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EFFECT OF WATER ON THE MOLECULAR STRUCTURE OF A PHOSPHATIDYLCHOLINE HYDRATE

RAMAN SPECTROSCOPIC ANALYSIS OF THE PHOSPHATE, CARBONYL AND CARBON-HYDROGEN STRETCHING MODE REGIONS OF 1,2-DIPALMITOYLPHOSPHATIDYLCHOLINE DIHYDRATE

ERNEST MUSHAYAKARARA, NORMAN ALBON and IRA W. LEVIN *

Laboratory of Chemical Physics, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)

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The Raman spectra of 1,2-dipalmitoylphosphatidylcholine (DPPC) dihydrate crystals were examined in (a) the 1250 and 1080 cm^{-1} PO_2^- antisymmetric and symmetric stretching mode regions, (b) the 1730 cm^{-1} carbonyl stretching mode region and (c) the 2800–3100 cm^{-1} C-H stretching mode interval. No evidence was observed for hydrogen bonding effects involving the nonester phosphate oxygen atoms. Spectra of the DPPC dihydrate crystal in the 1250 cm^{-1} region are compared to spectra of both anhydrous and successively hydrated systems and to spectra of crystalline glycerylphosphorylcholine. The splitting pattern in the 1240 to 1280 cm^{-1} interval for the DPPC dihydrate was interpreted in terms of the different bonding arrangements assumed by the PO_2^- groups in the two molecule asymmetric unit of the eight molecule unit cell. An unusual triplet is observed in the carbonyl stretching mode region around 1730 cm^{-1}. These features reflect differing bond environments, relative to the polar and hydrophobic regions of the bilayer, of the 1- and 2-chain carbonyl groups. Spectra for crystalline DPPC dihydrate in the 3000 cm^{-1} C-H stretching mode region suggest a hybrid chain packing lattice which is analogous to the packing arrangement determined for the isostructurally similar DMPC dihydrate species.

Introduction

Recently, Pearson and Pascher [1] presented an X-ray diffraction analysis for the bilayer structure of crystalline 1,2-dimyristoylphosphatidylcholine (DMPC) dihydrate. In their account the authors concluded that within the polar region of the crystal bilayer a strong hydrogen bonding environment exists for the nonester phosphate oxygen atoms [1]. In contrast, Raman [2] and ^{31}P-NMR [3] studies involving lipid-water interactions with polycrystalline samples of 1,2-dipalmitoylphosphatidylcholine (DPPC) provided no definitive evidence for hydrogen bonding to the phosphate moiety. These results suggested that the first two water molecules of hydration are not involved in PO_2^- hydrogen bonds [2] and that the addition of the third and fourth molecules of water most probably leads to a structural rearrangement of the entire headgroup [2,3]. Although the ^{31}P spectral effects were more plausibly interpreted in terms of a motional averaging of the PO_4^- group, the alternative hypothesis of hydrogen bonding to the phosphate oxygens could not be completely ex-

* To whom correspondence should be addressed.

cluded [3]. In order to further our understanding of the molecular complexities occurring in the various phosphatidylcholine-water phases, we examine in the present report the vibrational Raman spectra of highly purified crystalline DPPC dihydrate in the context of the molecular structure reported for the dihydrate of DMPC [1]. We emphasize specifically an analysis of the PO_2^- antisymmetric and symmetric stretching mode regions and note that the spectra are satisfactorily interpreted without invoking hydrogen bond considerations. Further, we examine the spectra of crystalline glycerylphosphorylcholine, a system in which the glycerol hydroxyl groups are reported to form intermolecular hydrogen bonds with the phosphate groups [4]. As in the case of the phosphatidylcholine dihydrate, the Raman spectra are not indicative of hydrogen bond formation. Finally, the Raman spectra for the carbonyl and carbon-hydrogen stretching mode regions for the dihydrate of DPPC are interpreted in terms of the molecular features previously elucidated for the isostructural DMPC dihydrate.

Experimental

1,2-Dipalmitoylphosphatidylcholine (DPPC) was prepared from highly purified palmitic acid and glycerylphosphorylcholine and further purified by the crystallization procedures previously described [5,6]. The samples were composed of small crystals of the dihydrate phase 2 [5]. An examination of aqueous dispersions of the crystalline dihydrate by high sensitivity differential scanning calorimetry revealed extraordinarily sharp transitions, indicating a purity greater than 99.94% [6]. Early details of the space group and cell dimensions [5] have been confirmed through extensive diffractometer data [7]. Since d_{001} DMPC + 5.1Å $= c/2$ DPPC, the present crystal is evidently isostructural with the reported DMPC dihydrate [1]. The doubling of the C axis is evidently due to an alternation in chain tilt which does not change the packing appreciably, as the tilt angle is small.

Raman spectra were recorded with a Spex Ramalog 6 spectrometer, equipped with plane holographic gratings, with approx. 3–4 cm^{-1} spectral resolution. Since the PO_2^- and C=O stretching modes exhibit quite weak Raman features, a moderately wide spectral band pass was utilized. Excitation radiation of approx. 200 mW at 514.5 nm was provided by a Coherent Model CR-12 argon ion laser. Spectral frequencies are reported to ± 2 cm^{-1}. Raman data were acquired with a Nicolet NIC-1180 data system interfaced to the spectrometer. Depending upon the spectral region under consideration, 3 to 40 scans, acquired at a rate of 1 cm^{-1}/s, were signal averaged. For spectroscopic viewing, the crystalline samples of the DPPC dihydrate were placed in a glass capillary tube, sealed and thermostatically maintained at 7.0 ± 0.1°C.

Results and Discussion

A. PO_2^- antisymmetric stretching modes: 1250 cm^{-1} region

Before we describe in detail the spectra for the phosphatidylcholine dihydrate system, we first review the Raman spectral observations for both anhydrous and hydrated polycrystalline DPPC [2]. For samples recrystallized from chloroform and then dried at 10^{-5} torr for 48 h to ensure the complete removal of water, the PO_2^- antisymmetric mode is located at 1253 cm^{-1} [2]. As described earlier, the addition of two molecules of water leaves the frequency unchanged, although the intensity of the spectral features decreases slightly upon addition of the second water molecule. If a strong hydrogen bond is formed with the phosphate oxygen, one would expect frequency decreases of the order of 20–80 cm^{-1} for the PO_2^- stretching modes [8]. As the third and fourth molecules of water are added, the frequency of the mode only decreases to 1243 cm^{-1}. Since this shift occurs concomitantly with an increase in the headgroup C-N stretching mode from 711 to 717 cm^{-1}, the change in frequency of the PO_2^- mode to 1243 cm^{-1} is interpreted in terms of a headgroup conformational rearrangement rather than a hydrogen bonding effect. Additional evidence that the 1243 cm^{-1} value for the antisymmetric PO_2^- mode arises from a change in the phosphate orientation lies in the following consideration. After heating anhydrous DPPC to approx. 130°C and slowly cooling to ambient temperatures, the 1253 cm^{-1} mode shifts to 1243 cm^{-1}, the same frequency observed after the addition of the third

and fourth molecules of water (Levin, I.W. and Bush, S.F., unpublished data). Since no water molecules have been added to the anhydrous, heated and annealed sample, the frequency shift clearly arises from a reorientation of the phosphate moiety as the sample changes its crystalline nature. Samples of oriented, dehydrated DPPC crystals have also been extensively examined by X-ray techniques (Albon, N., Biophys. J., submitted for publication). These studies indicate that the complete removal of water induces changes in packing which require different molecular conformations. The Raman data for polycrystalline DPPC samples are then consistent with the ^{31}P-NMR conclusion that the first four to five molecules of water induce a rotation of the PO_4^- group and probably do not participate in the formation of strong hydrogen bonds with the phosphate oxygen atoms. It is interesting to note that the frequency of the PO_2^- antisymmetric mode remains at 1243 cm^{-1} in DPPC dispersions composed of 30% water by weight. This observation suggests that further hydration of DPPC does not result in spectral changes that can be associated with hydrogen bond characteristics.

Figs. 1A and 1B display the PO_2^- antisymmetric stretching mode region determined in the present study for DPPC dihydrate and crystalline glycerylphosphorylcholine, respectively. For the phosphatidylcholine dihydrate three features appear at 1244, 1262 and 1276 cm^{-1}. Although the DPPC dihydrate has eight molecules per unit cell with two molecules per asymmetric unit [5], we would expect four spectral features corresponding to the differing bond arrangements of the two PO_2^- groups. Depending upon the strength of the coupling between molecules, these features may be further split by factor group considerations. (The 1262 cm^{-1} feature, for example, may be composed of two components.) Although only three primary Raman transitions are observed in the phosphate region, the fourth mode may be obscured by the intense 1296 cm^{-1} acyl chain CH_2 twisting transitions. It is tempting to associate the 1253 cm^{-1} frequency for the disordered anhydrous system whith the mean of the 1243 and 1262 cm^{-1} components observed in the dihydrate. Since the antisymmetric phosphate modes in the DPPC dihydrate crystal may be attributed to vibrational frequencies originating from conformational differences within the PO_2^- group, we find no compelling evidence to support the existence of strong hydrogen bonds in the phosphatidylcholine dihydrate system. In particular, no PO_2^- frequencies appear below approx. 1243 cm^{-1}, a frequency related to a change in geometry of the phosphate moiety. Further, we would expect to observe the same spectral behavior for a DMPC dihydrate crystal.

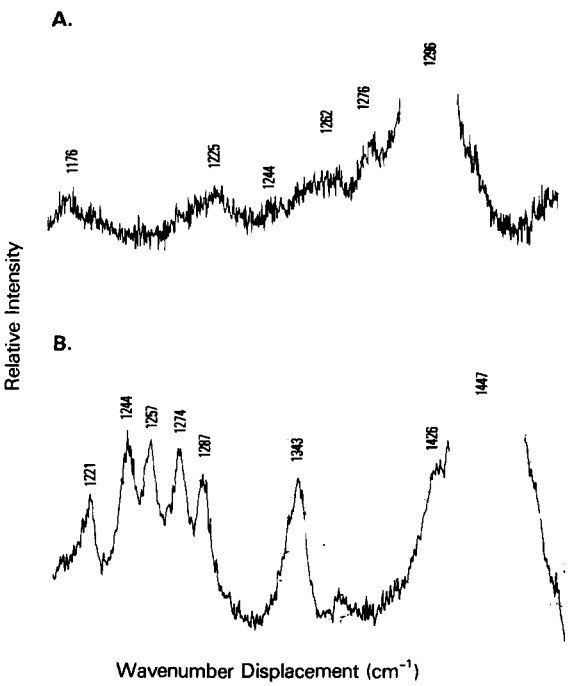

Fig. 1. Raman spectra of the PO_2^- antisymmetric stretching mode region of (A) crystalline DPPC dihydrate and (B) crystalline glycerylphosphorylcholine at 7°C.

For glycerylphosphorylcholine four vibrational transitions appear in the PO_2^- antisymmetric stretching mode region at 1244, 1257, 1274 and 1287 cm^{-1} (Fig. 1B). The feature at 1221 cm^{-1} in glycerylphosphorylcholine appears at approx. 1220 cm^{-1} in DPPC and is absent in dipalmitoylglycerol. A second prominent feature in this region appears at 1343 cm^{-1}. We tentatively assign the latter two modes at 1221 and 1343 cm^{-1} in glycerylphosphorylcholine to headgroup CH_2 twisting vibrations. Since the crystal analysis of glycerylphosphorylcholine indicates a unit cell consisting of two sets of equivalent molecules with

two molecules per set [4], we assign the two doublets at 1244, 1257 and 1274, 1287 cm^{-1} to factor group splittings for each set of equivalent molecules. (Note that each doublet is split by 13 cm^{-1}). Since one set of equivalent molecules possesses slightly longer PO_2^- bonds [4], we attribute the lower frequency 1244, 1257 cm^{-1} doublet to the vibrational distortions involving the longer P-O bond lengths. (For the two independent molecules forming the asymmetric unit, molecule one has P-O bond lengths of 1.497 Å, while molecule two has P-O bond lengths of 1.467 and 1.484 Å [4].

Abrahamson and Pascher [4] further postulate hydrogen bonds between a given P-O group and the glycerol hydrogen atoms from both a symmetrical and a *b* axis-translated molecule. Since the observed PO_2^- stretching mode frequencies do not correspond to expected hydrogen bonded values (vide supra), the suggestion that glycerylphosphorylcholine forms intermolecular hydrogen bonds through the glycerol moieties [4] must be considered with caution.

The PO_2^- symmetric stretching regions for the DPPC dihydrate and for glycerylphosphorylcholine are presented in Figs. 2A and 2B, respectively. These modes appear as weak transitions within the manifold of intense C-C stretching modes. The phosphate stretching mode is assigned to the feature at 1077 cm^{-1}, which is in good agreement with the 1081 cm^{-1} assignment for the PO_2^- symmetric stretching mode in lysophosphatidylcholine [9]. A suggestion of the second member of a correlation field doublet appears at 1072 cm^{-1}. The strong 1062 cm^{-1} C-C stretching mode precludes complete resolution of these features at this temperature. The phosphate symmetric stretching mode is not observed as a distinct feature in either polycrystalline DPPC at room temperatures or in aqueous dispersions of the phosphatidylcholines. At liquid nitrogen temperatures (approx. 80 K), however, the factor group components for anhydrous polycrystalline DL-DPPC and L-DPPC are observed clearly at 1077 and 1073 cm^{-1} and at 1082 and 1074 cm^{-1}, respectively (Levin, I.W. and Hill, I.R., unpublished data). Comparisons of the dihydrate crystals with anhydrous DPPC systems for this spectral interval again imply no strong hydrogen bonding characteristics.

Fig. 2B displays the PO_2^- symmetric stretching

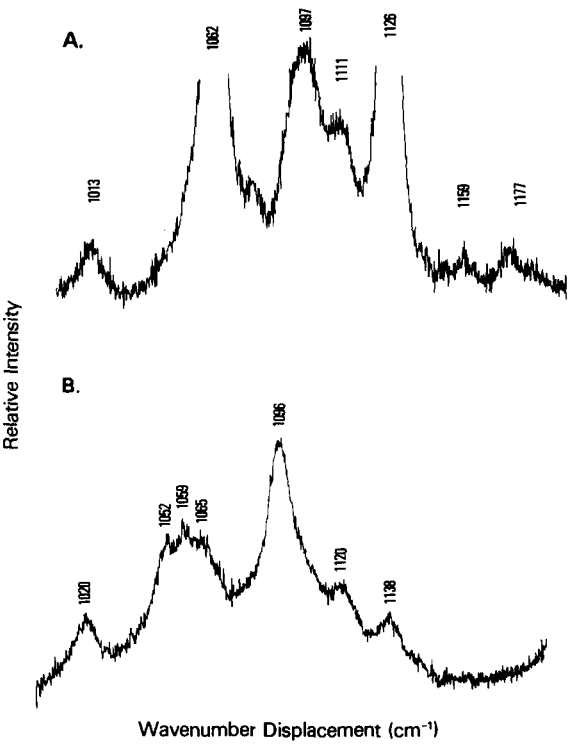

Fig. 2. Raman spectra of the PO_2^- symmetric stretching mode region of (A) crystalline DPPC dihydrate and (B) crystalline glycerylphosphorylcholine at 7°C.

mode region for glycerylphosphorylcholine. The broad feature in the 1050–1075 cm^{-1} interval probably represents the overlapping of several vibrational modes. The 1052 cm^{-1} mode, seen as a shoulder in DPPC and observed more clearly at 1051 cm^{-1} in dipalmitoylglycerol (spectra not shown) is assigned to an ester C-O stretching mode [9]. We tentatively associate the 1059 cm^{-1} frequency in Fig. 2B with either a second ester C-O mode or a headgroup C-C stretching vibration. The region from 1065–1075 cm^{-1} probably encompasses the PO_2^- symmetric stretching mode region. In comparing glycerylphosphorylcholine and DPPC spectra for this interval, these small frequency shifts for glycerylphosphorylcholine most likely reflect slight headgroup conformational changes rather than hydrogen bonding perturbations.

B. C=O stretching modes: 1730 cm^{-1} region

Fig. 3 displays the Raman spectrum of the

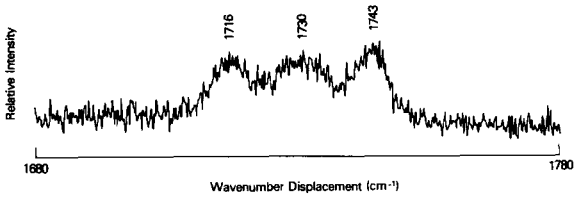

Fig. 3. Raman spectrum of the carbonyl stretching region of crystalline DPPC dihydrate at 7°C.

crystalline dihydrate in the C=O stretching mode region in the 1680–1780 cm^{-1} interval. This spectral pattern is quite different from the usual polycrystalline spectrum in which the components of a doublet are assigned to the 1- and 2-chain carbonyl groups, respectively [2,10]. The three prominent features at 1716, 1730 and 1743 cm^{-1} in Fig. 3 may be accounted for by the four conformationally inequivalent carbonyl groups of the two molecule asymmetric unit for the dihydrate [1]. As the DPPC dihydrate bilayers are structurally similar to those of the dihydrate of DMPC [1], the approx. 2.5 Å relative displacements for the two phosphatidylcholine molecules in the direction of the bilayer normal would have the effect of placing the carbonyl groups of one of the molecules closer to the polar region defined by the headgroups. With the carbonyl stretching frequency being determined by the dielectric constant of the surrounding medium [11], the carbonyl groups closer to the polar headgroup region would exhibit the lower frequency values. Since the two hydrocarbon chains within a molecule are offset by 2.7 Å [1] as a consequence of the bend in the 2-chain at the α-CH_2 group, the carbonyl group associated with 1-chain is placed more deeply within the hydrophobic region [10]. The 2-chain carbonyl group is then located closer to the polar region of the bilayer [10]. Thus, in reference to Fig. 3, we assign the highest frequency at 1743 cm^{-1} to the 1-chain carbonyl vibration of molecule A, since the C=O unit is located more deeply within the hydrophobic portion of the bilayer. The lowest frequency transition at 1716 cm^{-1} is then attributed to the 2-chain carbonyl group of molecule B; that is, this carbonyl moiety experiences the most polar environment. Since the central feature of the triplet at approx. 1730 cm^{-1} is broader than the flanking peaks at 1716 and 1743 cm^{-1} (see Fig. 3), we assign the 1730 cm^{-1} transition to an overlapping of the vibrational modes originating in the 1-chain carbonyl group of molecule B and the 2-chain carbonyl group of molecule A. As expected, there is no evidence of hydrogen bonding effects involving the interfacial carbonyl modes [2,10]. A hydrogen bonded carbonyl mode would occur at lower frequencies and exhibit a broadened band shape. For example, dipalmitoylglycerol, which should exhibit hydrogen bonding effects originating between the free OH and the 2-chain carbonyl group, shows a broadened, shifted Raman feature at 1704 cm^{-1} (Mushayakarara, E., Albon, N. and Levin, I.W., unpublished data).

C. C-H acyl chain stretching modes: 2800–3000 cm^{-1} region

Although the 2800–3000 cm^{-1} methylene and methyl C-H stretching region reflects a complex series of overlapping vibrational transitions, the peak height intensity ratios I_{2845}/I_{2880} for the CH_2 2845 cm^{-1} symmetric and 2880 cm^{-1} asymmetric stretching modes are particularly sensitive to chain packing characteristics [12,13]. For example, the peak height ratios determined from the Raman spectra [12] for a variety of *n*-alkane crystals range from approximately 0.35 for an orthorhombic structure (n-$C_{21}H_{44}$), 0.34 for a monoclinic strucutre (n-$C_{36}H_{74}$), 0.42 for a triclinic structure (n-$C_{20}H_{42}$) to 0.74 for an hexagonal arrangement (n-$C_{36}H_{74}$). Figs. 4A and 4B present the C-H region for the crystalline DPPC dihydrate system and for anhydrous, polycrystalline samples, respectively. While the I_{2845}/I_{2880} ratio of 0.72 for the polycrystalline material reflects the familiar hexagonal form, an intensity ratio of 0.55 for the dihydrate crystal (Fig. 4A) may imply a hybrid chain packing lattice analogous to that indicated by the X-ray analysis for the DMPC dihydrate [1]. In addition, a comparison of the I_{2845}/I_{2880} ratio for the DPPC dihydrate to the Raman ratio of 0.45 for crystalline dipalmitoylglycerol, whose acyl chains form an orthorhombic subcell (Refs. 7, 14 and unpublished Raman data, Mushayakarara, E., Albon, N. and Levin, I.W.), suggests for the dihydrate a packing situation having similar characteristics to an orthorhombic form. We emphasize that one must exert extreme caution in relating these

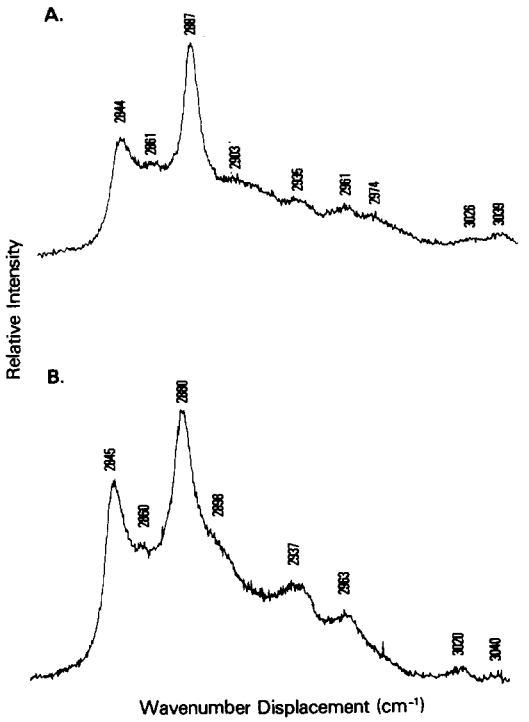

Fig. 4. Raman spectra of the C-H stretching region of (A) crystalline DPPC dihydrate at 7°C and (B) anhydrous, polycrystalline DPPC at room temperature.

ratios to a specific molecular ordering. For example, crystalline dipalmitoylglycerol and crystalline n-$C_{36}H_{74}$ are both monoclinic with identical orthorhombic perpendicular subcells. Further, the tilt angles are similar, although the monoclinic angle is different (Albon, N. and Craievich, A., J. Chem. Phys. Lipids, submitted for publication). Thus, the differences in peak height ratios of 0.45 and 0.34 for dipalmitoylglycerol and n-$C_{36}H_{74}$, respectively, suggests a sensitivity to extremely small packing differences. In comparing the dihydrate spectra to other systems, we note that the greater linewidth for the 2845 cm^{-1} transition relative to the 2880 cm^{-1} feature is also characteristic of an orthorhombic structure [13]. That is, the $\Delta\nu_{3/4}$ linewidths for hexagonal anhydrous DPPC in Fig. 4B are about 8 cm^{-1} for both the methylene stretching mode features; while for the dihydrate $\Delta\nu_{3/4}$ for the 2845 and 2880 cm^{-1} modes is approx. 11 and 6 cm^{-1}, respectively.

Pearson and Pascher [1] state that while the hybrid chain packing structure of alternately parallel and perpendicular chain planes in the DMPC dihydrate was only well defined near the carboxyl end, the chain planes orient, however, in a parallel, or triclinic manner, toward the methyl end. The presence of a weak transition at 2860 cm^{-1} for the DPPC dihydrate in Fig. 4A is consistent with an interpretation which allows for the presence of triclinic packing characteristics. Since interchain interactions of n-alkane triclinic crystals result in a doubling of the CH_2 symmetric stretching mode [12], the appearance of this 2860 cm^{-1} transition in phospholipid dispersions has been associated with a population of pairs of acyl chains oriented in a parallel or triclinic mode. The appearance of the 2860 cm^{-1} 'triclinic feature' in the spectrum for DPPC dihydrate (Fig. 4) suggests that this crystal also involves a packing form characteristic of a triclinic structure. Although the Raman data cannot describe the precise architectural arrangements for the hydrocarbon chains, contributions to the structure of the dihydrate crystal from both orthorhombic and triclinic chain packing modes are indicated.

In summary, vibrational Raman spectra of crystalline DPPC dihydrate provide no evidence for strong hydrogen bond formation with the headgroup PO_2^- moiety. Specifically, the spectral patterns observed for the PO_2^- antisymmetric stretching mode region around 1250 cm^{-1} do not exhibit frequency shifts indicative of phosphate hydrogen bonds. On the contrary, a comparison of the DPPC dihydrate spectra to spectra for anhydrous and successively hydrated crystalline systems suggests that the spectral features observed for the dihydrate crystal originate within the differing phosphate bond arrangements for the two lipid molecules forming the asymmetric cell. Further, the observed vibrational transitions for the PO_2^- symmetric stretching region are also consistent with the absence of hydrogen bonding effects entailing the phosphate oxygen atoms. The unusual spectral triplet observed in the 1730 cm^{-1} carbonyl stretching mode region reflects the packing characteristics of the four carbonyl groups involved in the asymmetric unit of the DPPC hydrate crystal. In particular, the relative locations of the 1- and 2-chain carbonyl groups with respect to the polar and hydrophobic regions of the crys-

talline bilayer account both for the overall splitting pattern and for the specific increase in broadness of the central 1730 cm^{-1} feature. Finally, comparisons of the C-H stretching mode spectra of the present system to Raman spectra for extended hydrocarbon chains in known crystal structures suggest for the DPPC dihydrate crystal a hybrid chain packing lattice similar to that determined by X-ray for DMPC dihydrate.

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