# Combined influence of cholesterol and synthetic amphiphillic peptides upon bilayer thickness in model membranes

Frank A. Nezil and Myer Bloom
Department of Physics, University of British Columbia, Vancouver, British Columbia, V6T-1W5, Canada

ABSTRACT Deuterium ($^2H$) NMR was used to study bilayer hydrophobic thickness and mechanical properties when cholesterol and/or synthetic amphiphillic polypeptides were added to deuterated POPC lipid bilayer membranes in the liquid-crystalline (fluid) phase. Smoothed acyl chain orientational order profiles were used to calculate bilayer hydrophobic thickness. Addition of 30 mol% cholesterol to POPC at 25°C increased the bilayer thickness from 2.58 to 2.99 nm. The peptides were chosen to span the bilayers with more or less mismatch between the hydrophobic peptide length and membrane hydrophobic thickness. The average thickness of the pure lipid bilayers was significantly perturbed upon addition of peptide only in cases of large mismatch, being increased (decreased) when the peptide hydrophobic length was greater (less) than that of the pure bilayer, consistent with the "mattress" model of protein lipid interactions (Mouritsen, O.G., and M. Bloom. 1984. *Biophys. J.* 46:141–153). The experimental results were also used to examine the combined influence of the polypeptides and cholesterol on the orientational order profile and thickness expansivity of the membranes. A detailed model for the spatial distribution of POPC and cholesterol molecules in the bilayers was proposed to reconcile the general features of these measurements with micromechanical measurements of area expansivity in closely related systems. Experiments to test the model were proposed.

## INTRODUCTION

Orientational order parameters of carbon-deuterium (CD) bonds in the hydrophobic milieu of model membrane lipids are important as they reflect the acyl chain ordering and disordering which dominates the thermodynamics of the gel-to-liquid crystalline (main) phase transition (Chapman, 1975). Deuterium nuclear magnetic resonance ($^2H$-NMR) has been used to determine the variation of the magnitude of the acyl chain orientational order parameters $|S_{CD}(n)|$ with carbonyl position $n$, often called the acyl chain "order profile" (Seelig and Seelig, 1977, 1980; Mantsch et al., 1977). It is well known that $|S_{CD}(n)|$ is sensitive to changes in cholesterol concentration, temperature, and headgroup substitution. A more recent empirical observation (Lafleur et al., 1990) is that, as each of these parameters is varied, the shape of the order profile changes in a manner that depends only on a single independent parameter, e.g., the average value of $|S_{CD}(n)|$ over the acyl chain, and not on which of the three perturbations is used to change $|S_{CD}(n)|$.

For an acyl chain containing $N$ carbon atoms,

$$\langle|S_{CD}|\rangle = \frac{1}{N-1}\sum_{2}^{N}|S_{CD}(n)|. \quad (1)$$

A semi-empirical relationship has been found to exist for fluid membranes between $\langle|S_{CD}|\rangle$, the bilayer hydrophobic thickness $d$, and its maximum thickness when the acyl chains are in the all-trans configuration, $d_0$:

$$d = d_0(\alpha\langle|S_{CD}|\rangle + \beta), \quad (2)$$

where the constants $\alpha$ and $\beta$ satisfy the constraint $\frac{1}{2}\alpha + \beta = 1$ (Ipsen et al., 1990). The fact that Eq. 2 is consistent with available measurements of $\langle|S_{CD}|\rangle$ using $^2H$-NMR, and $d$ using x-ray diffraction, suggests that $^2H$-NMR may be used to determine mechanical properties of biomembranes (Bloom et al., 1991). In this context, the observation that naturally occurring integral membrane proteins in fluid bilayers do not perturb $\langle|S_{CD}|\rangle$ has been interpreted theoretically, using the mattress model of protein-lipid interactions (Mouritsen and Bloom, 1984), as being due to the matching of hydrophobic regions of lipids and proteins via evolutionary processes in order to produce strain-free bilayers (Bloom and Mouritsen, 1988).

In this paper, we present the results of some experiments which test the concepts described above. In particular, we compare the hydrophobic matching of pure POPC bilayers (referred to as $B_1$), and bilayers containing 30 mol% cholesterol ($B_2$), to two $\alpha$-helical integral membrane peptides of different hydrophobic lengths: Lys$_2$-Gly-Leu$_m$-Lys$_2$-Ala-amide, with $m = 16$ or 24 (Davis et al., 1983, Huschilt et al., 1985), to be called $P_{16}$ and $P_{24}$ respectively. The monounsaturated POPC is representative of the dominant lipids in the plasma

0006-3495/92/05/1176/08 $2.00

membranes of eucaryotic cells having high cholesterol concentrations (Alberts et al., 1989; Bloom and Mouritsen, 1984; Bloom et al., 1991).

Using the relationship between hydrophobic bilayer thickness and orientational order expressed in Eq. 2, we shall show that the shorter peptide $P_{16}$ is well matched to the thin bilayer $B_1$ but too short for the "thick" bilayer $B_2$, while the longer peptide $P_{24}$ is well matched to $B_2$ but too long for $B_1$; these results are as anticipated by the mattress model. However, a more detailed analysis of the order profile and the temperature dependence of $\langle|S_{CD}|\rangle$ indicates subtleties in the spatial correlations of the lipids, peptides, and sterols in these membranes. A model will be proposed which addresses some of these issues. Further measurements of the type reported here, combined with x-ray, neutron diffraction and micromechanical measurements, will be required to provide a more detailed picture of the manner in which lipids, sterols, and proteins (or peptides) are organized in membranes.

## EXPERIMENTAL PROCEDURES

### Materials

Perdeuteriated palmitic acid was prepared by standard methods in our laboratory. Avanti Polar Lipids (Avanti Polar Lipids, Inc., Birmingham, AL) used this material to produce the single-chain perdeuteriated 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC-$d_{31}$) used here. Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). The amphiphilic cationic peptides $P_{16}$ and $P_{24}$ were synthesized by previously published methods (Davis et al., 1983).

### Sample preparation

The sample preparation procedure was a slightly modified version of already published methods (Huschilt et al., 1985). The modification simply involved measuring membrane components using dry weights rather than by titration and, after addition of the buffer (800 μl of 50 mM Hepes, 100 mM NaCl, and pH = 7.4, using deuterium depleted water from Sigma). The sample then underwent four cycles of freezing in liquid nitrogen followed by warming to room temperature and the samples were then centrifuged at 90,000 rpm at 25°C for 1 h and the pellet was transfered to an NMR tube. The amount of POPC-$d_{31}$ used was typically ~30 mg. The POPC-$d_{31}$:Cholesterol samples contained 30 mol% cholesterol and peptide-lipid samples contained 3.5 mol% peptide. The POPC-$d_{31}$ samples contained 4.8 mol% peptide.

### Nuclear magnetic resonance

$^2$H-NMR experiments were performed on a home-built spectrometer operating at 46 MHz for deuterons (Sternin, 1985). A quadrupolar echo sequence was used: $90_x$-$\tau$-$90_y$-$\tau$-echo, with $\tau$ = 60 μs and a repeat time of 150 ms. The 90° pulse length was 4.0 μs. Spectra were recorded with a 2 μs dwell time and at least 50,000 transients collected for signal averaging. Signals were detected in quadrature with phase cycling (Davis, 1979; Rance and Byrd, 1983), and temperature was controlled using a Bruker model BV-T1000 temperature controller (Bruker Instruments, Inc., Billerica, MA).

## RESULTS

Fig. 1 presents the $^2$H-NMR spectra and their depaked forms (Sternin et al., 1983), obtained at 25°C for the various cases of peptide-lipid hydrophobic matching and mismatching in the POPC-$d_{31}$ ($B_1$) and POPC-$d_{31}$: cholesterol ($B_2$) samples. Comparing the unperturbed bilayers (i.e., no peptide) we observe the familiar dramatic increase in the quadrupolar splittings which occurs with the addition of cholesterol to a pure phospholipid system. We also note that addition of the (relatively) well-matched peptide to each of $B_1$ and $B_2$ (i.e., samples $B_1P_{16}$ and $B_2P_{24}$, respectively) has relatively little effect on the spectrum compared to the cases of mismatch ($B_1P_{24}$ and $B_2P_{16}$). For the latter cases we see that adding $P_{16}$ decreases the quadrupolar splittings of $B_2$ whereas adding $P_{24}$ results in increased splittings for $B_1$. The small isotropic peak in Fig. 1 *c* is insignificant.

The comments of the last paragraph also apply to the depaked spectra of Fig. 1, however, the spectral differences of interest are more pronounced. We use these depaked spectra to calculate the smoothed order profiles plotted in Fig. 2. This figure demonstrates that

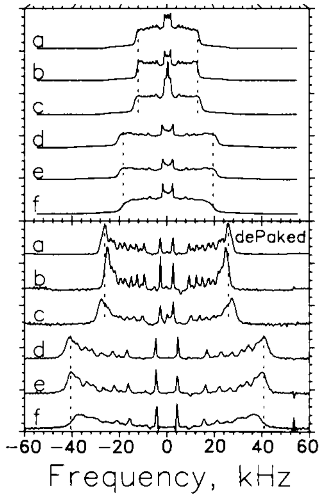

FIGURE 1 $^2$H-NMR spectra (above), and depaked spectra (*below*), from quadrupolar echo experiments with $\tau$ = 60 μs at 25°C for various cases of hydrophobic match/mismatch. The upper three spectra in each case show bilayer $B_1$ (POPC-$d_{31}$) with no peptide added (*a*); with 6.3 mol% $P_{16}$ (*b*); with 4.8 mol% $P_{24}$ (*c*). The lower three spectra in each case are for bilayer $B_2$ (POPC-$d_{31}$:cholesterol 7:3 M:M) with no peptide added (*c*); with 3.5 mol% peptide $P_{24}$ (*d*); and with 3.5 mol% peptide $P_{16}$ (*d*). The vertical dashed lines are for visual aid only.

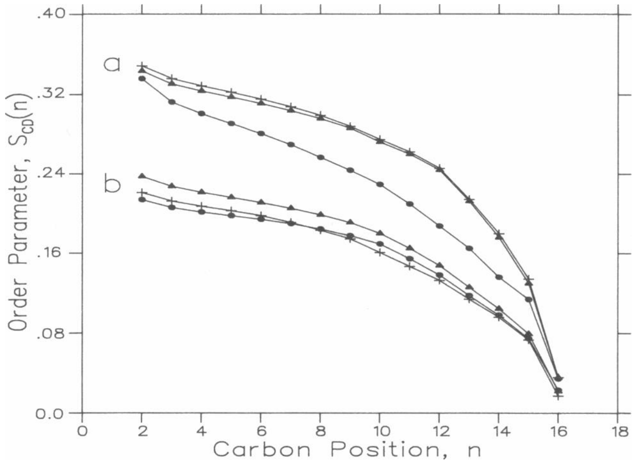

FIGURE 2 Smoothed acyl chain orientational order profiles calculated from the depaked spectra of Fig. 1. Parts *a* (bilayer $B_2$) and *b* (bilayer $B_1$) each display three order profiles corresponding to addition of peptide: no peptide (+); peptide $P_{16}$ (○); and peptide $P_{24}$ (△).

mismatch between hydrophobic lengths $d_p$ and $d_l$, for the peptides and lipids respectively, is correlated with a systematic increase (for $d_p > d_l$) or decrease (for $d_p < d_l$) in $|S_{CD}(n)|$. Using Eq. 2, a corresponding increase or decrease in the lipid-peptide bilayer hydrophobic length, $d_{lp}$, is obtained. There is relatively little change in $|S_{CD}(n)|$ upon addition of a matching peptide ($d_p \approx d_l$).

Similar order profiles were obtained at several temperatures for samples $B_1$ and $B_2$, and for the corresponding cases of greatest mismatch: $B_1P_{24}$ and $B_2P_{16}$, respectively. The thickness in these cases, as calculated from Eq. 2, are plotted as a function of temperature in Fig. 3. The solid lines are linear least squares fits to the data with the following slopes (in units of Å/°C): $-0.05 \pm 0.01$ for $B_1$; $-0.06 \pm 0.01$ for $B_1P_{24}$; $-0.11 \pm 0.01$ for $B_2$; and $-0.10 \pm 0.01$ for $B_2P_{16}$.

## DISCUSSION

### $^2$H NMR time and distance scales

It is well known that any spectroscopic technique has some intrinsic time scale $\tau_S$ associated with it. In the case of $^2$H NMR in fluid membranes, $\tau_S \geq 10^{-4}$ s (Devaux, 1983; Seelig and Seelig, 1980; Bloom and Smith, 1985), which is approximately equal to the inverse of the spectral width. Because of the relatively large lateral diffusion constant in fluid membranes (typically, $D \approx 4 \times 10^{-12}$ m$^2$s$^{-1}$), the distance diffused by molecules during $\tau_S$, $L_d \approx (4D\tau_S)^{1/2} \approx 400$ Å, is quite large and represents an intrinsic distance scale for $^2$H NMR in fluid membranes (Bloom et al., 1991). For this reason, the spectra described below should be interpreted as averages over domains having a size greater than several hundred Angstroms, which is more than ten times greater than the average separation of neighboring peptide molecules. Thus, the $^2$H NMR spectra only provide information on the average change in bilayer hydrophobic thickness, due to the peptide-lipid interaction, and not on the correlation length of the peptide-induced perturbation of the bilayer chacteristics. We will not discuss here the information on correlation length provided by peptide-induced orientation-dependent spin–spin relaxation effects. We have shown elsewhere (Nezil et al., 1991) that such effects do not modify significantly the interpretation given below of the spectral changes induced by the presence of the peptide molecules.

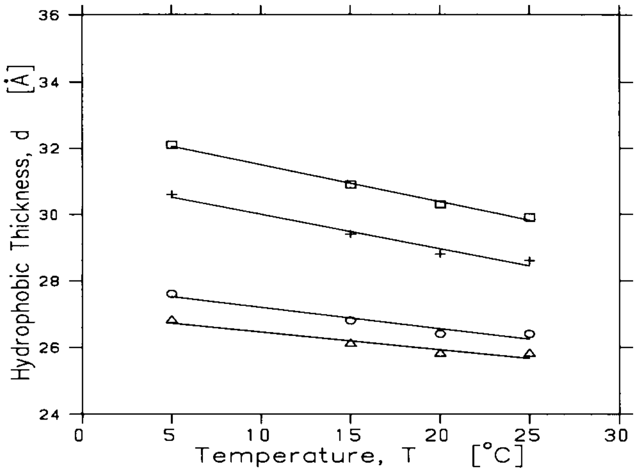

FIGURE 3 Bilayer hydrophobic thickness versus temperature for samples $B_1$ (△), $B_1P_{24}$ (○), $B_2$ (□), and $B_2P_{16}$ (+); and linear least squares fits to this data.

### Orientational order and bilayer thickness

The calculated bilayer hydrophobic thickness (see Eq. 2) for each of the smoothed order profiles of Fig. 2 is listed in Table 1. There is good agreement for the calculated

TABLE 1 **Bilayer hydrophobic thickness***

| Bilayer type | Calculated‡ thickness, Å | | |
|---|---|---|---|
| | no peptide | $P_1$ | $P_2$ |
| $B_1$ (POPC-$d_{31}$) | 25.8 | 25.8 | 26.4 |
| $B_2$ (POPC-$d_{31}$:Chol.) | 29.9 | 28.6 | 29.8 |

*At temperature $T = 25$°C. ‡Eq. 2 has been used to calculate $d$ with $d_0 = 39.4$ Å, as deduced by Marčelja for DPPC (Marčelja, 1974).

thickness of pure POPC-$d_{31}$, 25.8 Å, with the published value of 26 Å for (buffered) DPPC in the $L_\alpha$-phase (Lewis and Engelman, 1983). Some information on electron density profiles for egg PC-cholesterol mixtures is available from x-ray diffraction experiments (McIntosh et al., 1989). These samples are similar to POPC-cholesterol mixtures because most of the egg PC molecules also consist of a single, monounsaturated acyl chain. The x-ray diffraction measurements on a 33 mol% cholesterol mixture at 21°C gives a headgroup separation of 40 Å. This is in agreement with our measurement of a bilayer thickness of 29.9 Å for 30 mol% cholesterol at 25°C (see Table 1) when allowing a reasonable thickness of 5 Å for the headgroup region. Encouraged by these correlations, we examine the implications of our NMR measurements in the rest of this paper under the assumption that Eq. 2 is valid for such mixtures. It would, of course, be desirable to check the connection between Eq. 2 and x-ray diffraction experiments on the same material.

An important consequence of Eq. 2 is that the well established increase in $\langle|S_{CD}|\rangle$ upon addition of cholesterol means that cholesterol appreciably increases the $L_\alpha$ phase average bilayer hydrophobic thickness. Table 1 lists this increase as 4.1 Å for addition of 30 mol% cholesterol to a POPC bilayer at 25°C.

The peptides used here have a well defined, rigid, α-helical structure with hydrophobic lengths of $d_p \approx 24$ Å for $P_1$ and $d_p \approx 36$ Å for $P_2$ (Davis et al., 1983; Huschilt et al., 1985). Comparing $d_p$ with the unperturbed thicknesses, $d_l$, of the peptide-free bilayers in Table 1, we see that, in all cases, $d_p \neq d_l$, i.e., only different degrees of mismatching are considered here. In general, the addition of peptides with $d_l > d_p$ ($d_l < d_p$) results in a decreased (increased) average thickness of the bilayer, as expected. The magnitude of mismatch is not exactly correlated with the resultant change in thickness: for the case of greatest mismatch, $d_p - d_l \approx$ 10 Å for $P_{24}$ in $B_1$, the change in thickness is +0.6 Å; yet, for the lesser degree of mismatch $d_l - d_p \approx 6$ Å for $P_{16}$ in $B_2$, the thickness change is −1.3 Å. Thus, the perturbation due to mismatch is not symmetrical with respect to the sign of the mismatch: the "short" peptide in the "thick" bilayer is more effective than the "long" peptide in the "thin" bilayer in changing the bilayer thickness. In view of the observations in earlier studies (Davis et al., 1983) that these peptides are fully incorporated into lipid bilayers, we believe that the origin of this asymmetry is to be found in the physical aspects of the lipid-peptide interaction and not in any problem associated with peptide solubility.

The results given here for peptide $P_{24}$ in bilayer $B_1$ are similar to those obtained in previous studies on peptides in phospholipid or potassium-palmitate bilayers (Huschilt et al., 1985), but our study of peptides in $B_2$ represents the first results on matching in membranes containing large amounts of cholesterol.

The approximate matching of $P_{16}$ to $B_1$ and $P_{24}$ to $B_2$, and mismatch of $P_{16}$ to $B_2$ and $P_{24}$ to $B_1$, is in qualitative agreement with the predictions of the mattress model (Bloom and Mouritsen, 1984). To interpret our results quantitatively other considerations become important including thermally excited thickness fluctuations of the bilayers (see, e.g., Brochard and Lennon, 1975; Bloom et al., 1991) and the flexible coupling of the positively charged lysines (at neutral pH) to the peptide backbone via four methylene groups. It seems to us that the main features of the lipid-peptide interaction, including the lack of symmetry associated with mismatch, are likely to be interpretable in terms of reasonable physical models in which the physical considerations described above are included. Along these lines, the lack of symmetry with respect to the sign of the mismatch can probably be explained in terms of the flexible coupling of the positive charges of the lysines. For example, the four methylene groups of $P_{16}$ in the $B_2P_{16}$ membrane are probably totally extended with the $P_{16}$ peptide oriented parallel to the bilayer normal in order that the charges reach the water region; this is to be compared with the $B_1P_{24}$ membrane where the $P_{24}$ peptide, being too long for the thickness of $B_1$, can tilt relative to the surface normal.

## Unusual features of the results

There are two main features of our results which are unusual and indicative of subtleties in the arrangement ("packing") of the POPC, cholesterol, and peptide molecules in the bilayers and that will require further experimental investigation to clarify.

The first feature concerns the shape of the order profiles, $|S_{CD}(n)|$ versus $n$: the three-component bilayers differ markedly from either the one-component (POPC) or two-component (POPC:cholesterol and POPC: peptide) bilayer systems studied here. This is seen from Figs. 2 and 4. Whereas the change in $|S_{CD}(n)|$ upon addition of cholesterol to the pure POPC bilayer monotonically decreases for increasing $n$ (a similar result applies to temperature changes [Lafleur et al., 1990]), the corresponding change in $|S_{CD}(n)|$ upon addition of $P_{16}$ to $B_2$ goes through a maximum near $n = 12$. These features are especially evident in Fig. 4 which is based on measurements of the order parameter, $|S_{CD}(n, T)|$, at several temperatures between 5°C and 25°C. In Fig. 4, we have plotted the variation with chain position $n$ of the "difference order parameter" $\Delta S^{\gamma,\delta}(n, T)$:

$$\Delta S^{\gamma,\delta}(n, T) = |S^{\gamma}_{CD}(n,T)| - |S^{\delta}_{CD}(n, T)|, \qquad (3)$$

where the superscripts γ, δ specify the two different

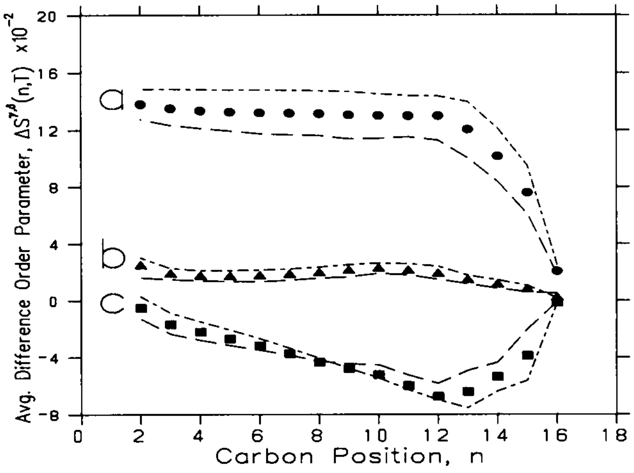

FIGURE 4 The difference order parameter, $\Delta S^{\gamma,\delta}(n, T)$ (see text) has been averaged over the temperature range 5–25°C for various cases: $\gamma = B_2$, $\delta = B_1$ (a); $\gamma = B_1P_{24}$, $\delta = B_1$ (*b*); $\gamma = B_2P_{16}$, $\delta = B_2$ (*c*). The long-dashed lines represent $\Delta S^{\gamma,\delta}(n, T)$ at temperature $T = 25°C$ while the other dashed lines are for $T = 5°C$.

samples for which the difference in order is being demonstrated. The dashed lines in Fig. 4 represent the difference order profiles at the highest (25°C) and lowest (5°C) temperatures used while the solid symbols represent the average difference order profile over this temperature range. It is perhaps worth noting that the $n = 12$ position on the acyl chain roughly corresponds to the methylene position to which cholesterol is predicted to penetrate into the bilayer (based upon the all-*trans* configuration for the acyl chains near the cholesterol). The data in Fig. 4 suggest that the change in the equilibrium thickness upon perturbation of the thick bilayer by the short peptide is correlated with an increasing acyl chain disorder. The change is maximal in the region near the hydrophobic termination of the cholesterol molecules. The presence of cholesterol and the short peptide in a phospholipid mixture together represent two competing forces effecting the thickness of the bilayer: the results of Fig. 4 suggest that the influence of the peptides in making the membrane thinner cannot be pictured simply as a cancellation of the thickening effect of the cholesterol molecules.

The second feature that requires interpretation is that the expansivity of the bilayer hydrophobic thickness, i.e., $\dot{\alpha}_d = (1/d)(\partial d/\partial T)$, is increased (in magnitude) by a factor of approximately two (see Fig. 3) upon addition of cholesterol, independent of peptide inclusion. The values of $|\dot{\alpha}_d|$ for the four samples are given: $2.1 \times 10^{-3}$ °C$^{-1}$ ($B_1$); $2.4 \times 10^{-3}$ °C$^{-1}$ ($B_1P_{24}$); $3.6 \times 10^{-3}$ °C$^{-1}$ ($B_2$); and $4.1 \times 10^{-3}$ °C$^{-1}$ ($B_2P_{16}$). (Note that $\dot{\alpha}_d < 0$). For example, in Table 1 of (Needham et al., 1988), it is seen that for DMPC at 35°C, $\dot{\alpha}_A = (4.2 \pm 0.2) \times 10^{-3}$ °C$^{-1}$, while a sample of DMPC:cholesterol containing 40 mol% cholesterol gives $\dot{\alpha}_A = 2.3 \times 10^{-3}$ °C$^{-1}$ at 35°C. This is to be compared with the results for the area expansivity $\dot{\alpha}_A = (1/A)(\partial A/\partial T)$ which, according to micromechanical measurements (Needham et al., 1988), is always substantially decreased upon the addition of cholesterol. This is difficult to reconcile because the volume expansivity $\dot{\alpha}_V = (1/V)(\partial V/\partial T)$ is usually expected to be extremely small in relation to the area expansivity, i.e., making the identification $V = Ad$, and $\dot{\alpha}_V \approx 0$ these simple considerations imply $\dot{\alpha}_A \approx -\dot{\alpha}_d$. This is in contradiction with our results and the results of (Needham et al., 1988) which taken together show that upon addition of cholesterol the change in $|\dot{\alpha}_d|$ is increased while the change in $|\dot{\alpha}_A|$ is decreased.

To interpret these unusual features of our experimental results, we propose a simple, but speculative, model for characterizing the spatial distribution of phospholipid and cholesterol molecules in bilayer membranes composed of such lipids. We then describe some experiments that are capable of testing the model. We wish to emphasize that we are not yet able to compare the actual magnitudes of $\dot{\alpha}_d$ and $\dot{\alpha}_A$ because we do not have any micromechanical measurements of $\dot{\alpha}_A$ in the POPC and POPC:cholesterol samples used here for the $\dot{\alpha}_d$ measurements. Rather, we are trying to deal with the comparison of the changes induced in $|\dot{\alpha}_A|$ and $|\dot{\alpha}_d|$ by cholesterol, i.e., the observation that $|\dot{\alpha}_d|$ increases while $|\dot{\alpha}_A|$ decreases upon the addition of cholesterol. We believe that this represents a real anomaly that must be explained in terms of changes in the geometrical distribution of cholesterol in the lipid bilayer as the temperature is varied.

## Molecular packing considerations

It is clear that some drastic over-simplification of molecular packing has been made in relating the membrane volume V to the product of membrane thickness *d*, as determined from $\langle |S_{CD}| \rangle$ measurements using Eq. 2, with membrane area *A*, as obtained from micromechanical measurements (Needham et al., 1988). Although *A* is unambiguously the sum of the projections of the areas of all the phospholipid and cholesterol molecules on the membrane surface, the quantity *d* is an effective phospholipid hydrophobic thickness (a distance approximately equal to the average separation of the glycerol backbones of the phospholipid molecules of the two leaflets of the membranes). In writing $V = Ad$, it is implicitly assumed that the cholesterol molecule maintains a constant depth in the membrane relative to the glycerol backbone of the phospholipid molecule as the temperature and peptide concentration are varied. Another questionable assumption, implicit in neglecting changes in $\dot{\alpha}_V$ relative to changes in $\dot{\alpha}_A$ and $\dot{\alpha}_d$, is that there are no temperature-dependent packing problems associated with the different shapes of the phospholipid and cholesterol molecules. Also, the micromechanical measurements used to determine $\dot{\alpha}_A$ are performed on unilamellar vesicles under tension whereas the measurements of $\dot{\alpha}_d$ are performed on multilamellar vessicles in their relaxed state.

A central feature of the model we now propose is that the average position of the cholesterol molecules relative to the phospholipid-water interface is allowed to vary with temperature. With this model, we are able to interpret qualitatively the observation that $\dot{\alpha}_A + \dot{\alpha}_d \neq 0$ in phospholipid-cholesterol mixtures in terms of a nonnegligible volume expansivity $\dot{\alpha}_V$ and the variation with temperature of the small fraction of the cholesterol that is on average transferred outside the bilayer. It also has the potential to explain the change in shape of the order profile, although we have not addressed this problem quantitatively.

## Membrane model for lipid-cholesterol mixtures

Suppose that the membrane is composed of $N_L$ phospholipid and $N_C$ cholesterol molecules of average cross-sectional areas $A_L$ and $A_C$, and volumes $V_L$ and $V_C$, respectively. Then, if a fraction $f$ of each cholesterol molecule protrudes, on average, beyond a lipid-fixed reference point (e.g., such as the position of the glycerol backbone within the membrane) into the aqueous region (see Fig. 5), we may write the following equations for the total area $A$ and total volume $V$ of the membrane in terms of the above quantities and the apparent phospholipid thickness $d$ which we assume may be related to the $^2$H-NMR measurements of orientational order via Eq. 2,

$$A = N_L A_L + N_C A_C, \tag{4}$$

and

$$V = Ad + fN_C V_C. \tag{5}$$

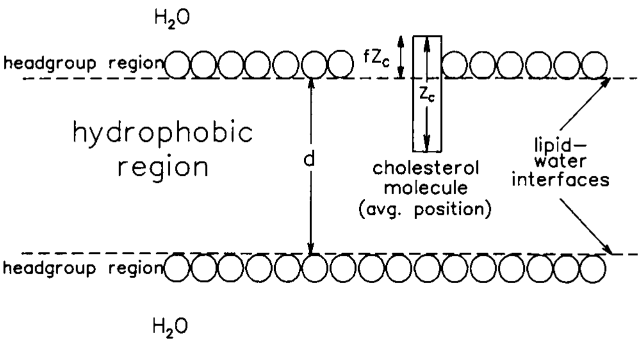

FIGURE 5 Diagram showing a cholesterol molecule in a phospholipid bilayer used for proposed theoretical model (see text). The cholesterol is depicted in its average position with respect to the lipid water interface. $Z_C$ is the length of the cholesterol molecule having area $A_C$; $f$ is the fraction of the cholesterol molecule protruding from the hydrophobic interior; and $d$ is the hydrophobic thickness of the bilayer.

It is then easy to show that the assumption of small protruding cholesterol volume ($fN_C V_C \ll V$) gives

$$\frac{df}{dT} \approx \left(\frac{N_L V_L + N_C V_C}{N_C V_C}\right)(\dot{\alpha}_V - \dot{\alpha}_A - \dot{\alpha}_d). \tag{6}$$

A very rough estimate of $df/dT$ may be made for $|\dot{\alpha}_V| \ll |\dot{\alpha}_A + \dot{\alpha}_d|$ from our measured values of $\dot{\alpha}_d$ and representative measurements of $\dot{\alpha}_A$ from micromechanical measurements (Needham et al., 1988). The experimental observation that the changes in expansivity in going from $B_1$ to $B_2$ corresponds to $|\Delta\dot{\alpha}_A| < |\Delta\dot{\alpha}_d|$ yields (for $N_L/N_C = 7/3$, $V_C/V_L \approx 0.6$ and $\dot{\alpha}_A + \dot{\alpha}_d \approx -10^{-3}/°C$) $df/dT \approx +0.5\%°C^{-1}$. This small displacement of the cholesterol molecules relative to the phospholipids, corresponding to ~0.07 Å/°C should be detectable with specially designed neutron diffraction experiments, as described below. We note that the increased protrusion of cholesterol molecules from the membrane surface with increasing temperature can be interpreted in a natural way as a consequence of the increase in lateral pressure on cholesterol from the neighboring phospholipid molecules as the temperature is increased.

The experimental observations could also be understood, in principle, if $df/dT = 0$ providing that the excess volume associated with the packing of mixtures of phospholipid and cholesterol molecules changes with temperature such that $\dot{\alpha}_V \approx \dot{\alpha}_A + \dot{\alpha}_d \approx -10^{-3}/°C$. It seems unlikely that $\dot{\alpha}_V < 0$ so that the interpretation in terms of a nonzero value of $df/dT$ is favored.

The model described above draws attention to the desirability of correlating the results of NMR experiments with those of micromechanical experiments such as those developed by Evans and his collaborators (Bloom et al., 1991; Needham et al., 1988). In relation to the studies reported here, micromechanical experiments on POPC:cholesterol mixtures are planned but have not yet been carried out. Other measurements, such as x-ray diffraction to determine the electron density variation in the bilayer, will also be needed to test the model just described. Especially useful would be neutron diffraction experiments similar to those previously carried out to determine the spatial distribution of different segments of the acyl chains and polar headgroups of phospholipid molecules in bilayer membranes (Büldt et al., 1979; Zaccai et al., 1979). These experiments exploit contrast methods using partially deuterated molecules and would make it possible to measure changes in the relative positions of phospholipid and cholesterol molecules in membranes.

## CONCLUDING REMARKS

In this paper, measurements of the orientational order profile of the palmitoyl chains of POPC have been used to explore several aspects of the hydrophobic thickness of POPC. Increased average chain orientational order $\langle|S_{CD}|\rangle$ was observed upon addition of 30% cholesterol in agreement with many previous observations (see e.g., Lafleur et al., 1990) and this was interpreted quantitatively in terms of increased bilayer hydrophobic thickness $d$ using a previously developed linear relationship, Eq. 2, between $d$ and $\langle|S_{CD}|\rangle$. The influence of amphiphilic polypeptides of different hydrophobic lengths on the average orientational order of pure POPC bilayers and those containing 30% cholesterol was found to be in qualitative agreement with the predictions of the mattress model of protein-lipid interactions (Mouritsen and Bloom, 1984). This represents the first experimental check of the mattress model in membranes containing cholesterol and serves to confirm, at least qualitatively, the utility of Eq. 2 for mixtures of phospholipids and cholesterol.

The experimental results were also used to examine the combined influence of the polypeptides and cholesterol on the orientational order profile and thickness expansivity of the membranes. When the thickness expansivity was compared to published area expansivity measurements of related, but not identical, systems it was found necessary to take into account details of the manner in which lipid and cholesterol molecules are geometrically arranged (packed) in the bilayer to reconcile the different types of mechanical measurements. In particular, with the present interpretation of the orientational order in terms of bilayer thickness, it is necessary to explain why the magnitude of the thickness expansivity is increased upon addition of cholesterol while the area expansivity is decreased. To test whether the model proposed for this reconciliation is correct, it would be necessary to carry out other experiments, especially x-ray and neutron diffraction measurements, on lipid-cholesterol mixtures.

The fact that cholesterol doubles the rate of thermal expansivity of the bilayer hydrophobic thickness may well have interesting consequences in real biological systems: e.g., because permeability is a decreasing function of bilayer thickness, therefore, presence of cholesterol implies a more rapid physical response of the bilayer permeability to changes in temperature. This could be biologically advantageous by providing a cell with increased isolation from its environment at vulnerable times of lowered temperature when many enzyme-related cellular functions, which also have a strong temperature dependence, become inactive.

The authors would like to thank M. Roux for his generous aid in the sample preparation and E. Evans and J. Ipsen for helpful discussions.

The financial support of the Natural Sciences and Engineering Research Council of Canada (NSERC) is gratefully acknowledged.

*Received for publication 11 June 1991 and in final form 4 November 1991.*

## REFERENCES

Alberts, A., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. Molecular Biology of the Cell (Second Edition). Garland Publishing, New York.

Bloom, M., and I. C. P. Smith. 1985. Manifestations of lipid-protein interactions in deuterium NMR. *In* Progress in Protein-Lipid Interactions. A. Watts and J.J.H.H.M. Depont, editors. Elsevier North Holland, Amsterdam. 61–88.

Bloom, M., and O. G. Mouritsen. 1988. The evolution of membranes. *Can. J. Chem.* 66:706–712.

Bloom, M., E. Evans, and O. G. Mouritsen. 1991. Physical properties of the fluid lipid-bilayer: a perspective. *Quart. Rev. Biophys.* 24:293–397.

Brochard, F., and J. F. Lennon. 1975. Frequency spectrum of the flicker phenomenon in erythrocytes. *J. Physique.* 36:1035–1047.

Büldt, G., H. U. Gally, J. Seelig, and G. Zaccai. 1979. Neutron diffraction studies on phosphatidylcholine model membranes: head group conformations. *J. Mol. Biol.* 134:673–691.

Chapman, D. 1975. Phase transitions and fluidity of lipids and cell membranes. *Q. Rev. Biophys.* 8:185–235.

Davis, J. H. 1979. Deuterium magnetic resonance of the gel and liquid crystalline phases of dipalmitoylphosphatidylcholine. *Biophys. J.* 27:339–358.

Davis, J. H., D. M. Clare, R. S. Hodges, and M. Bloom. 1983. Interaction of a synthetic amphiphillic polypeptide and lipids in a bilayer structure. *Biochemistry.* 22:5298–5305.

Devaux, P. F. 1983. ESR and NMR studies of lipid-protein interactions in membranes. *In* Biological Magnetic Resonance, Vol. 5. L. J. Berliner and J. Reuben, editors. Plenum Publishing Corp., New York. 183–299.

Dill, K. A., and P. J. Flory. 1980. Interphases of chain molecules: monolayers and lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* 77:3115–3119.

Huschilt, J. C., R. S. Hodges, and J. H. Davis. 1985. Phase equilibria in an amphiphillic peptide-phospholipid model membrane by deuterium nuclear magnetic resonance difference spectroscopy. *Biochemistry.* 24:1377–1386.

Ipsen, J. H., G. Karlstrom, O. G. Mouritsen, H. Wennerstrom, and M. H. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta.* 905:162–172.

Ipsen, J. H., O. G. Mouritsen, and M. Bloom. 1990. Relationships between lipid membrane area, hydrophobic thickness, and acyl chain orientational order. The effects of cholesterol. *Biophys. J.* 57:405–412.

Lafleur, M., P. R. Cullis, and M. Bloom. 1990. Modulation of the orientational order profile of the lipid acyl chain in the $L_\alpha$ phase. *Eur. Biophys. J.* 19:55–62.

Lafleur, M., B. Fine, E. Sternin, P. R. Cullis, and M. Bloom. 1989. Smoothed orientational order profile of the lipid bilayers by $^2$H-nuclear magnetic resonance. *Biophys. J.* 56:1037–1041.

Lewis, B. A., and D. M. Engelman. 1983. Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vessicles. *J. Mol. Biol.* 166:203–210.

Mantsch, H. H., H. Saito, and I. C. P. Smith. 1977. Deuterium magnetic resonance. Applications in physics, chemistry, and biology. *Prog. Nucl. Magn. Reson. Spectrosc.* 11:211–272.

Marčelja, S. 1974. Chain ordering in liquid crystals. II. Structure of bilayer membranes. *Biochim. Biophys. Acta.* 367:165–176.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1989. Cholesterol modifies the short-range repulsive interactions between phosphatidylcholine membranes. *Biochemistry.* 28:17–25.

Mouritsen, O. G., and M. Bloom. 1984. Mattress model of protein-lipid interactions in membranes. *Biophys. J.* 46:141–153.

Needham, D., T. J. McIntosh, and E. Evans. 1988. Thermomechanical and transitionproperties of dimyristoylphosphatidylcholine/cholesterol bilayers. *Biochemistry.* 27:4668–4673.

Nezil, F. A., C. Morrison, K. P. Whittall, and M. Bloom. 1991. Relaxation-spectra in model membranes using deuterium nuclear magnetic resonance. *J. Mag. Res.* 93:279–290.

Rance, M., and R. A. Byrd. 1983. Obtaining high-fidelity spin—½ powder spectra in anisotropic media: phase-cycled Hahn echo spectroscopy. *J. Magn. Reson.* 52:221–240.

Seelig, A., and J. Seelig. 1977. Effect of a single *cis*-double bond on the structure of a phospholipid bilayer. *Biochemistry.* 16:45–50.

Seelig, J., and A. Seelig. 1980. Lipid conformation in model membranes. *Quart. Rev. Biophys.* 13:19–61.

Sternin, E., M. Bloom, and A. L. MacKay. 1983. De-pake-ing of NMR spectra. *J. Magn. Reson.* 55:274–282.

Sternin, E. 1985. Data acquisition and processing: a systems approach. *Rev. Sci. Instrum.* 56:2043–2049.

Zaccai, G., G. Büldt, A. Seelig, and J. Seelig. 1979. Neutron diffraction studies on phosphatidylcholine model membranes II. Chain conformational and segmental disorder. *J. Mol. Biol.* 134:693–706.