CONFORMATIONAL NONEQUIVALENCE OF CHAINS 1 AND 2 OF DIPALMITOYL PHOSPHATIDYLCHOLINE AS OBSERVED BY RAMAN SPECTROSCOPY

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ABSTRACT Raman spectroscopic data indicate that the conformations of the two hydrocarbon chains of dipalmitoyl phosphatidylcholine in aqueous dispersions of the lipid differ significantly. The compounds 1-palmitoyl, 2-palmitoyl-d_{31}-3-*sn*-phosphatidylcholine and 1-palmitoyl-d_{31}, 2-palmitoyl-3-*sn*-phosphatidylcholine were synthesized. Aqueous dispersions of these phospholipids display very similar phase behavior, with both premelting and melting transitions at nearly identical temperatures, midway between the comparable transition temperatures of undeuterated and completely deuterated dipalmitoyl phosphatidylcholine. We have monitored the state of chains 1 and 2 of these molecules simultaneously and independently by Raman spectroscopy. Raman difference spectra taken between samples of the two compounds under identical conditions show significant features. We attribute these spectral differences to nonequivalent conformations of the fatty acyl chains attached at positions 1 and 2 on the glycerol backbone. Below the pretransition the conformation of chain 2 is, on average, slightly less all-*trans* than is the chain at position 1. There is some evidence that the conformations of the terminal methyl group of the two chains are significantly different at low temperatures.

INTRODUCTION

Although much is known about the structure of the phospholipid bilayer, there are certain details that remain unresolved. Among these is the packing of the hydrocarbon chains, even in model systems as simple as aqueous dispersions of dipalmitoyl phosphatidylcholine (DPPC). For example, Sundaralingam (1) has shown by potential energy minimization calculations that two equally stable conformations of the glycerol backbone exist, either or both of which could exist in phospholipid bilayers. From deuterium nuclear magnetic resonance (NMR) experiments on DPPC bilayers at temperatures above the melting transition, Seelig and Seelig (2) have proposed that fatty acyl chain 1 is relatively perpendicular to the plane of the bilayer, but that chain 2

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exists in either of two conformations of equal stability in which the first two carbons near the glycerol backbone are nearly parallel to the plane of the bilayer. Subsequent evidence of differing hydration of ester carbonyls (3) has added further weight to the belief that the conformations of the two hydrocarbon chains are not the same.

Unfortunately, most NMR techniques are difficult to apply to studies of phospholipid conformations below the phase transition temperature because of severe line broadening. Raman spectroscopy suffers no such limitations—most bands sharpen at lower temperatures—yet there has been no evidence from Raman data that chains 1 and 2 differ in conformation. Raman bands from the two chains overlap too closely to be readily differentiated.

Recently, it has been shown that deuterocarbons are excellent substituents for phospholipid membrane structural studies (4–6).[1] Deuteration of the acyl chains of the lipid molecule does not change the phase behavior of the phospholipid dispersion other than by lowering the temperatures of the premelting and melting transitions by approximately 5°C. Building upon our best understanding of the Raman spectra of both DPPC and its chain-perdeuterated analog DPPC-d_{62} (4, 7, 8), we were able to formulate a procedure by which to determine structural differences between chains 1 and 2 of DPPC by Raman spectroscopy.

Two partially deuterated DPPC's were synthesized, both of which contained one completely deuterated palmitic acid chain—one with the deuterated chain in the 1 position and the other with the deuterated chain in the 2 position. As the only difference between these two compounds is the position of the deuterated and nondeuterated chains, and as the melting points of the two compounds are identical, any difference in the Raman spectra of the two compounds taken under identical conditions must originate in differences in conformation of the 1 and 2 chains.

MATERIALS AND METHODS

All phospholipids used in this study were pure L-isomer. The lecithin 1,2-palmitoyl-d_{62}-3-*sn*-phosphatidylcholine (DPPC-d_{62}) was purchased from Lipid Specialties, Inc., (Boston, Mass.); palmitic acid-d_{31} from Merck Sharp & Dohme Canada Ltd. (Montreal, Canada); palmitic acid, sodium palmitate, palmitoyl lysolecithin, and phospholipase A were purchased from Sigma Chemical Co. (St. Louis, Mo.). The compound 1-palmitoyl, 2-palmitoyl-d_{31}-3-*sn*-phosphatidylcholine (HD-DPPC) was synthesized by acylation of lysopalmitoyl phosphatidylcholine, by the acylation procedure of Robles and van den Berg (9) using the fatty acid anhydride of palmitic acid-d_{31}. Reaction products were purified first on silica gel, and then on Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N.J.) in ethanol at 37°C. Thin-layer chromatography showed only one spot. Purity was further confirmed by differential scanning calorimetry (DSC), which demonstrated a sharp main endothermic transition (width of <2°), and a distinct premelting transition. To synthesize 1-palmitoyl-d_{31}, 2-palmitoyl-3-*sn*-phosphatidylcholine (DH-DPPC), DPPC-d_{62} was first cleaved with phospholipase A, removing chain 2 and leaving palmitoyl-d_{31} lysolecithin. The lysolecithin was then purified on Sephadex LH-20. Acylation with palmitic anhydride and purification was as described above.

[1] Klump, H. H., B. P. Gaber, W. L. Peticolas, and P. Yager. The thermodynamic properties of mixtures of deuterated and undeuterated dipalmitoyl phosphatidylcholines. Manuscript in preparation.

Differential scanning calorimetry of DH-DPPC yielded a sharp melting point and a premelting transition at temperatures nearly identical to that for HD-DPPC. Dispersions were prepared as in reference 8.

DSC was performed on the Privalov differential adiabatic scanning microcalorimeter (10). Raman spectra, data collection, manipulation, and plotting were performed as described previously (4, 7). Spectra were collected with slit widths at 150 μm to prevent spurious difference peaks due to changes in apparent line width. Grating ghosts were subtracted from absolute spectra before further computation. Melting curves on these compounds were produced as we have previously described (4), with the exception that slit widths were set at 200 μm, giving an average spectral resolution of better than 5 cm^{-1}.

Because of differences in the homogeneity of dispersions from sample to sample, it is not possible to use a water-soluble internal standard as an intensity reference, nor is it possible to use a lipid-soluble internal standard, as any such compound would perturb the structure of the phospholipids (11). Consequently, the C—N stretching band at 716 cm^{-1} was chosen as internal standard for normalization of spectra to be compared. The differences between the two compounds DH-DPPC and HD-DPPC are sutble, and these should not manifest themselves as differences in the conformation of the head group, particularly if spectra of the two compounds at the same temperature are compared. However, it should be kept in mind that the normalization procedure is not definitive and care should be taken to avoid overinterpreting the difference spectra shown subsequently.

RESULTS AND DISCUSSION

When examined by DSC, main phase transitions occurred at 39.1°C for HD-DPPC and 39.2°C for DH-DPPC, and the pretransitions at 30.9°C and approximately 30°C, respectively. Full details will be published elsewhere (see footnote 1). Dispersions of both compounds demonstrate behavior intermediate between that of DPPC and DPPC-d_{62}. As it has been shown that DPPC and DPPC-d_{62} mix ideally in aqueous dispersions (4, 12, and footnote 1), the conformation of pure bilayers of each compound must be identical. Transition temperatures observed for HD-DPPC (12) and DH-DPPC are nearly identical to those observed for a 1:1 mixture of DPPC and DPPC-d_{62}, so the conformations of the bilayers in dispersions of our synthetic lecithins must be nearly identical to those found for DPPC or DPPC-d_{62} at comparable temperatures relative to their transition temperatures. One may also compare the structure of a dispersion of HD-DPPC to one of DH-DPPC at nearly any temperature considering the similarities of their two melting temperatures.

Fig. 1 (*a*, *b*, and *c*) displays Raman spectra of a dispersion of HD-DPPC at 30° ($T_{pre} < T < T_m$), at 50° ($T > T_m$), and the difference between these two spectra in three regions. The difference spectra, then, show the conformational changes which occur upon melting of the dispersion. Both the absolute and difference spectra may be analyzed by comparison with the spectra of DPPC and DPPC-d_{62} (4, 7).

The Raman spectrum of HD-DPPC in Fig. 1*a* (and of DH-DPPC as well) is composed of bands from the glycerophosphorylcholine head group, a fatty acyl chain, and a perdeuterated fatty acyl chain. Both absolute and "melting difference" spectra resemble a superposition of the corresponding spectra for DPPC (7) and DPPC-d_{62} (4), although neither the spectra of HD-DPPC nor of DH-DPPC can be completely synthesized by a linear combination of spectra of DPPC and DPPC-d_{62}. Loss of crystal

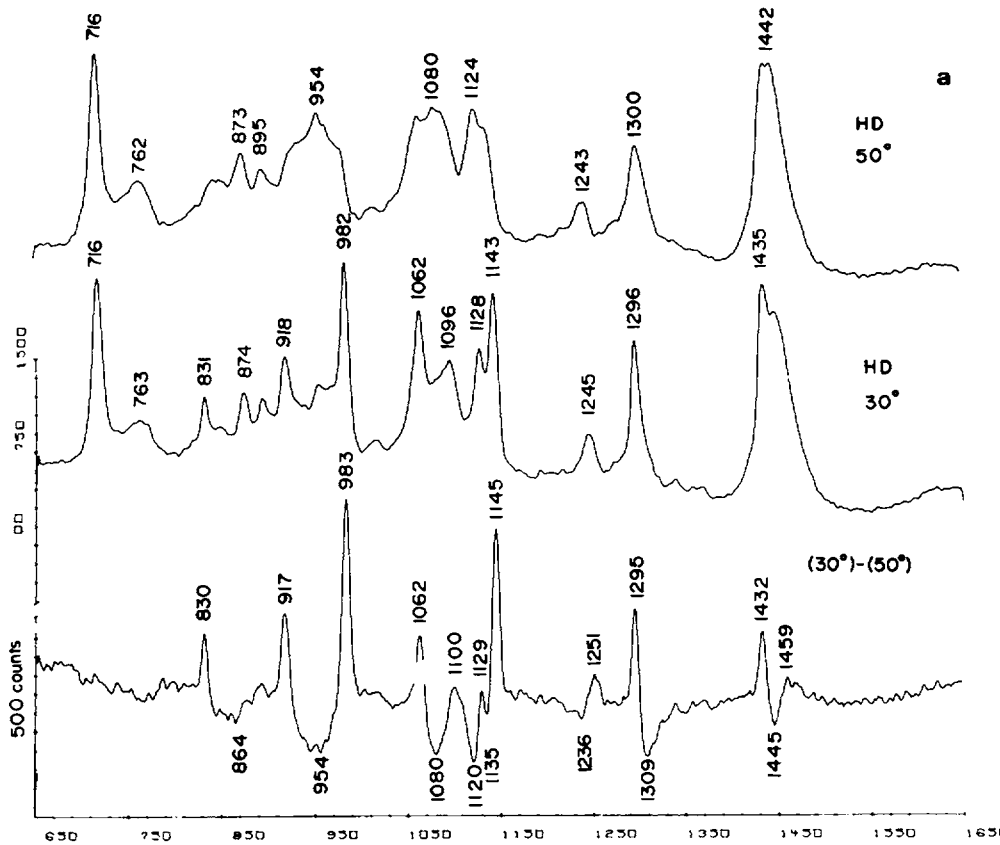

FIGURE 1 (a) HD-DPPC spectra at 30°C, 50°C, and the difference between the absolute spectra. The pair of absolute spectra is to scale, but the difference spectrum is, as shown, vertically expanded. The same experiment as in Fig. 1*b* and *c*. (b) The C—D stretching region of HD-DPPC at 30°C, 50°C, and the difference between the absolute spectra. The pair of absolute spectra is to scale, but the difference spectrum is, as shown, vertically expanded. The same experiment as in Fig. 1*a* and *c*. (c) The C—H stretching region of HD-DPPC at 30°C, 50°C, and the difference between the absolute spectra. The pair of absolute spectra is to scale, but the difference spectrum is, as shown, vertically expanded. The same experiment as in Fig. 1*a* and *b*.

mode splitting existing in bilayers of singly substituted lecithins only partially accounts for this fact.

In Fig. 1*b* the "melting difference" is comparable to that seen for DPPC-d_{62} (4), and for DH-DPPC as well. Any differences between these absolute spectra and those of DPPC-d_{62} are too subtle to be observed without differential techniques.

Although the "melting difference" spectrum in Fig. 1*c* resembles that of DPPC, an obvious difference between the C—H stretching region of HD-DPPC at 30°C and the DPPC spectrum taken at a comparable temperature (7) is apparent. There is substantially less intensity at 2,860 cm^{-1} in both HD-DPPC and DH-DPPC than in DPPC. The difference is even more pronounced at lower temperature (compare the absolute spectra in Fig. 3*c* with Fig. 2 of reference 7). This confirms our conclusion

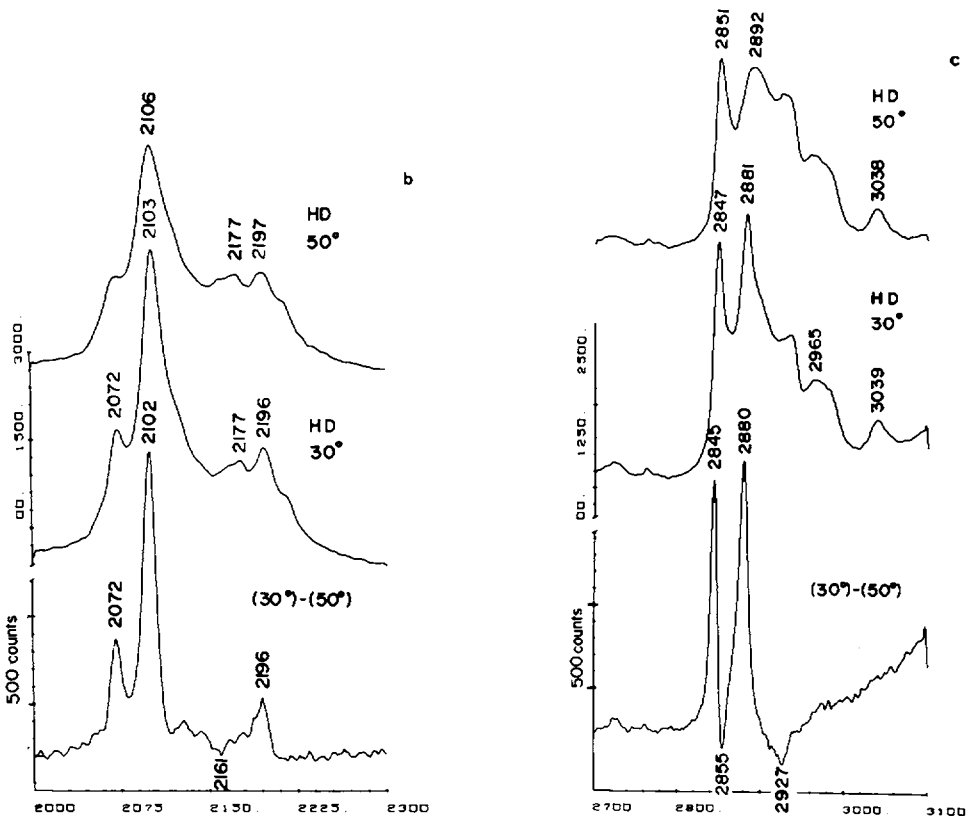

FIGURE 1 *b* and *c*

that intensity at 2,860 cm^{-1} is contributed, in low-temperature spectra of DPPC, by crystal mode interactions between adjacent hydrocarbon chains (7), similar to those seen in triclinic phases of hydrocarbons. Hydrocarbon and deuterocarbon chains alternate throughout the bilayer in these dispersion samples reducing interchain interaction as seen by the 2,860 cm^{-1} band (7) and the ratio of the 2,881 to 2,845 cm^{-1} bands (8) at all temperatures below T_m. The "premelting" spectra (i.e., the [10°]–[30°] difference spectrum) of both HD-DPPC and DH-DPPC do not show a drop in intensity of a band at 2,860 cm^{-1} as is observed for DPPC, because the interaction has been "diluted out" by the deuterocarbon chains.

Fig. 1 demonstrates that one may simultaneously monitor the conformations of chains 1 and 2 of DPPC by Raman spectroscopy. Whereas the region shown in Fig. 1*a* contains many overlapping bands, the entire spectrum shown in Fig. 1*b* is derived from the perdeuterated chain 2 of the phospholipid, and the vast majority of the intensity seen in the spectrum in Fig. 1*c* is from the protiated chain in position 1. The changes that occur in the C—H stretching region from the head group of the phos-

pholipid molecule are extremely small and consist of a decrease of intensity at 2,885 cm^{-1} and a slight increase at 2,900 cm^{-1} (4). In Fig. 1*c* no difference attributable exclusively to the head group can be observed.

The single sharp melting transition seen by differential scanning calorimetry for both DH-DPPC and HD-DPPC (see footnote 1) suggests that both chains 1 and 2 of the labeled phosphatidylcholines "melt" at the same temperature. This is confirmed by the Raman melting curves for DH-DPPC (Fig. 2). These data, similar to those created for DPPC-d_{62} (4), demonstrate that one can observe the melting of the two chains simultaneously and independently from a single sample. For chain 2 the skeletal optical mode band at 1,062 cm^{-1} shows a discontinuity at the premelting transition. Thus the number of allowed *gauche* bonds in chain 2 must be greater than in chain 1 in the phase that exists between T_{pre} and T_m. However, the premelt has almost no effect on the relative peak intensity of the CH_2 asymmetric stretch of chain 2 of DH-DPPC at 2,880 cm^{-1}. This is not the case for DPPC (7, 8) for which a distinct premelt occurs in the CH_2 stretch melting curve. The chain-chain lateral vibrational interaction, the strength of which is known to undergo modification at the premelt in DPPC (8), is largely diluted by the presence of deuterated 1 chains.

The 983 cm^{-1} CD_2 scissoring mode of deuterated chain 1 of DH-DPPC does show

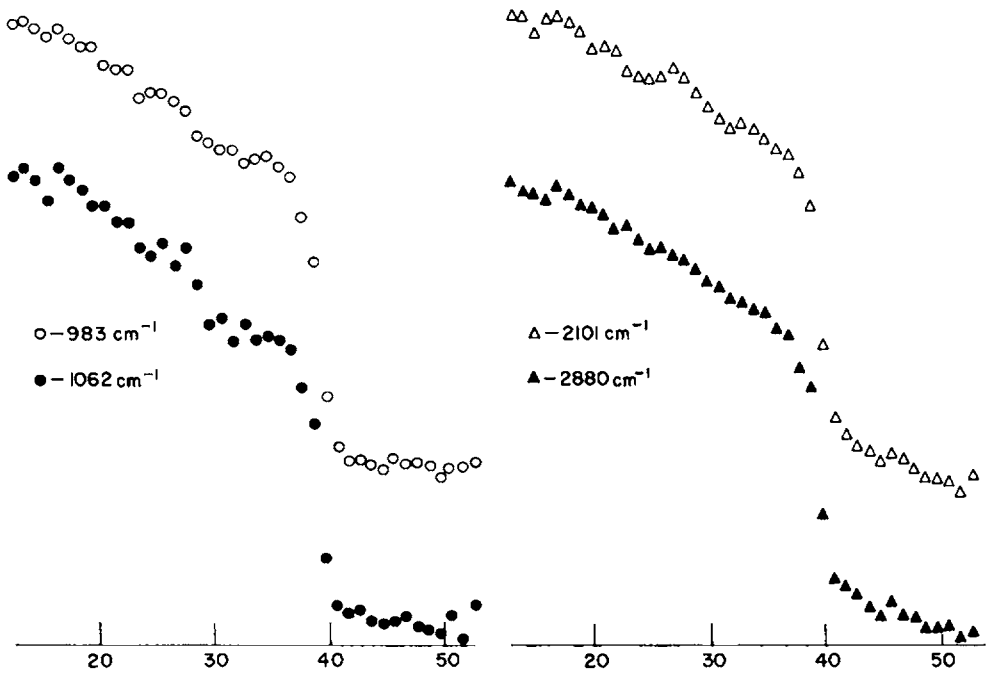

FIGURE 2 Melting curves of four Raman bands of DH-DPPC. The magnitudes of the changes are not to scale, as each of the four sets of points was arbitrarily scaled with the lowest and highest points as references, and superimposed to create the figure. Decrease in height of the curve is proportional to a decrease in the appropriate band relative to the C—N stretch.

an inflection at the premelt. As an inflection of similar magnitude has been seen for the 983 cm^{-1} band of DPPC-d_{62} (4), the inflection is probably not ascribable to alterations in chain-chain interaction between deuterated chains. Rather, there must be sufficient coupling between the CD_2 scissoring motion and C—C vibrations of the chains so that the inflection at the premelt results from an increase in number of *gauche* bonds in chain 1 as well.

Seen in Fig. 3*a*, *b*, and *c* are the spectra of HD-DPPC and DH-DPPC dispersions at 10°C, and the difference between these two spectra. In the difference spectra a positive deviation of a hydrocarbon band indicates stronger Raman scattering at that frequency for hydrocarbons in the 1 position than for hydrocarbons in the 2 position. Conversely, a positive deflection for a deuterocarbon band indicates stronger Raman scattering for a deuterocarbon in the 2 position. The presence of a complex pattern of sharp bands in the HD-DH spectra indicates that chains 1 and 2 of DPPC are conformationally nonequivalent. We have compared the HD-DH difference spectra at 10° to the "melting difference" spectra of Fig. 1 as a basis for analyzing the differences in static conformations between chains 1 and 2, using the terminology "more liquidlike" or "less liquidlike" to describe the qualitative differences observed.

In Fig. 3*a* there is no difference between the CH_2 scissoring bands (ca. 1,450 cm^{-1}) of the two compounds. For DPPC, as well as model compounds such as the *n*-alkanes, the scissoring modes, like the C—H stretching modes, are sensitive to changes of both inter- and intrachain interaction occurring upon premelting and melting (7) as a result of vibrational coupling between adjacent chains. The previously mentioned dilution of vibrational interactions with neighboring hydrocarbon chains may result in the insensitivity of this band. However, a fairly weak inflection in the CH_2 twist band centered at 1,300 cm^{-1} is seen. By comparison with Fig. 1*a*, this change in the 1,300 cm^{-1} region appears comparable to that seen upon melting and indicates that hydrocarbon chain 2 in DH-DPPC is more "liquidlike" than the hydrocarbon chain in HD-DPPC. The origin of the downward deflection at 1,306 cm^{-1} in Fig. 3 may be seen by careful inspection of the CH_2 twist band of DH-DPPC, which shows a definite shoulder at approximately 1,306 cm^{-1}. The corresponding change on melting of HD-DPPC (see upper trace of Fig. 1*a*) is a broadening of the 1,300 cm^{-1} band.

Three important hydrocarbon bands—the all-*trans* skeletal vibrations at 1,129 cm^{-1} and 1,062 cm^{-1} and the *gauche* marker near 1,080 cm^{-1} (7, 8, 13)—are strongly perturbed in melting difference spectra (e.g., Fig. 1*a*). The bands are nearly absent in the HD-DH spectrum, with the exception of a weak positive peak at 1,131 cm^{-1}. This latter band may arise because of a longer all-*trans* segment in chain 1. The order parameter S_{trans} for hydrocarbon chains (8) can be determined for HD-DPPC and DH-DPPC dispersions at 10°C relative to solid HD-DPPC. For HD-DPPC S_{trans} = 0.87 (identical to DPPC) (8) and for DH-DPPC S_{trans} = 0.74, indicating that the number of bonds in all-*trans* segments is lower in chain 2 than in chain 1 by 1–1.5 bonds. In both chain positions the *gauche* bonds are likely to be constrained to be near an end of the chain.

The CD_2 twisting mode at 917 cm^{-1} in HD-DPPC is seen at 913 cm^{-1} in DH-DPPC

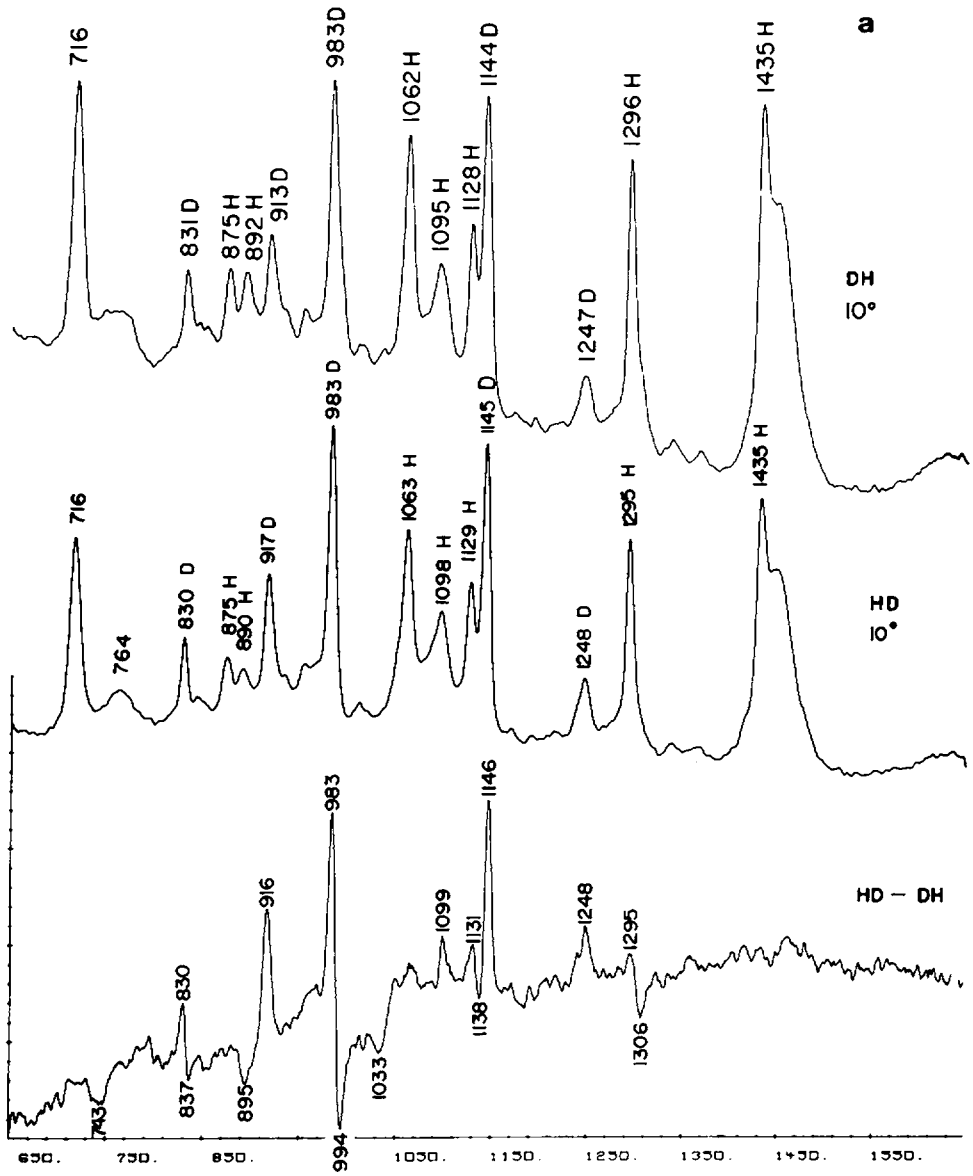

FIGURE 3 (a) Spectra of DH-DPPC and HD-DPPC dispersions at 10°C, and the difference between these spectra. The two absolute spectra are not to scale, and the difference spectrum has been substantially vertically expanded. Peaks uniquely ascribable to either hydrocarbons or deuterocarbons are so labeled. (b) Spectra of DH-DPPC and HD-DPPC dispersions at 10°C, and the difference between these spectra. The two absolute spectra are not to scale, and the difference spectrum has been substantially vertically expanded. (c) Spectra of DH-DPPC and HD-DPPC dispersions at 10°C, and the difference between these spectra. The two absolute spectra are not to scale, and the difference spectrum has been substantially vertically expanded.

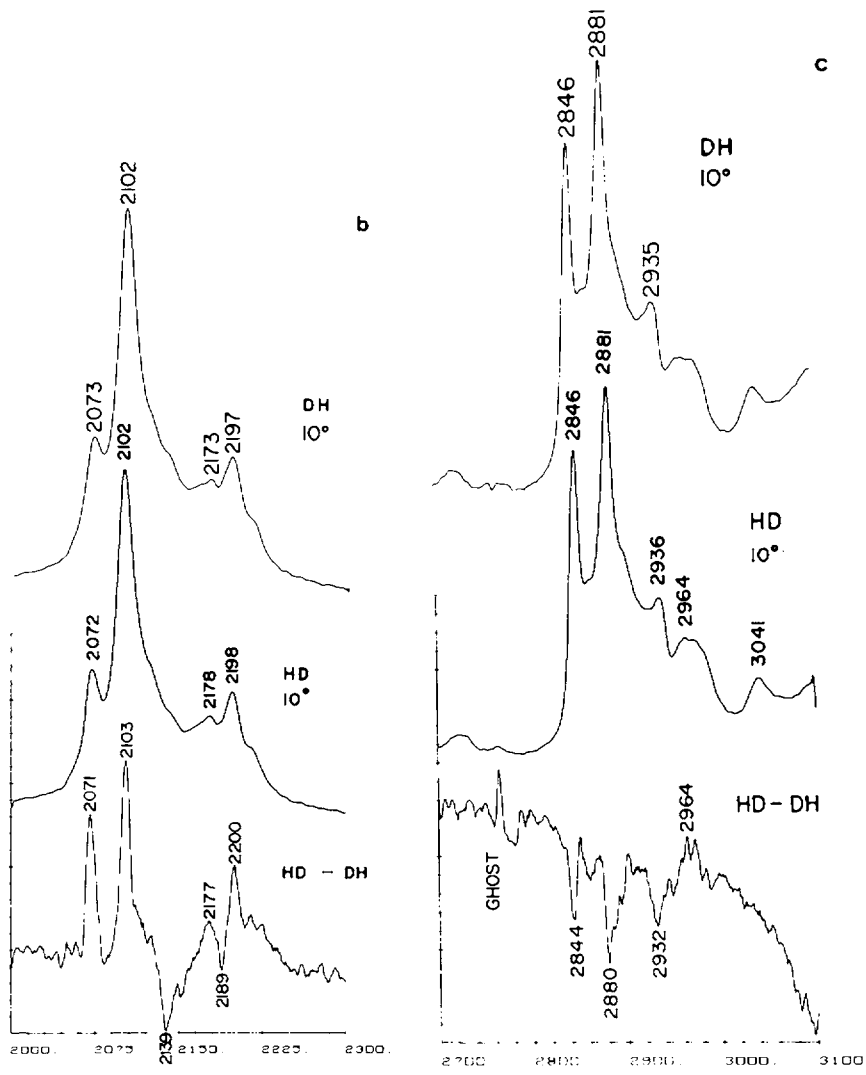

FIGURE 3 *b* and *c*

(see Fig. 3*a*). This shift appears in the difference spectrum as a trough at 895 cm^{-1} and a peak at 916 cm^{-1}. This band normally shifts to higher Δcm^{-1} upon melting (4), which again indicates that chain 2 is more "liquidlike" than chain 1, but a note of caution must be added. The largest grating ghost produced by our monochromator falls at 896 cm^{-1}, so Raman bands at this Δcm^{-1} shift are susceptible to artifacts.

Two prominent features in the HD-DH difference spectrum require special attention. One is the distinctive derivative-type band with a positive deflection at 983 cm^{-1} and a negative deflection at 994 cm^{-1}. There are no hydrocarbon bands at this frequency, and in DPPC-d_{62} the 984 cm^{-1} band is substantially broader than in either

HD-DPPC or DH-DPPC (see reference 4 for comparison). This band is known to shift to lower frequency upon melting of both hexadecane-d_{34} and DPPC-d_{62} (4), so the shape of this difference band in the HD-DH difference spectrum is consistent, although the analogy with the melting difference spectrum (Fig. 1*a*) is inexact. The sharp negative deflection at 994 cm^{-1} in the HD-DH data is absent in Fig. 1*a*, and in melting difference spectra for DPPC-d_{62} (4) as well. Upon isolation of hexadecane-d_{34} in a matrix of hexadecane,[2] an *n*-alkane model system, the position of the 984 cm^{-1} band shifts continuously downward by up to 10 cm^{-1}. The band is, therefore, sensitive to crystal interactions.

Although the strong difference band at 1,146 cm^{-1} supports the contention that the conformations of the two chains are not identical, the sign of the band is difficult to reconcile with the interpretation developed so far. Intense positive difference bands are characteristic of both HD-DPPC (Fig. 1*a*) and DPPC-d_{62} (4) melting difference spectra, implying that chain 2 of HD-DPPC is more "solid" than chain 1 of DH-DPPC. Like the 1,131 cm^{-1} hydrocarbon band, the 1,146 cm^{-1} deuterocarbon band is partially a skeletal optical mode, deriving intensity from all-*trans* segments. But the 1,146 cm^{-1} mode also contains a considerable contribution from CD_2 scissoring and other motions,[3] so peak height at 1,148 cm^{-1} need not be directly correlated with the all-*trans* segment length.

Although the HD-DH difference spectrum in Fig. 3*b* is not identical to the melting difference spectrum observed in Fig. 1*b*, there are similarities, even though the relative intensities of the 2,071 and 2,103 cm^{-1} bands are quite different. The bands at 2,071 and 2,139 cm^{-1} represent the CD_3 stretching mode split by Fermi resonance (4). Unlike hydrocarbon methyl vibrations, the CD_3 stretches of the terminal methyls of deuterocarbons appear to be quite sensitive to perturbations of the end group (4). Fig. 3*b* suggests that the environments of the terminal methyls of the two fatty acyl chains are different, and that chain 1 is more "liquidlike" than chain 2 near the methyl terminus.

The spectra seen in Fig. 3*c* are consistent with the data observed in the 1,450 cm^{-1} region of Fig. 3*a*; that is, the differences between the hydrocarbon bands as shown in those regions normally sensitive to "lateral" hydrocarbon chain packing, are small.

CONCLUSIONS

We have shown that the Raman spectra of the two compounds we have synthesized differ. No artifacts appear to be able to account for the full range of spectral differences observed. The most obvious artifact would result from incorrect or incomplete synthesis of the partially deuterated DPPCs. These include acyl migration of the deuterated and undeuterated chains, but rearrangement of the hydrocarbon and deuterocarbon chains during synthesis in either compound (or both) would merely

[2]Yager, P. Unpublished observations.
[3]Fanconi, B. Private communication.

result in a decrease of the difference signal, rather than generation of spurious differences.

An alternate possibility is that during the synthesis of HD-DPPC, for example, some "double deuteration" occurred. Under such conditions, one would expect that HD-DPPC would have an excess of perdeuterated fatty acyl chains. In this case difference spectra normalized to the C—N stretch would leave all of the deuterated bands positive and all the undeuterated bands negative. Such is not the case. Further, the isotopic purity of the starting materials was such that there was little likelihood that differing degrees of deuteration of the individual "perdeuterated" fatty acyl chains could generate difference signals.

The sharp transition observed in DSC of these samples (see footnote 1) rules out contamination by small amounts of fatty acid and lysolecithin. They would produce appreciable broadening of the endothermic transitions, and certain diminution of the pretransition (11). Those differences that appear must be structurally significant.

Some general conclusions may be drawn from the Raman data: Both chains have nearly the same fraction and distribution of *trans* bonds and, below T_m, neither has a large concentration of close-coupled *gauche* rotamers. Thus, both chains are extended and have superficially similar geometries. The chains differ in the geometry with which they terminate near the ester linkage and at the bilayer center.

The contribution to the HD-DH difference spectrum of deuterocarbon bands appears disproportionately large. We have earlier noted that deuterocarbons, and in particular DPPC-d_{62} (4), are more sensitive than hydrocarbons to the conformation of the terminal methyl groups of the hydrocarbon chains, whereas in these difference spectra we see little or no change in the hydrocarbon CH_2 scissoring and stretching regions. This indicates that the "lateral" packing environments of chains 1 and 2 are relatively equivalent, but the environments of the terminal methyl groups differ. As the deuterocarbon chains are, then, most sensitive to the interface between the two monolayers that compose the phospholipid bilayer structures in these dispersion samples, it may be that the chain 1 terminal methyl group is more strained than the other by its contact with the opposing bilayer surface. The 1 chain, although it is probably more all-*trans* near the glycerol backbone, is distorted near the terminal methyl group, whereas the 2 chain, though more distorted near the glycerol backbone, is more all-*trans* near its terminal methyl group.

The Raman data may be rendered more complex because, if Seelig and Seelig (2) are correct in their analysis of data above T_m, the glycerol backbone may, at temperatures below T_m, exist in two different conformations. The present comparison is, then, not between two homogeneous populations but is in fact a superposition of four difference spectra. Certain of these spectral changes, such as the shoulder observed in the DH-DPPC spectrum at approximately 1,306 cm^{-1}, may represent a contribution from a component in which chain 2 is in a particularly "*gauche*-like" conformation.

Even though HD-DPPC and DH-DPPC are well suited for this type of Raman experiment, other uses for similar compounds are easily imagined. Such compounds

allow one, by Raman spectroscopy, to monitor the conformations and environments of chains 1 and 2 of a phospholipid simultaneously and separately. Such a "probe molecule" would, for example, be of great utility for the study of binding interactions between membrane proteins that show specificity as to the nature of the hydrocarbon chain.

It has been brought to our attention by a referee that our conclusions are similar to those of Dennis et al. (14, 15) who, in lipid-detergent mixed micelles, showed the nonequivalence of fatty acyl chains using NMR techniques.

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