at the lipid/water interface [48]. If indeed TMA-DPH is localized at the lipid/water interface, then our results indicate that the phase transition in this region is equally as sensitive to pressure as that which occurs in the acyl side-chain region. The volume changes for the transitions in each region are also similar (Table I).

The effects of pressure on the differential tangents of DPH in DMPC and DPPC vesicles are shown in Fig. 11. Perhaps the most important point here is that the pressure profiles are nearly mirror images of the temperature profiles found earlier [11]. These temperature profiles are shown as an insert in Fig. 11. At temperatures below the

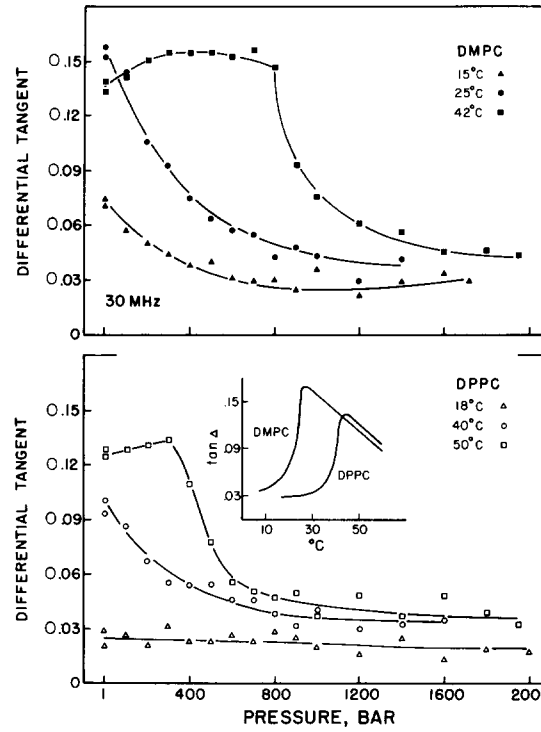

Fig. 11. Pressure-dependent differential tangents for DPH in DMPC and DPPC vesicles. The inset shows the temperature-dependent values of tan Δ [11].

phase-transition temperature, the values of tan Δ do not increase with pressure. This is because the rotational motions are already highly hindered at these temperatures, and any further increase in order does not affect the values of tan Δ. Furthermore, compared to bilayers above their transition temperatures, the ordered phase vesicles are relatively incompressible [24]. At temperatures above T_c the values of tan Δ are seen to decrease with increasing pressure. This is because the pressure causes the formation of the ordered phase, in which the rotational motions of DPH are strongly hindered. Note that, at higher temperatures, higher pressures are needed to decrease than tan Δ values to those representative of the gel phase.

The tan Δ values were used to calculate the limiting anisotropy (r_∞) of DPH in DMPC and DPPC as a function of pressure. These results are depicted in Fig. 12. The most striking result is that increased pressures result in r_∞ values comparable to those below the transition temperatures of these

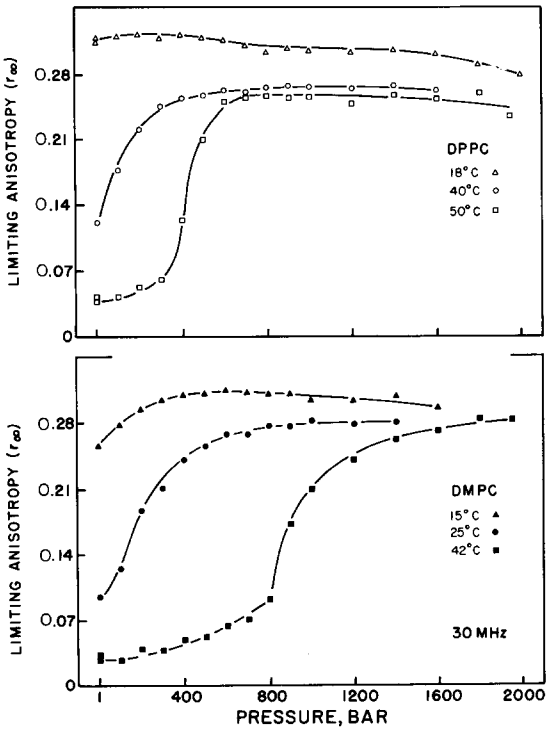

Fig. 12. Effects of pressure on the limiting anisotropies (r_∞) of DPH in DMPC and DPPC vesicles.

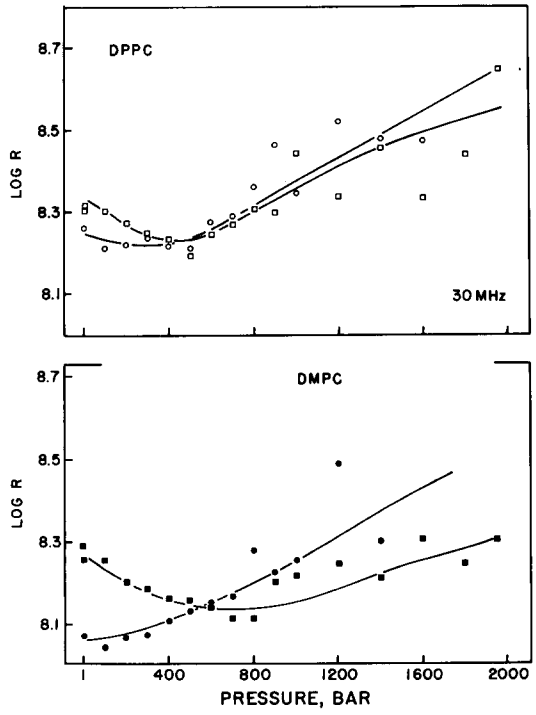

Fig. 13. Effects of pressure on the rotational rates of DPH in DMPC (●, 25°C; ■, 42°C) and DPPC (○, 40°C: □, 50°C) vesicles.

lipids. Since r_∞ is a measure of the order parameter of the acyl side-chain region, these results indicate that increased pressures cause this region to be ordered in a manner clearly similar to that which occurs on decreasing the temperature.

From these same data (Figs. 5, 8, 9, 11) we calculated the rotational rates of DPH (Fig. 13). In general, increased pressures resulted either in no change in this rate or in an increased rate of DPH rotations. The increase in order which occurs upon increasing the pressure does not slow the rate of DPH rotation. Apparently, the larger amplitude motions which occur at temperatures above T_c are more sensitive to temperature and pressure than the smaller motions which occur below the phase transition. This is not unreasonable because in one case the DPH is diffusing among disordered side chains, whereas in the other case it is oscillating between the ordered side chains.

Unsaturated and cholesterol-containing vesicles

We also studied the effect of pressure on vesicles composed of the unsaturated lipid DOPC and on cholesterol-containing vesicles (DMPC/cholesterol, 7 : 1 molar ratio). Fluorescence lifetimes at various pressures are shown in Fig. 14. The lifetimes for DPH decrease smoothly with pressure, which is in contrast to those found for the saturated phosphatidylcholines (Fig. 6). These results are not surprising since neither DOPC nor DMPC/cholesterol vesicles are expected to display a sharp phase transition at these pressures and tempera-

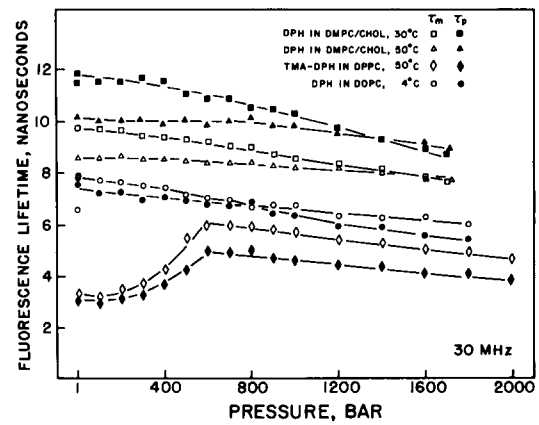

Fig. 14. Pressure-dependent lifetimes of DPH and TMA-DPH-labeled lipid vesicles.

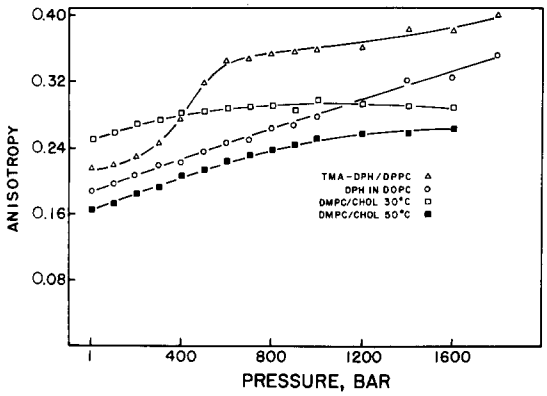

Fig. 15. Pressure-dependent anisotropies of DPH- and TMA-DPH-labeled vesicles.

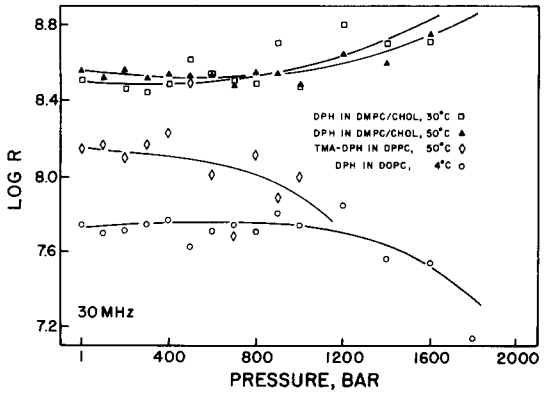

Fig. 18. Pressure-dependent rotational rates for DPH and TMA-DPH in lipid vesicles.

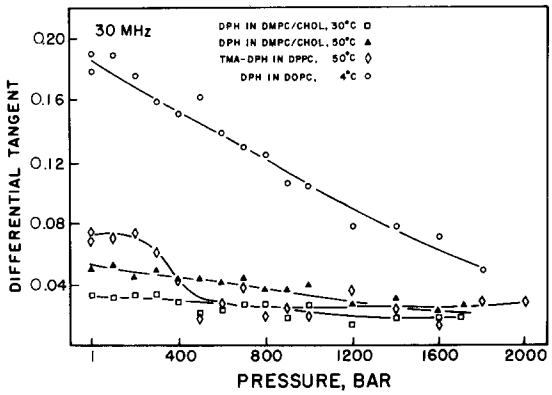

Fig. 16. Pressure-dependent differential tangents of DPH- and TMA-DPH-labeled vesicles.

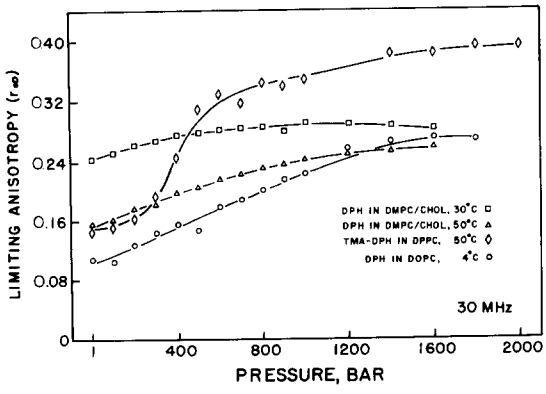

Fig. 17. Pressure-dependent limiting anisotropies (r_∞) for DPH and TMA-DPH in lipid vesicles.

tures. Complementary results were found for the steady-state anisotropies (Fig. 15) and the differential tangents (Fig. 16). The anisotropies increased and the differential tangents decreased uniformly with increasing pressure. These data were used to calculate r_∞ and R for DPH in these vesicles (Figs. 17 and 18). As the pressure is increased the probe motion, as revealed by r_∞, increases gradually for DPH in both the DOPC and the DMPC/cholesterol vesicles. The lack of a sharp increase in r_∞ for DPH in DOPC indicates that it does not undergo a phase transition at these temperatures and pressures. The pressure-dependent increase in r_∞ appears to level off at the higher pressures, which probably indicates that the vesicles become less compressible at these higher pressures. The DPH rotational rates are shown in Fig. 18. These rates are initially higher in DMPC/cholesterol than in DOPC. The rotational rate in DOPC appears to decrease slightly with pressure, whereas this rate seems to increase with pressure in the DMPC/cholesterol vesicles. However, we note that precise quantitation of these rates is difficult when r_∞ is comparable in magnitude to the steady-state anisotropy [11].

TMA-DPH-labeled DPPC vesicles

We also examined the effects of pressure on DMPC vesicles, as revealed by the charged probe TMA-DPH (Figs. 14–18). The pressure-dependent data for TMA-DPH are comparable with the temperature-dependent data reported previously [47].

In particular, the pressure-induced reversal of the DPPC phase transition results in an increase in its fluorescence lifetime (Fig. 14), an increase in anisotropy (Fig. 15) and a decrease in tan Δ (Fig. 16). Although the lifetime of TMA-DPH does not increase at 1 atm when the temperature is lowered, the change observed with increasing pressure is not precisely comparable. Hence, the pressure and temperature profiles of the lifetime (as for DPH) are not precisely 'mirror images'. Given the sensitivity of the TMA-DPH lifetime to polar and 'protic' solvents [47], we suspect that the initial increase indicates that the probe is becoming less accessible to the interfacial region. Above 600 bar, the gradual decline in τ_p and τ_m suggests the same sort of quenching process as occurs for DPH in the various bilayers. Further interpretation of these data would require a more detailed understanding of the effect of solvent polarity, 'proticity', temperature and viscosity on the lifetimes of these probes.

Perhaps most significant are the values of r_∞ observed for TMA-DPH at high pressures. These are comparable to those observed below the transition temperature. Hence, pressure appears to reverse the temperature-induced disorder in DPPC vesicles in both the acyl side-chain and the lipid/water interfacial region of lipid bilayers.

Discussion

The physiological effects of pressure include stimulation and depression of the nervous system [49,50], convulsions and paralysis, and reversal of the effects of anesthetics [51] and other agents [52]. Because of the low pressures at which these effects occur, it seemed likely that they originated with the effects of pressure on the structural and/or dynamic properties of the neural membranes. Indeed, the effects of pressure on the phase transitions of membranes has been correlated with the enzymatic activities of membrane-bound proteins [53–55]. For these reasons it is of interest to develop methods which can reveal the detailed effects of pressure on biological membranes. In this report we demonstarte that the technique of differential polarized phase fluorometry can reveal the effects of pressure on both the rotational rate and the limiting anisotropy of a membrane-bound fluorophore. These parameters reflect the dynamic properties and the order parameter of the lipid bilayers. These are the parameters which are likely to reveal the effects of anesthetics on membranes. For instance, inhalation anesthetics are known to broaden the phase transition [56,62] or, alternatively stated, to decrease the cooperativity of this transition. With regard to the pressure reversal of anesthesia it is not agreed whether pressure restores the original phase transition of bilayers [57] or simply increases the central temperature of the broadened transition [58]. Indeed, the mechanism of pressure reversal of anesthesia may be different for various anesthetics and may depend upon the lipid composition of the membrane. Since the differential-phase method can reveal the effects of pressure on the phase transition, this method may also clarify the effects of pressure on anesthetic-membrane interactions.

On a more fundamental level it is interesting to note that the effects of pressure on the conformation of the alkyl chains in membranes is opposite to that observed in simple alkane solvents. In the solvents increased pressures result in a higher percentage of *gauche* isomers [59,60]. Evidently, these isomers are of smaller volume relative to the *trans* isomers. In contrast, for membranes, increased pressure results in a higher percentage of the all-*trans* isomers of the alkyl side chains. Apparently, the minimum volume is obtained by a side-to-side arrangement of the all-*trans* isomers.

And, finally, it is worthwhile to note some of the advantages of combining high-pressure and fluorescence measurements for studies of biological macromolecules. The sensitivity and non-invasiveness of fluorescence techniques makes them useful for studies, even up to the Mbar range. More valuable is the well-known sensitivity of fluorescence to processes with rates comparable to the fluorescence decay rates. These include many processes of biological interest. When this ability to glean dynamic information is combined with the gentle, reversible perturbation produced by pressure, one has a useful tool for examining biological molecules. For instance, it has been pointed out by Heremans et al. [63] that one can fix the phase state of the membrane independent of temperature, which can be important for the study of membrane-bound proteins.

Note added in proof (Received June 14th, 1983)

While this manuscript was in press, Chong et al. [65] published the first differential phase measurements of DPH under pressure, studying its behavior in goldfish neural membranes. It is interesting to note that they also observed an increase in the rotational rate of DPH in membranes under pressure, but explained their result in terms of a slip-stick mechanism of DPH motion. The Appendix by Weber explains his birefringence correction for the differential phase angles in detail.

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