Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2082–2086, March 1990
Biophysics

Characterization of the transverse relaxation rates in lipid bilayers

(deuterium NMR/bilayer membrane/director fluctuations)

PAULA I. WATNICK, PHOEBE DEA*, AND SUNNEY I. CHAN†

Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125

Communicated by Harden M. McConnell, December 26, 1989

ABSTRACT **The ^{2}H NMR transverse relaxation rates of a deuterated phospholipid bilayer reflect slow motions in the bilayer membrane. A study of dimyristoyl lecithin specifically deuterated at several positions of the hydrocarbon chains indicates that these motions are cooperative and are confined to the hydrocarbon chains of the lipid bilayer. However, lipid head group interactions do play an important role in modulating the properties of the cooperative fluctuations of the hydrocarbon chains (director fluctuations), as evidenced by the effects of various lipid additives on the ^{2}H NMR transverse relaxation rates of the dimyristoyl lecithin bilayer.**

Many motional modes are accessible to the hydrocarbon chains of a lipid molecule in a bilayer membrane. Unimolecular motions such as bond vibration, bond rotation, and trans–gauche isomerizations occur at rates faster than 1×10^9 s^{-1} (1). In addition, evidence for motions involving more than one lipid molecule has been presented (2–5). The frequency of these motions decreases from 1×10^9 s^{-1} to 1×10^4 s^{-1} as more lipid molecules become involved in the motion. These motions may be visualized as fluctuations of the hydrocarbon chains in a direction perpendicular to the bilayer normal. If the amplitudes of such fluctuations are sufficiently large, concerted chain motion results. These collective motions are often referred to as director fluctuations.

An increase in the density of slow cooperative motions has been postulated upon the addition of proteins to a lipid bilayer (6–9). Long-range protein–protein interactions have been observed in lipid bilayers (10, 11). It has been proposed that a protein embedded in a bilayer membrane can communicate its presence to distant proteins by lipid–protein interactions. Since this lipid–protein interaction decays only gradually with distance from the lipid–protein interface, the spectral density of the director fluctuations can be modified dramatically. The addition of protein to a bilayer membrane also increases the permeability of the lipid bilayer to water, ions, and small water-soluble organic molecules (12). The disruption of cooperative motions in the bilayer may be responsible for this. Finally, the activity of membrane-bound transporters and enzymes is dependent on the lipid environment (13). In biological membranes, the coupling of cooperative motions to membrane-bound transporters or enzymes may enhance or inhibit the conformational fluctuations necessary to the function of these proteins.

^{2}H NMR relaxation measurements provide an ideal means of observing slow motions in lipid bilayers (14–16). ^{2}H is a quadrupolar nucleus, and the quadrupolar interaction dominates other spin interactions in the ^{2}H NMR relaxation expression. In addition, the lipid hydrocarbon chains may be deuterated at a single position allowing selective observation of a particular region of the bilayer.

It has been proposed that the spin–spin relaxation rate (T_2^{-1}) is sensitive both to cooperative motions in the lipid bilayer and to changes in these motions upon addition of a solute lipid or protein. After standard treatments for nematic liquid crystals, the following expression has been obtained for the contribution of director fluctuations to T_2^{-1} (17–19):

$$T_2^{-1}(\text{director fluctuations}) \propto \sin^2(2\beta) S_{CD}^2 \int_{-\infty}^{+\infty} \langle \delta\beta(0)\delta\beta(\tau)\rangle d\tau. \quad [1]$$

Here, β is the angle between the director and the external magnetic field, S_{CD} is the order parameter for the given deuteron, and $\langle \delta\beta(0)\delta\beta(\tau)\rangle$ is the correlation function describing the director fluctuations. Thus, if director fluctuations are present, Eq. 1 predicts a dependence of T_2^{-1} on S_{CD}^2 and β. Because liposomal bilayers are curved structures, however, the presence of significant lateral diffusion may complicate the angular dependence of T_2^{-1}. If the rate of lateral diffusion is comparable to T_2^{-1}, an average over β is necessary. The limits of this averaging may be expressed as $\beta \pm \Delta\beta$, where the magnitude of $\Delta\beta$ will depend both on the rate of lateral diffusion of the lipid molecules within each monolayer as well as the radius of curvature of the liposome. In the limit that lateral diffusion is complete over the time scale of T_2^{-1}, $\langle \sin^2(2\beta)\rangle = 16/30$, and the angular dependence of T_2^{-1} vanishes. However, when the rate of lateral diffusion is rapid compared to T_2^{-1}, the physics is modified (20), the effects of director fluctuations are masked, and no angular dependence of T_2^{-1} on β is expected.

In this paper, we use the angular dependence of T_2^{-1} and the dependence of T_2^{-1} on the order parameter to characterize the nature of slow motions and to determine the contribution of director fluctuations to T_2^{-1} in a dimyristoyl lecithin (DML) bilayer. The effects of distearoyl lecithin (DSL), myristic acid (MA), cholesterol, phytol, and chlorophyll a (Chl-a) on the T_2^{-1} of the DML bilayer are also determined. The structures of these lipids are shown in Fig. 1. These lipids were chosen to probe the relative propensity of the various regions of the bilayer towards cooperative distortions.

EXPERIMENTAL

Materials and Methods. DSL and perdeuterated DML ([$^2H_{54}$]DML) were purchased from Avanti Polar Lipids. Cholesterol and phytol were purchased from Sigma. [6,6-$^2H_2$]Myristic acid ([6,6-$^2H_2$]MA) was synthesized by MSD Isotopes.

DML deuterated at the 9 and 10 positions on both chains ([9,9′,10,10′-$^2H_4$]DML) was synthesized as described previously (4). [(2,2,2′,2′)-$^2H_4$]DML was synthesized by attaching MA deuterated at the 2 position to a glycerophosphocholine

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DML, dimyristoyl lecithin; DSL, distearoyl lecithin; MA, myristic acid; Chl-a, chlorophyll a; T_2^{-1}, spin–spin relaxation rate.

*Present address: Department of Chemistry and Biochemistry, California State University at Los Angeles, Los Angeles, CA 90032.
†To whom reprint requests should be addressed.

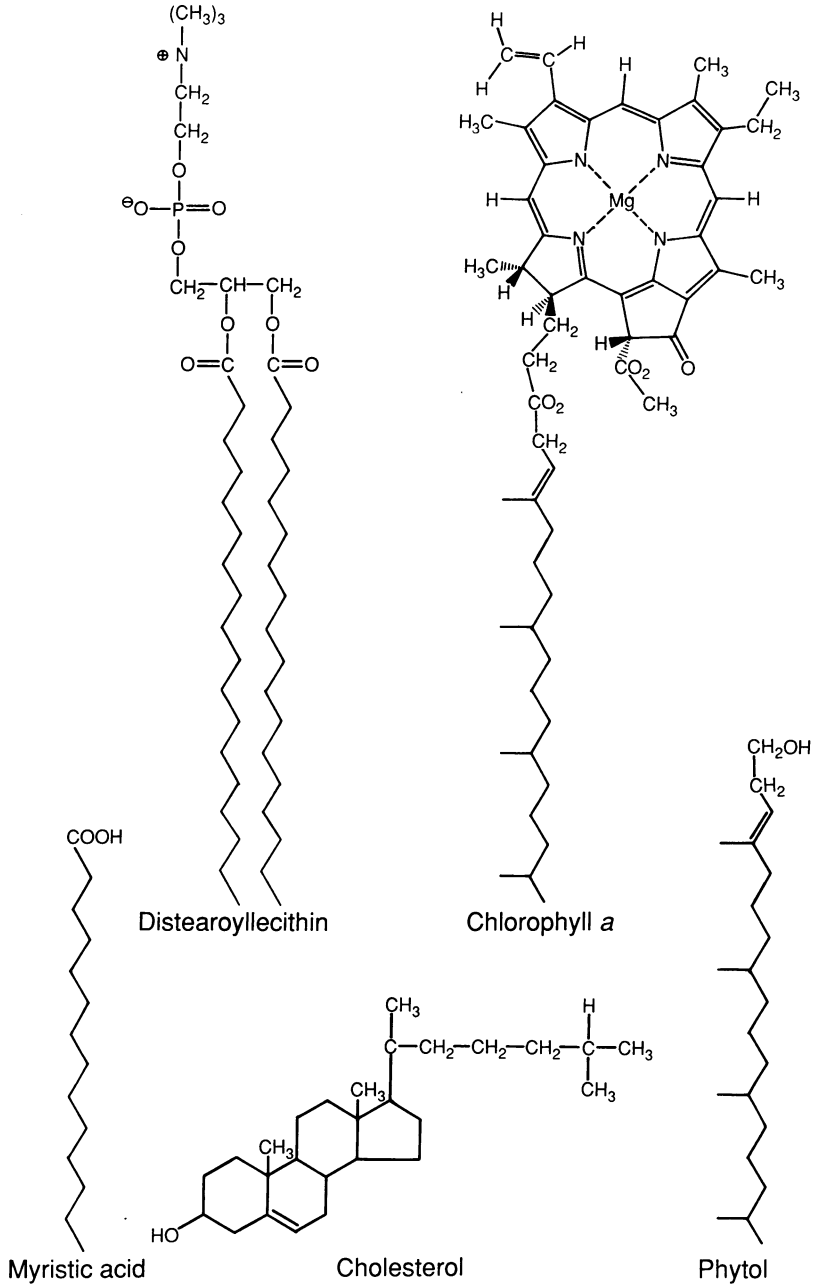

FIG. 1. Structures of DSL, Chl-a, MA, cholesterol, and phytol.

head group. The procedure of Regen *et al.* (21) was used. The MA used in the [2,2,2′,2′-$^2H_4$]DML synthesis was deuterated at the 2 position by the procedure of Aasen *et al.* (22).

Multilamellar dispersions were formed using the following protocol. DML powder or a mixture of lipids was dissolved in chloroform and then transferred to a 5-mm sample tube. Most of the chloroform was then evaporated under a stream of nitrogen. The last traces of the solvent were removed by drying the sample under high vacuum for at least 8 hr. The dried lipid films were hydrated with 25 mM Tris·HCl (pH 7.0). The sample tube was then sealed. The lipid systems were dispersed by alternately vortex mixing and warming to just above the gel-to-liquid crystalline phase transition temperature of the phospholipid. The vortex mixing was continued until a uniform suspension was formed (≈5 min).

Experiments using [2,2,2′,2′-$^2H_4$]DML were done within hours of hydration. This was necessary because deuterons at the two position are fairly acidic. Over a period of days, these deuterons exchange quite rapidly with protons in the aqueous environment.

All lipid mixtures contained less than 20% lipid by weight with respect to water. Experiments were performed at 303 K, with the exception of that involving the [9,9′,10,10′-$^2H_4$]DML sample containing 50 mol % DSL in lipid, which was run at 313 K. All samples were observed well above their gel-to-liquid crystalline phase transition temperatures.

Instrumentation. NMR relaxation experiments were conducted on a Bruker model WM 500 spectrometer. The transmitter pulse was attenuated to 1 V peak/peak and used to drive an Amplifier Research model 200L amplifier. The amplified pulse was fed into a home-built high-power deuterium probe. This arrangement provided a 3.5-μs 90° pulse.

The pulse sequence and data analysis programs used to determine the T_2 values of these lipid systems have been described (23).

RESULTS

A multilamellar dispersion of perdeuterated DML yields a powder-type spectrum with similar S_{CD} values for deuterons

at positions 3–10 along the lipid hydrocarbon chain (24). Accordingly, we have measured the dependence of T_2^{-1} on β (see Eq. 1) in a sample of [9,9′,10,10′-$^2H_4$]DML at several positions in the ^2H NMR spectrum. These results are summarized in Fig. 2, both as a function of β and resonant frequency. The angular dependence of T_2^{-1} showed a minimum at the 90° singularities and increased toward the center of the spectrum, following the trend predicted in Eq. 1. Because an explicit dependence of T_2^{-1} on β was observed, the spatial averaging of β associated with lateral diffusion on the timescale of T_2^{-1} must be small.

Deuterons undergoing cooperative motions at different positions on the same lipid chain are expected to experience the same director fluctuations. To determine the dependence of T_2^{-1} on S^2_{CD}, as predicted by the model of director fluctuations, T_2^{-1} values were measured at several resolvable singularities in the pure [$^2H_{54}$]DML system. A graph of T_2^{-1} versus S^2_{CD} for this system is shown in Fig. 3. The line drawn through the points was calculated using a linear least-squares fit program. The equation describing this line is

$$T_2^{-1} = (11{,}926 \pm 306)\text{s}^{-1} \cdot S^2_{CD} + (329 \pm 7)\text{s}^{-1}. \quad [2]$$

Thus, a good fit was obtained corresponding to a linear dependence of T_2^{-1} on S^2_{CD} for the various deuterons along the lipid chain. The contribution of motions other than director fluctuations—i.e., uncorrelated chain motions—to T_2^{-1} is given by the y intercept in Eq. 2.

The nature of the motions near the lipid head group can be gleaned from the relaxation rates observed in [2,2,2′,2′-$^2H_4$]DML. The one-dimensional spectrum of [2,2,2′,2′-$^2H_4$]DML is shown in Fig. 4. Each peak is labeled with its experimental T_2^{-1} value. The extreme orientation of the multilayers in the magnetic field in this sample allowed resolution of four signals corresponding to the four deuterium labels in this lipid. Previous NMR studies have shown (24) that the inner two powder patterns correspond to the lipid chain nearest to the head group, while the outer two correspond to the lipid chain farthest from the head group. The T_2^{-1} values of [2,2,2′,2′-$^2H_4$]DML are plotted against S^2_{CD} in Fig. 5. The line calculated for the perdeuterated system is included for comparison. The data fit the following straight line

$$T_2^{-1} = (2535 \pm 346)\text{s}^{-1} \cdot S^2_{CD} + (344 \pm 14)\text{s}^{-1}. \quad [3]$$

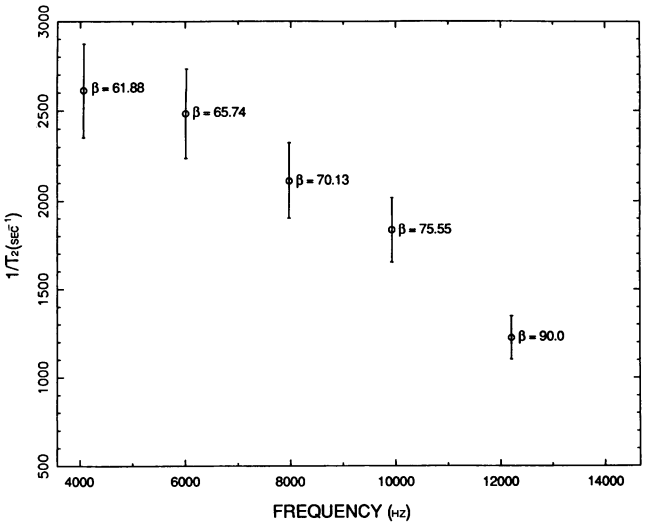

FIG. 2. Dependence of T_2^{-1} on the orientation of the [9,9′,10,10′-$^2H_4$]DML bilayer director with respect to the external magnetic field. β denotes the angle between the director and the external magnetic field.

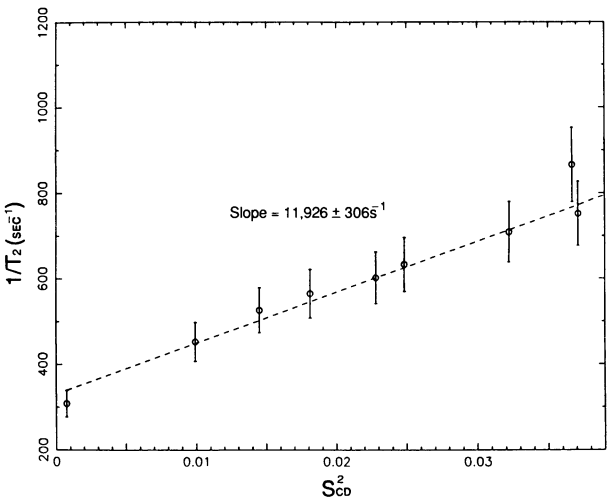

FIG. 3. T_2^{-1} versus S^2_{CD} for [$^2H_{54}$]DML multilayers. Data are fitted to a straight line with slope 11,926 ± 306 s^{-1} and y intercept 329 ± 7 s^{-1}.

The slope of this line is considerably smaller than that of the perdeuterated lipid. This suggests that the cooperative motions observed from C_3 to C_{10} of the lipid chain do not extend to the 2 position. Thus the C_2–C_3 bond must provide the anchor for the cooperative chain fluctuations. On the other hand, the intercepts in Eqs. 2 and 3 are essentially identical. Thus, as expected, noncooperative contributions to T_2^{-1} are similar at all chain positions.

The possible effects of added solute lipids on the cooperative motions in the DML bilayer have been investigated by comparing the T_2^{-1} (at 90°) of the pure [$^2H_{54}$]DML bilayer with the relaxation rates measured for [9,9′,10,10′-$^2H_4$]DML bilayers containing various concentrations of DSL, cholesterol, phytol, or Chl-a, as well as the T_2^{-1} of [6,6-$^2H_2$]MA in an unlabeled DML bilayer. This exercise has led to a classification of the experimental systems into two groups. Systems in group A exhibit a dependence of the relaxation rates versus S^2_{CD} identical to the pure DML system (Fig. 6*a*). These include 20 mol % [6,6-$^2H_2$]MA in DML (pH 7.4), [9,9′,10,10′-$^2H_4$]DML containing 30 mol % cholesterol, 8 and 23 mol % phytol, and 50 mol % DSL (313 K). Group B included various concentrations of Chl-a in [9,9′,10,10′-$^2H_4$]DML. The dependence of T_2^{-1} values versus S^2_{CD} values in these systems (Fig. 6*b*) does not follow Eq. 2. Certain structural features of the lipids appear to have large effects on the director motions in the bilayer.

DISCUSSION

Director Fluctuations in the Pure DML Bilayer. The model of director fluctuations predicts both an angular dependence of T_2^{-1} on β and a linear relationship of T_2^{-1} with S^2_{CD}. The theoretical angular dependence of T_2^{-1} is given by $\sin^2(2\beta)$. This function is zero at $\beta = 90°$ but rises steeply for smaller and larger values of β, peaking at 45° and 135°. Whereas the nonzero experimental value obtained for the director contribution to T_2^{-1} at $\beta = 90°$ indicates that motions such as lateral diffusion are occurring, $\Delta\beta$ need only be 20°–30° to give rise to the experimental value observed. The T_2^{-1} at the 90° orientation in the ^2H NMR spectrum of [9,9′,10,10′-$^2H_4$]-DML is around 1000 s^{-1} whereas the y intercept of the best line fit to the perdeuterated data is about 300 s^{-1}. Thus, two-thirds of the T_2^{-1} relaxation at $\beta = 90°$ is due to director motion. Our experimental results indicate that, indeed, director fluctuations are the dominant cause of T_2^{-1} relaxation in lipid bilayers and the time scales of these motions must span a range and extend to 10^{-4} s or longer.

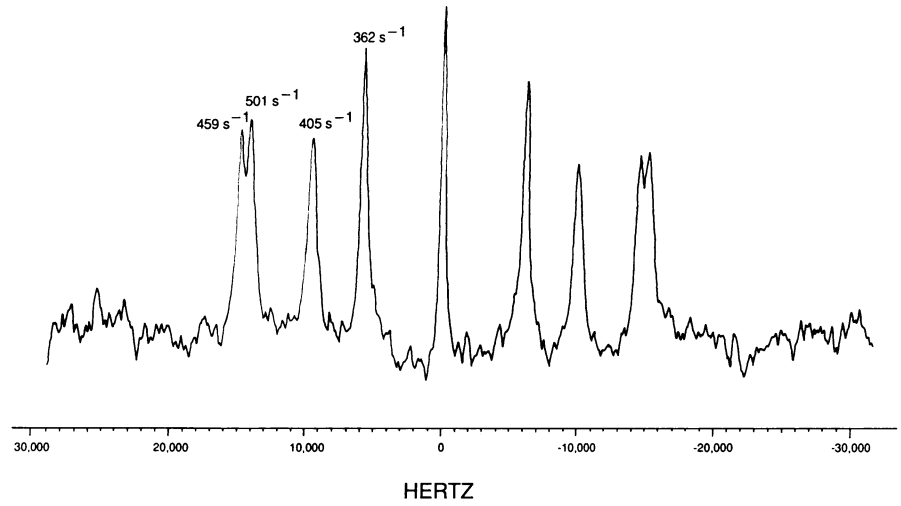

FIG. 4. One-dimensional ^{2}H NMR spectrum of [2,2,2',2'-$^2H_4$]DML multilayers. An oriented powder pattern is observed for each of the four deuterons. Each powder pattern is labeled with its corresponding T_2^{-1} at $\beta = 90°$.

A comparison of the T_2^{-1} versus S^2_{CD} plots for deuterons near the lipid headgroup with those for the lipid chain shows that the slope for the deuterons in [2,2,2',2'-$^2H_4$]DML is about 5 times smaller than that for the perdeuterated chain (Fig. 5). Since the slope is directly proportional to the correlation function for director fluctuations and reflects the density of slow cooperative motions in the bilayer, our results suggest that the C_2–C_3 bond of the acyl chains provide the anchor for the cooperative chain motions in the lipid bilayer.

Lipid Mixtures. In the plot of T_2^{-1} versus S^2_{CD} for the lipid systems classified under group A (Fig. 6*a*), most of the T_2^{-1} values fall near the line described by Eq. **2**. This suggests that the contribution of director fluctuations to T_2^{-1} in these mixed systems is similar to the contribution in the pure DML bilayer. For Chl-a in DML [i.e., systems in group B (Fig. 6*b*)], there is no correlation between the line of Eq. **2** and the T_2^{-1} of the mixed systems. The T_2^{-1} values of these systems increase abruptly with increasing Chl-a concentration without exerting a noticeable effect on S_{CD}. Addition of Chl-a must therefore increase the density of slow correlated motions in the bilayer.

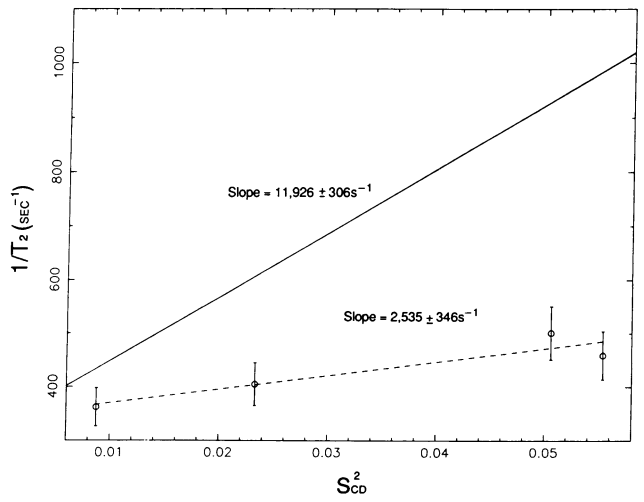

FIG. 5. Comparison of T_2^{-1} versus S^2_{CD} between [$^2H_{54}$]DML (solid line) and [2,2,2',2'-$^2H_4$]DML (dashed line) multilayers. The data for each system are fitted to a straight line. Although the *y* intercepts of these lines are comparable, the slope of the line for the [2,2,2',2'-$^2H_4$]DML system is about 20% that of [$^2H_{54}$]DML.

We now discuss these results in terms of the structures of the various lipids to identify certain features that are important in influencing the director fluctuations in a lipid bilayer.

Coexisting DML and DSL Domains. Differential scanning calorimetry and freeze–fracture electron microscopy experiments have shown DML and DSL to be only slightly miscible in the gel state and nonideally miscible in the liquid crystalline state (25–27). In these experiments, the system was studied at 313 K, a temperature below the gel-to-liquid crystalline phase-transition temperature of DSL but above the phase transition temperature of DML. Since this lipid mixture behaves as a group A system, one may conclude that the liquid crystalline domains have very little gel character, and the coupling between the liquid crystalline- and gel-state

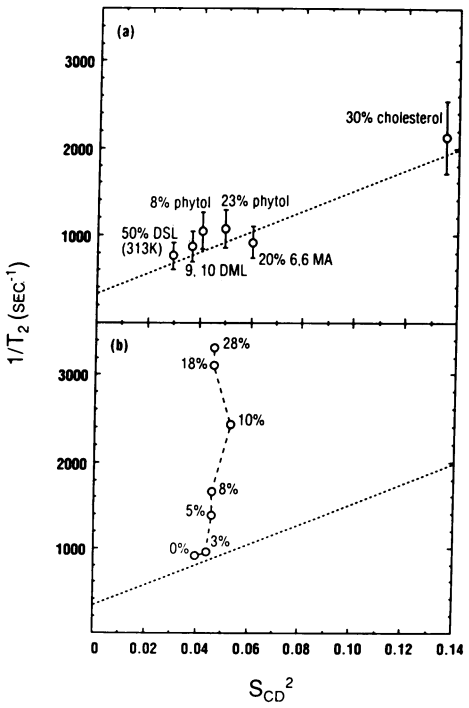

FIG. 6. Comparison of the plots of T_2^{-1} versus S^2_{CD} for lipids classified under group A (*a*), as identified, and the Chl-a/[9,9', 10,10'-$^2H_4$]DML system (*b*), each point identified with the stoichiometric Chl-a concentration. The best line for the [$^2H_{54}$]DML data is included for comparison.

domains must be weak. The rigid gel-state boundaries, therefore, do not interfere with motions in the liquid crystalline patches of DML, suggesting that the correlation length (i.e., the distances over which cooperativity extends) must be much smaller than the size of the liquid crystalline domains.

[6,6-$^2H_2$]MA, Phytol, and Cholesterol in DML. [6,6-$^2H_2$]MA, phytol, and cholesterol are three lipids with small neutral head groups. At pH 7.4, MA is a neutral fatty acid with a hydrocarbon chain identical to that of DML (28, 29). MA will contribute bulk to the hydrocarbon region of the bilayer, simulating the effect of close packing of lipid chains. The phytol head group consists of an alcohol functionality. A methyl group protrudes from every fourth carbon of the saturated hydrocarbon chain, differing somewhat from the nonbranched hydrocarbon chain of DML. An -OH functionality at the end of the cyclopentanophenanthrene ring constitutes the hydrophilic portion of cholesterol. The hydrocarbon portion of cholesterol includes three six-membered rings and one five-membered ring. These cyclic structures represent a significant departure from the linear saturated chains of DML.

Our T_2^{-1} results show that all three of these lipids exert very little effect on the cooperative motions in the DML bilayer. Significant differences between the hydrocarbon region of DML and that of an added lipid, therefore, have little effect on the cooperative motions in the bilayer. Furthermore, lipids with small head groups do not interfere with cooperative motions in the bilayer.

Phytol and Chl-a in DML. The Chl-a head group is composed of a porphyrin moiety with a central magnesium atom. Attached to the ring is a 17-carbon phytol chain with methyl groups protruding from every fourth carbon atom. Phytol has a hydrocarbon chain identical to that of Chl-a; however, it has only a small -OH head group.

A comparison of the effects of phytol and Chl-a on T_2^{-1} underscores the importance of the head group in bilayer motions. Bilayer motions are not perturbed by addition of the phytol chain. This contrasts strongly with the change in motion in the bilayer upon the addition of Chl-a. Because phytol and Chl-a differ only in the head-group region, one must conclude that head-group interactions play an important role in bilayer motion. In our earlier work (4), we proposed that the addition of Chl-a to the DML bilayer plasticizes the hydrocarbon region of the bilayer and disrupts the size of the cooperative domains.

CONCLUSIONS

The work described here reveals several characteristics of the motions that affect T_2^{-1} in DML bilayers. Both cooperative fluctuations and uncorrelated motions contribute to T_2^{-1}. In cooperative motions of the hydrocarbon chains, the head group and the C_2–C_3 bond of the acyl chain serve as an anchor about which these fluctuations occur. In mixed lipid/DML systems, lipids with small head groups do not perturb the cooperativity of director fluctuations in the DML bilayer; neither do lipids with very different hydrophobic moieties. Lipids with large head groups, however, interfere greatly with these cooperative motions.

We are indebted to M. Dea, H. Eckert, T. Handel, A. Nayeem, and J. Yesinowski for helpful discussions and assistance. This work was supported by Grants GM-22432 (to S.I.C.), GM-36132 (to P.D.), and RR-08101 (to P.D.) from the National Institutes of General Medical Sciences, U.S. Public Health Service; and Petroleum Research Fund Grant 20212-B4 (to P.D.) administered by the American Chemical Society. P.I.W. was a recipient of National Research Service Award T32 GM07616 from the National Institutes of General Medical Sciences. Access to the Southern California Regional NMR Facility at Caltech, supported by Grant CHE84-40137 from the National Science Foundation, for the NMR experiments is gratefully acknowledged. This is contribution 7974 from the Division of Chemistry and Chemical Engineering.

1. Peng, Z., Simplaceanu, V., Lowe, I. J. & Ho, C. (1988) *Biophys. J.* **54**, 81–95.
2. Brown, M. F. (1982) *J. Chem. Phys.* **77**, 1576–1599.
3. Cornell, B. A. & Pope, J. M. (1980) *Chem. Phys. Lipids* **27**, 151–164.
4. Watnick, P. I., Dea, P., Nayeem, A. & Chan, S. I. (1987) *J. Chem. Phys.* **86**, 5789–5800.
5. Rommel, E., Noack, F., Meier, P. & Kothe, G. (1988) *J. Phys. Chem.* **92**, 2987–2990.
6. Cornell, B. A., Sacre, M. M., Peel, W. E. & Chapman, D. (1978) *FEBS Lett.* **90**, 29–35.
7. Rice, D. & Oldfield, E. (1979) *Biochemistry* **18**, 3272–3279.
8. Cornell, B. A., Hiller, R. G., Raison, J., Separovic, F., Smith, R., Vary, J. C. & Morris, C. (1983) *Biochim. Biophys. Acta* **732**, 473–478.
9. Devaux, P. F., Hoatson, G. L., Favre, E., Fellmann, P., Farren, B., Mackay, A. L. & Bloom, M. (1986) *Biochemistry* **25**, 3804–3812.
10. Pearson, L. T., Chan, S. I., Lewis, B. A. & Engelman, D. M. (1983) *Biophys. J.* **43**, 167–174.
11. Pearson, L. T., Edelman, J. & Chan, S. I. (1984) *Biophys. J.* **45**, 863–871.
12. Cheifetz, S., Boggs, J. M. & Moscarello, M. A. (1985) *Biochemistry* **24**, 5170–5175.
13. Carruthers, A. & Melchior, D. L. (1984) *Biochemistry* **23**, 6901–6911.
14. Seelig, J., Tamm, L., Humel, L. & Fleischer, S. (1981) *Biochemistry* **20**, 3922–3932.
15. Siminovitch, O. J., Ruocco, M. J., Olejniczak, E. T., Das Gupta, S. K. & Griffin, R. G. (1985) *Chem. Phys. Lett.* **119**, 251–255.
16. Williams, G. D., Beach, J. M., Dodd, S. W. & Brown, M. F. (1985) *J. Am. Chem. Soc.* **107**, 6868–6873.
17. Doane, J. W. & Johnson, D. L. (1970) *Chem. Phys. Lett.* **6**, 291–295.
18. Doane, J. W., Tarr, C. E. & Nickerson, M. A. (1974) *Phys. Rev. Lett.* **33**, 620–624.
19. Pace, R. J. & Chan, S. I. (1982) *J. Chem. Phys.* **76**, 4228–4240.
20. Bloom, M. & Sternin, E. (1987) *Biochemistry* **26**, 2101–2105.
21. Regen, S., Yamagushi, K., Samuel, N. K. P. & Singh, M. (1983) *J. Am. Chem. Soc.* **105**, 6345–6356.
22. Aasen, A. J., Lauer, W. M. & Holman, R. T. (1970) *Lipids* **5**, 869–877.
23. Mueller, L. & Chan, S. I. (1983) *J. Chem. Phys.* **78**, 4341–4348.
24. Seelig, A. & Seelig, J. (1974) *Biochemistry* **13**, 4839–4845.
25. Mabrey, S. & Sturtevant, J. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3862–3866.
26. Van Dijck, P. W. M., Kaper, A. J., Oonk, H. A. J. & De Gier, J. (1977) *Biochim. Biophys. Acta* **470**, 58–69.
27. Luna, E. J. & McConnell, H. M. (1978) *Biochim. Biophys. Acta* **509**, 462–473.
28. Ptak, M., Egret-Charlier, M., Sanson, A. & Bouloussa, O. (1980) *Biochim. Biophys. Acta* **600**, 387–397.
29. Schullery, S. E., Seder, T. A., Weinstein, D. A. & Bryant, D. A. (1981) *Biochemistry* **20**, 6818–6824.