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Effects of Cholesterol on Conformational Disorder in Dipalmitoylphosphatidylcholine Bilayers. A Quantitative IR Study of the Depth Dependence†

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ABSTRACT: A method originally proposed by Snyder and Poore [(1973) *Macromolecules 6*, 708-715] as a specific probe of trans-gauche isomerization in hydrocarbon chains and recently applied [Mendelsohn et al. (1989) *Biochemistry 28*, 8934-8939] to the quantitative determination of phospholipid acyl chain conformational order is utilized to monitor the effects of cholesterol at various depths in dipalmitoylphosphatidylcholine (DPPC) bilayers. The method is based on the observation that the CD_2 rocking modes from the acyl chains of specifically deuterated phospholipids occur at frequencies in the Fourier transform infrared spectrum which depend upon the local geometry (trans or gauche) of the C-C-C skeleton surrounding a central CD_2 group. Three specifically deuterated derivatives of DPPC, namely, 4,4,4′,4′-d_4 DPPC (4-d_4 DPPC), 6,6,6′,6′-d_4 DPPC (6-d_4 DPPC), and 12,12,12′,12′-d_4 DPPC (12-d_4 DPPC), have been synthesized, and the effects of cholesterol addition at 2:1 DPPC/cholesterol (mol:mol) on acyl chain order at various temperatures have been determined. At 48 °C, cholesterol inhibits gauche rotamer formation by factors of ~9 and ~6 at positions 6 and 4, respectively, of the acyl chains, thus demonstrating a strong ordering effect in regions of the bilayer where the sterol rings are presumed to insert parallel to the DPPC acyl chains. In contrast, the ability of the sterol to order the acyl chains is much reduced at the 12-position. The sterol demonstrates only a slight disordering of phospholipid gel phases. Finally, the contributions of different classes of gauche conformers to the spectra have been determined. Kinks and single gauche bends dominate the disordering process as the temperature is increased, although acyl chains with multiple gauche forms are noted at low temperatures. These data are discussed in terms of current models for the effect of cholesterol on phospholipid order and phase properties.

Despite two decades of biophysical studies aimed toward an understanding of cholesterol/phospholid (PL)[1] interaction [for a recent review, see Presti (1985)], a complete description of how PL motion and order are altered by the sterol has not yet been achieved. A plethora of spectroscopic methods such as electron spin resonance (ESR) (Hubbell & McConnell, 1971; Boggs & Hsia, 1972; Marsh & Smith, 1973; Delmelle et al., 1980), Raman (Lippert & Peticolas, 1971; Mendelsohn, 1972), Fourier transform infrared (FT-IR) (Umemura et al., 1980), and ^2H NMR (Stockton et al., 1976) spectroscopies have led to the generally accepted conception that cholesterol fluidizes and/or disorders phospholipids below their gel-liquid-crystal phase transition temperature (T_m) while rigidifying phospholipids at temperatures above T_m. A simple, specific structural model (Rothman & Engelman, 1972; Hubbell & McConnell, 1971) has been proposed in which PL acyl chain conformational disorder is restricted by the sterol ring structure for a distance of about 11 Å into the bilayer from the interfacial region. In contrast, those acyl chain segments that protrude beyond this bulky region of sterol (i.e., toward the bilayer center) are thought to possess enhanced conformational flexibility due to poor packaging between themselves and the chain region of the sterol.

In addition to the above microscopic description of cholesterol-induced alterations in PL structure and order, the phase properties of PL/cholesterol mixtures have been widely probed. High-sensitivity differential scanning calorimetry (DSC) (Mabrey et al., 1978) has demonstrated the coexistence of two domains in mixtures of saturated phosphatidylcholines (PCs) and cholesterol. One corresponds to a relatively pure PC phase, the second to a binary cholesterol/PC complex. The

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; 4,4,4′,4′-d_4 DPPC, 4-d_4 DPPC; 6,6,6′,6′-d_4 DPPC, 6-d_4 DPPC; 10,10,10′,10′-d_4 DPPC, 10-d_4 DPPC; 12,12,12′,12′-d_4 DPPC, 12-d_4 DPPC; FT-IR, Fourier transform infrared; ESR, electron spin resonance; T_m, gel-liquid-crystal phase transition temperature; PL, phospholipid; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

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detailed data interpretation has remained controversial (Estep et al., 1978, 1979; Blume, 1980), although phase diagrams have been proposed (Lentz et al., 1980; Lundberg et al., 1982). Special properties observed at 20, 33, and 50 mol % have been interpreted as arising from gel-state phase boundaries. McConnell and his collaborators (Rubenstein et al., 1979; Owicki & McConnell, 1980; Recktenwald & McConnell, 1981; Rice & McConnell, 1989) have produced evidence from a variety of experimental approaches for microscopic phase separation between cholesterol-rich and cholesterol-poor areas of bilayer membranes.

Lacking in the current description is a quantitative estimate of the effect of cholesterol on phospholipid order and motion. Such information is essential for critical testing and refinement, at the microscopic level, of models for sterol insertion and interactions. The reasons for this deficiency have been discussed elsewhere (Mendelsohn et al., 1989). Basically each spectroscopic technique contributes information on its own time scale to a composite picture of membrane organization. For example, the most powerful method available for membrane dynamics, ^2H NMR, yields order parameters that are averaged by all motions faster than about a microsecond. The contribution of any single motion to the spectrum, especially trans–gauche isomerization (the fastest motion available to the acyl chains), is therefore difficult to elucidate.

We have recently (Mendelsohn et al., 1989) applied to the problem of phospholipid conformational order a vibrational spectroscopic method developed by Snyder and co-workers (Snyder & Poore, 1973; Maroncelli et al., 1985a,b; Shannon et al., 1989) for the determination of conformational order in alkanes. The approach requires the measurement of CD_2 rocking modes in the FT-IR spectra of specifically deuterated phospholipids. The local conformation of a C–C–C skeleton immediately surrounding a central CD_2 group partially determines the CD_2 rocking frequencies. Integrated band intensities thus yield a quantitative measure of the fraction of gauche rotamers at a particular chain position. In the current study, this approach has been applied to the problem of conformational disorder in 1,2-dipalmitoylphosphatidylcholine (DPPC)/cholesterol bilayers at depths in the membranes corresponding to the 4-, 6-, and 12-positions of the DPPC acyl chains.

Experimental Procedures

Synthesis of Specifically Deuterated Phospholipids. Syntheses of 4-d_4 DPPC, 6-d_4 DPPC, and 12-d_4 DPPC were modifications (H. F. Schuster, S. S. Hall, and R. Mendelsohn, unpublished results) of the procedures of Tulloch (1979), scaled to yield 2–3 g of each phospholipid. The various derivatives were fully characterized by NMR, mass spectrometry, and FT-IR.

Sample Preparation. Twelve milligrams of deuterated lipid and 3 mg of cholesterol were added to a culture tube. This mixture was then dissolved in a minimal amount of chloroform, which was then evaporated under a stream of dry nitrogen. The resulting film was placed in an evacuated desiccator overnight to ensure complete solvent removal. D_2O (18 μL) was added to the tube, which was then sealed. The tube was held at 55 °C, with frequent vortex mixing, for 1.5–2 h to ensure complete mixing and hydration. Samples were placed between two AgCl windows, contained and sealed with a 6-μm spacer. The circumference of this assembly was wrapped with Teflon tape, forming an added seal against dehydration. The window assembly was inserted into a variable-temperature Harrick cell. Sample temperatures were controlled with a Haake circulating water bath and monitored with a thermocouple placed adjacent to the point where IR radiation was focused. Temperatures are estimated to be accurate to ±1 °C.

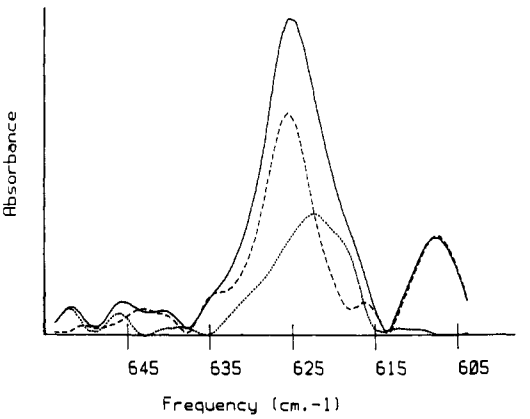

FIGURE 1: Three stages of the spectral subtraction process for 12-d_4 DPPC at 49 °C. The background spectrum, d_0 DPPC/cholesterol/D_2O (dashed), is subtracted from the sample spectrum, containing deuterated lipid (solid), to yield the resultant (dotted).

FT-IR Data Acquisition. Spectra were acquired with a Mattson Sirius 100 FT-IR spectrometer (Mattson Instruments, Madison, WI) equipped with a HgCdTe detector (1 × 1 mm) fabricated (Infrared Associates, Cranbury, NJ) to give enhanced response near 620 cm^{-1}; 2000 scans of sample and backgound were co-added and apodized with a triangular function. Spectrometer resolution was 4 cm^{-1}. Interferograms were zero filled (two levels) and fast Fourier transformed, yielding data encoded every 1 cm^{-1}. Band positions had an uncertainty of less than ±0.1 cm^{-1}.

FT-IR Data Reduction. The CD_2 rocking modes discussed in this study appear between 660 and 600 cm^{-1} as weak features on an intense D_2O libration background. Typically, this sloping background was 0.8–1.2 absorbance units under conditions described above. Background subtraction is necessary for two reasons—first, because the strongest band in this spectral region, the 622-cm^{-1} all-trans marker, is about 7 milliabsorbance units at 34 °C and, second, for removal of weak cholesterol spectral bands which overlap the all-trans marker. Spectra of DPPC-d_0/cholesterol mixtures, prepared by using molar proportions identical with those of the deuterated sample and studied under the same path length and temperature conditions, are used as a backgrounds for spectral subtraction.

Spectral subtraction is carried out by first fitting a cubic polynomial function (J. W. Brauner, unpublished results) to the sloping background of each sample and background spectrum between 665 and 595 cm^{-1}. The function was subtracted from the spectra to give spectra with little, if any, base-line slope or curvature. Background spectra are then scaled by using a cholesterol band centered at 609 cm^{-1} and subtracted from sample spectra at corresponding temperatures. Final base-line correction, if necessary, is carried out by using an algorithm supplied with the spectrometer. Figure 1 illustrates sample (solid line), background (dashed line), and resultant spectra (dotted line) from a spectral subtraction of 12-d_4 DPPC at 49 °C.

Incomplete removal of cholesterol bands overlapping the 622-cm^{-1} marker made curve fitting for quantitative analysis of band areas necessary (see dotted spectrum, Figure 1). Band profiles constructed from addition of individual Gaussian–Lorentzian bands were compared with experimental spectra by using an interactive algorithm written specifically for this purpose (J. W. Brauner, unpublished results). The area of of the 622-cm^{-1} marker was computed by using the same

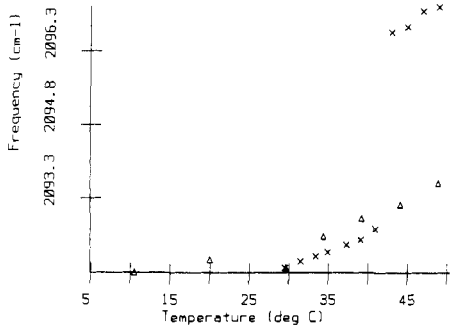

FIGURE 2: Melting profiles of 6-d_4 DPPC/D_2O (×) and 6-d_4 DPPC/cholesterol/D_2O (△) constructed from the CD_2 symmetric stretching frequency. The sharp break in the profile of 6-d_4 DPPC is the main gel–liquid-crystal phase transition.

algorithm; this result, in turn, was used in eq 1 (see Theory) for calculating relative populations of various conformers. The weak (3×10^{-4} absorbance units at 34 °C in 6-d_4 DPPC) 646- and 652-cm^{-1} conformational markers were minimally overlapped by other spectral features, and the areas of these bands were computed directly by using Simpson's rule. Variation of base-line parameters used in Simpson's rule calculations led to variations in band areas; error bars for these areas were estimated by calculation of percentage difference between areas calculated with differing base-line parameters.

Theory

The theory (Snyder & Poore, 1973) required for analysis of the CD_2 rocking modes has been outlined previously (Mendelsohn et al., 1989); a brief summary is included for convenience. Maroncelli et al. (1985a,b) demonstrated that the rocking mode frequency of an isolated CD_2 group in a hydrocarbon chain is sensitive to the conformation of C–C bonds in the immediate vicinity of the group. Trans or gauche bonds adjoining the CD_2 group are underlined; thus, tt indicates that the CD_2 group adjoins a trans bond pair, while tg indicates a trans–gauche conformation. CD_2 probe frequencies depend upon the conformation of C–C bonds adjacent to the tt or tg bond groups. If the CD_2 group adjoins two trans bonds (tttt conformation), the CD_2 rocking frequency occurs at 622 cm^{-1}. Gauche conformers of the type g′tgt (kink) or ttgt (single gauche bend) occur at 652 cm^{-1}, while those of the ttgg or g′tgg types are observed at 646 cm^{-1}. Maroncelli et al. (1985a,b) have further shown that the unit absorptivities of the various conformers away from the ends of the chain are the same. Thus, the fraction of gauche rotamers is given by

$$f_g = [I(652) + I(646)]/[I(622) + I(652) + I(646)] \quad (1)$$

where I is the band area of the frequency given in parentheses.

Results

Two spectral regions, arising from acyl chain C–D stretching (2300–2000 cm^{-1}) and rocking modes (660–600 cm^{-1}), respectively, are used in this work to probe phospholipid structure. The symmetric and asymmetric stretching frequencies, near 2100 and 2300 cm^{-1}, respectively (Mendelsohn & Maisano, 1978; Bansil et al., 1980; Cameron et al., 1981), are qualitative markers of lipid phase state and gauche rotamer formation. The symmetric stretch is used in the current study to monitor the thermotropic behavior of deuterated DPPC derivatives in the presence and absence of cholesterol. Typical melting curves for hydrated pure 6-d_4 DPPC and a 2:1 6-d_4 DPPC/cholesterol mixture are shown in Figure 2. The cooperative gel–liquid-crystal transition at 41 °C, evident in the

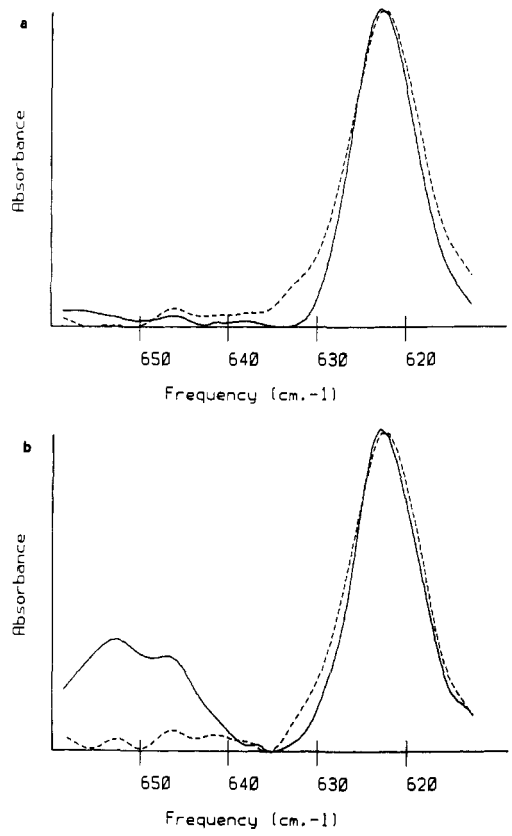

FIGURE 3: (a) Spectra of the CD_2 rocking region of 6-d_4 DPPC/D_2O with (---) and without (—) cholesterol at 34 °C. (b) Spectra of the CD_2 rocking region of 6-d_4 DPPC/D_2O with (---) and without (—) cholesterol at 49 °C.

melting curve of the pure phospholipid, is replaced by a profile in which frequency is essentially constant from 10 to 30 °C and then increases monotonically from 30 to 50 °C. There is no indication of a residual cooperative melting event at the 6-position of the acyl chain. The frequency for the 2:1 DPPC/cholesterol mixture increases from 2091.8 cm^{-1} at 10.6 °C to 2093.6 cm^{-1} at 49.0 °C, in marked contrast to the much larger increase observed for pure 6-d_4 DPPC from 2091.9 cm^{-1} at 29.6 °C to 2097.2 cm^{-1} at 49.0 °C. It is also evident from Figure 2 that CD_2 stretching frequencies measured at temperatures between 29.7 and 49.0 °C in the presence of cholesterol undergo a monotonic increase similar to those for the pure lipid in the gel phase; that is, the temperature-induced formation of gauche rotamers at the 6-position occurs at approximately the same rate in DPPC/cholesterol and pure DPPC below the phase transition. Furthermore, the CD_2 symmetric stretching frequencies for the DPPC/cholesterol mixture observed at temperatures ranging from 29 °C to the melting point of the pure lipid, 41 °C, are much closer to those of the pure lipid in the gel phase than that in the liquid-crystalline phase. Since this frequency qualitatively monitors acyl chain disorder, it is concluded that cholesterol strongly inhibits gauche rotamer formation, i.e., exhibits a strong ordering effect, at the 6-position. The investigation of the CD_2 rocking modes permits the quantitative characterization of the above phenomenon, at several depths in the bilayer.

Typical spectra of the CD_2 rocking region for the 6-d_4 and 12-d_4 DPPC derivatives are shown in Figures 3 and 4, while calculated percentages of gauche rotamers at various temperatures for all derivatives studied are listed in Table I. All data in Figures 3 and 4 have been base-line corrected and flattened as detailed under Experimental Procedures. Spectra of the CD_2 rocking region obtained at 34 °C for 6-d_4 DPPC

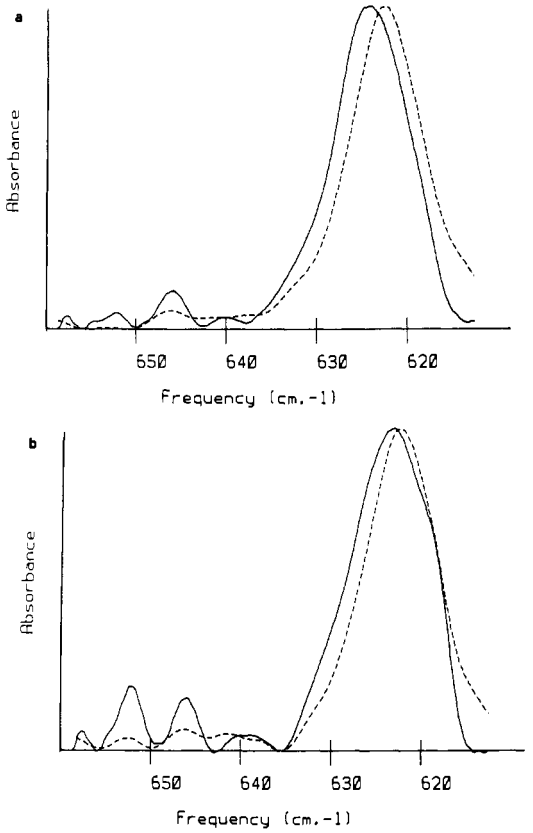

FIGURE 4: (a) Spectra of the CD_2 rocking region of 12-d_4 DPPC/D_2O (—) and 6-d_4 DPPC/D_2O (---) in the presence of cholesterol at 34 °C. (b) Spectra of the CD_2 rocking region of 12-d_4 DPPC/D_2O (—) and 6-d_4 DPPC/D_2O (---) in the presence of cholesterol at 49 °C.

Table I: Comparison of Percent of Total Gauche Conformers for Specifically Deuterated DPPC Derivatives in the Presence or Absence of Cholesterol

	temp (°C)			
derivative	5.6	20	34	49
4-d_4[a]		7.6	10.9 ± 6.1	20.7 ± 4.2
4-d_4/cholesterol			1.5 ± 1.0	3.7 ± 1.0
6-d_4[a]			1.7 ± 0.3	32.3 ± 2.3
6-d_4/cholesterol			2.6 ± 1.0	3.6 ± 1.0
10-d_4[a]			2.5 ± 1.0	19.7 ± 0.8
12-d_4/cholesterol	4.5 ± 1.0	7.5 ± 1.0	6.7 ± 2.0	10.6 ± 2.0

[a] Data from Mendelsohn et al. (1989).

both in the absence and in the presence of cholesterol are given in Figure 3a. The corresponding spectra at 49 °C are shown in Figure 3b. Three main spectral features, arising from the CD_2 rocking modes of particular conformations (see Theory) are observed. The 622-cm^{-1} band[2] arising from trans segments is the strongest feature in all spectra. Most notable in Figure 3 is the suppression of gauche rotamer formation at 49 °C by

[2] A reviewer has pointed out, and we have observed, the presence of small variations in the position of the trans marker band at 622 cm^{-1} (see Figures 3 and 4). There are several possible origins for such effects. Differences in the factors used for background subtractions in the various spectra could easily lead to slightly changed band positions. We note that the background spectrum at 622 cm^{-1}, which is curved and temperature dependent, is 100–500 times stronger than the peaks plotted in Figures 3 and 4, so that perfect compensation is exceptionally difficult, especially in samples containing cholesterol. The latter has a band near 626 cm^{-1}. While thermal and intermolecular effects may also contribute to this small variation, they are (i) expected to be small over the rather limited temperature range reported in the current work and (ii) neither distinguishable nor separable from the aforementioned difficulties with background subtraction.

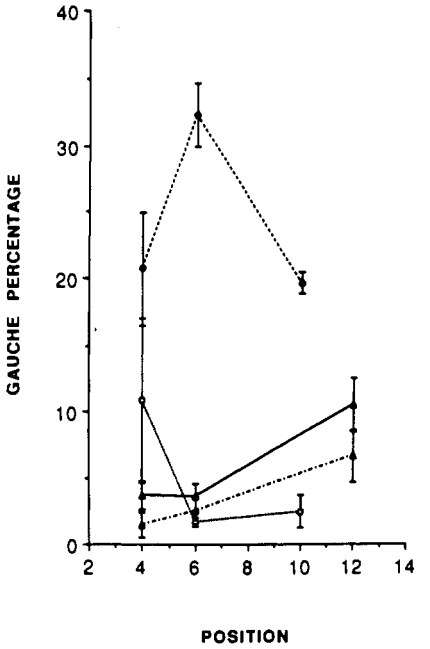

FIGURE 5: Graphical summary of results showing the effect of cholesterol on the ordering of the acyl chains of DPPC as a function of both temperature and depth in the bilayer. Error bars shown are in accord with the data in Table I. Data points acquired at the same temperature and in either the presence or absence of cholesterol are connected. Solid circles connected by a dashed line represent DPPC/D_2O at 48.5 °C; circles connected by a dotted line represent DPPC/D_2O at 34.0 °C; triangles connected by a solid line represent DPPC/cholesterol/D_2O at 48.5 °C; and triangles connected by a dot-dash line represent DPPC/cholesterol/D_2O at 34.0 °C.

cholesterol as revealed by the relative weakness of the gauche features at 646 and 652 cm^{-1} in the cholesterol-containing sample. Calculation of the band areas and application of eq 1 yields the percentage of gauche rotamers. As shown in Table I, these are reduced by a factor of 9—from 32% to 3.6% at 49 °C. The position dependence of the ordering effect of cholesterol is directly illustrated in Figure 4. Spectra of 6-d_4 and 12-d_4 DPPC, each with 33 mol % cholesterol, are shown for 34 °C in Figure 4a, and for 49 °C in Figure 4b. At 34 °C, 12-d_4 DPPC contains 6.7% gauche rotamers; 6-d_4 DPPC contains 2.6%. The differences are even more pronounced at 49 °C, where 12-d_4 DPPC has 10.6% gauche rotamers and 6-d_4 DPPC has 3.6%. The data are summarized graphically in Figure 5, which indicates the percentage of gauche rotamer formation as a function of acyl chain position at 34 and 49 °C in the presence and absence of cholesterol. Also given in Figure 5 for comparison are gauche rotamer values for 10-d_4 DPPC in the absence of cholesterol.

Finally, from the intensity of the gauche marker bands at 652 and 646 cm^{-1}, it is clearly seen from Figure 4a that those rotamers giving rise to the 646-cm^{-1} band, namely, the multiple gauche class (see Discussion), are the predominant form at 34 °C. Comparison with the high-temperature data shown in Figure 4b shows that the 652-cm^{-1} band, arising from kinks and single gauche bends (see Discussion), increases in intensity (relative to the 622-cm^{-1} marker) at a faster rate than the 646-cm^{-1} feature.

Discussion

As noted in the introduction, while the qualitative effects of cholesterol on the order and fluidity of saturated PCs have generally been agreed upon, the quantitative changes on acyl chain conformational order produced by the sterol have yet to be determined. As demonstrated in a previous paper (Mendelsohn et al., 1989), acyl chain CD_2 rocking modes are

sensitive, nonperturbing structural probes of phospholipid acyl chain conformational order. The experiments described herein have addressed (Table I; Figure 5) the following issues:

(1) What are the fractions of trans and gauche conformers present in DPPC acyl chains at various temperatures when cholesterol is present in the mixture?

(2) What is the depth dependence of the trans/gauche ratio along the acyl chain?

(3) How do these results compare with those obtained in the absence of cholesterol; i.e., what is the quantitative effect of cholesterol on acyl chain conformation?

As in our previous paper (Mendelsohn et al., 1989), two assumptions have been made in the data analysis:

(1) The relative trans/gauche absorptivities of the CD_2 rocking modes calculated by Maroncelli et al. (1985a,b) are transferrable from alkanes to phospholipid acyl chains. In addition, it is assumed that the relative extinction coefficients of these modes are not sensitive to the presence of cholesterol in the bilayer. As the CD_2 rocking modes are highly localized, and thus, not sensitive to bulk phase structure, this assumption seems appropriate.

(2) CD_2 rocking modes are sensitive only to trans–gauche isomerization because the rapid time scale of molecular vibrations "uncouples" these vibrational frequencies from slower motions detected in NMR and other studies.

The widely accepted molecular description of cholesterol insertion is derived from the ESR experiments of Hubbell and McConnell (1971), who probed the depth dependence of acyl chain order parameters in egg lecithin/cholesterol mixtures. A structural model followed (Rothman & Engelman, 1972; Hubbell & McConnell, 1971) in which the acyl chains are thought to be fairly rigid for a region up to approximately seven to eight carbon atoms from the fatty acid-glycerol linkage. The probability of gauche rotamer formation increases rapidly beyond this range toward the bilayer center. In a more general sense, Rothman and Engelman (1972) have suggested that the often quoted "intermediate fluid condition" in which biological membranes are thought to exist is derived from a cholesterol-induced restriction of disorder of the upper half of the chains coexisting with bilayer regions possessing much motional freedom toward the bilayer center.

The results reported here begin to quantitatively define acyl chain order within the context of the above structural model. The ability of cholesterol to strongly hinder gauche rotamer formation at the 4 and 6 acyl chain positions is clearly demonstrated. Gauche rotamer formation is much less hindered at the 12-position (Table I), consistent with the structural model which suggests that the sterol ring system does not penetrate deeply enough into the bilayer to hinder acyl chain isomerization at that location. Thus, methylene groups near the terminal end of the acyl chain have greatly increased probability of gauche rotamer formation.

Two additional effects are evident from the IR spectral data. First, as expected, the disorder as measured from the percentage of gauche conformers in the acyl chains of the 12-d_4 derivative increases with increasing temperature. Such an effect is not consistently detected at current signal to noise levels in either the 6-d_4 or 4-d_4 derivative, as the weak intensities ($2–5 \times 10^{-4}$ absorbance unit) make quantitative parameters difficult to extract.

Second, the current FTIR experiments yield information concerning the types of gauche conformers present. As the 646/652-cm^{-1} doublet is most intense in the 12-d_4 derivative, we emphasize this molecule in the following discussion. The relative intensity of the 646-cm^{-1} band to that of the all-trans 622-cm^{-1} marker is much greater at low temperature than in either the 4-d_4 or 6-d_4 derivative. As seen in Figure 4, the relative intensity at 652 cm^{-1} increases much more rapidly with increasing temperature than that of the 646-cm^{-1} component of the doublet. The former arises from chains containing tg′tgt type kinks or ttgt type single gauche bends, while the 646-cm^{-1} component arises from conformers containing multiple gauche bends, such as tg′tggt. Thus, we conclude that multiple gauche forms are already present toward the bilayer center at low temperatures. These may arise from poor acyl chain packing in areas where the flexible sterol side chain is located and are rather weakly affected by temperature.

In contrast, those conformers that give rise to the 652-cm^{-1} marker band (including kinks and single gauche bends), and are suggested (Jackson, 1976) to be the main source of disordering induced in acyl chains during melting events, appear to form much more easily as temperature is increased and are the main source of conformational disordering. We note that the average value of the band area ratio $I(652)/I(622)$ increases sharply from 0.017 below 39 °C to 0.058 above 39 °C. In contrast, the corresponding values for the $I(646)/I(622)$ marker are 0.046 (below) and 0.050 (above). This substantial difference may be a consequence of kinks inducing a much smaller disruption of chain packing.

Despite numerous investigations of DPPC/cholesterol phase behavior, the details of the phase diagram for this important system remain unclear. Recently, Ipsen et al. (1987) have used both thermodynamic and microscopic models to propose a DPPC/cholesterol phase diagram in which a single homogeneous phase exists at 33 mol % cholesterol between 20 and 50 °C. Results reported herein lend credence to this suggestion. For example, if a 1:1 DPPC/cholesterol complex were coexistent with a relatively pure DPPC phase, the observed gauche fraction at the 6-position at 49 °C should have been approximately 0.16 (50% of the molecules having a 0.32 gauche fraction, as observed for pure 6-d_4 DPPC, and 50% having near zero disorder in the DPPC/cholesterol complex), instead of the observed 0.036 ± 0.010.

The current study amply demonstrates the power of the CD_2 probe method for the study of membrane order, affording a means to quantitatively map acyl trans–gauche isomerization at particular depths in the bilayer. As noted elsewhere (Mendelsohn et al., 1989), other experimental approaches used to date have not been successfully used for this purpose. For example, order parameters derived from ESR or NMR techniques are sensitive to all motions occurring on a time scale faster than that characteristic of the method. In contrast, the approach described here does not rely upon assumptions about the various types of motions undergone on the time scale of the experiment by individual phospholipid molecules; the spectral analysis is straightforward, following the seminal investigations of Snyder and his students, because the rocking modes studied are sensitive only to trans–gauche isomerization in the acyl chains, at least over the small temperature ranges probed in the current experiments. Spectra have been acquired with as little as 5 mg of scarce deuterated sample. Although the conformational marker bands are very weak, they are, as demonstrated here, observable with current IR technology, and it should be possible to extend this approach to study the effect of other molecules such as peptides on lipid conformational order.

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Fragmentation of Isoaspartyl Peptides and Proteins by Carboxypeptidase Y: Release of Isoaspartyl Dipeptides as a Result of Internal and External Cleavage†

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ABSTRACT: Isoaspartate-containing versions of sea urchin sperm-activating peptide, δ sleep-inducing peptide, and lactate dehydrogenase (231–242) were cleaved at internal sites by carboxypeptidase Y. Cleavage occurred between the isoaspartate and the preceding amino acid, and it was accompanied by sequential digestion of amino acids from the two resulting carboxyl termini. Because the isoaspartyl bonds were not cleaved, isoaspartyl dipeptides were among the final products. The rate of release of isoaspartyl dipeptides was different for the three peptides, a 24-h digestion yielding 0.32 mol of isoaspartylglycine/mol of isoaspartyl sperm-activating peptide, 0.50 mol of isoaspartylalanine/mol of isoaspartyl δ sleep-inducing peptide, and 1.15 mol of isoaspartylserine/mol of isoaspartyl lactate dehydrogenase (231–242). The different rates could be explained by the slow cleavage of amino acids preceded by glycine. Isoaspartyl dipeptides were not detected in digests of the corresponding aspartate- or asparagine-containing forms of the peptides. Release of isoaspartyl dipeptides by carboxypeptidase Y was used to demonstrate the presence of isoaspartylglycine sequences in deamidated adrenocorticotropin (0.54 mol/mol), in a mixture of tryptic fragments of base-treated calmodulin (0.20 mol/mol), and in a mixture of tryptic fragments of base-treated triosephosphate isomerase (0.08 mol/mol). These results confirm earlier work suggesting that isoaspartylglycine formation is prevalent in proteins exposed to alkaline conditions. They also provide a methodology that should prove useful in the characterization of natural substrates for protein L-isoaspartyl methyltransferase.

Isoaspartyl (β-aspartyl) linkages form spontaneously in proteins and peptides following deamidation of certain asparagines or isomerization of certain aspartates (Gråf et al., 1971; Geiger & Clarke, 1987; Johnson et al., 1989b). When these atypical linkages, which are characterized by an extra carbon in the peptide backbone and a free α-carboxylic acid, are introduced into synthetic peptides, the peptides can be methylated by a protein carboxyl methyltransferase that shows no ability to methylate corresponding peptides containing

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