Components of the Carbonyl Stretching Band in the Infrared Spectra of Hydrated 1,2-Diacylglycerolipid Bilayers: A Reevaluation

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ABSTRACT Previous vibrational spectroscopic studies of solid acyl-alkyl and diacyl phosphatidylcholines suggested that the *sn*1- and *sn*2- carbonyl stretching modes of 1,2-diacylglycerolipids have different absorption maxima. To address the assignment of *sn*1- and *sn*2- carbonyl stretching modes of hydrated 1,2-diacylglycerolipids, aqueous dispersions of 1-palmitoyl-2-hexadecyl phosphatidylcholine (PHPC), 1-hexadecyl-2-palmitoyl phosphatidylcholine (HPPC), 1,2-dipalmitoylphosphatidylcholine (DPPC), as well as hydrated samples of unlabeled, *sn*1-^{13}C=O-labeled, *sn*2-^{13}C=O-labeled, and doubly ^{13}C=O-labeled dimyristoylphosphatidylcholine (DMPC) were examined by Fourier transform infrared spectroscopy. The ester carbonyl stretching ($\nu_{C=O}$) bands of HPPC and PHPC each exhibit maxima near 1726 cm^{-1} and appear to be a summation of three subcomponents with maxima near 1740 cm^{-1}, 1725 and 1705–1711 cm^{-1}. In contrast, the $\nu_{C=O}$ band of DPPC exhibits its maximum near 1733 cm^{-1} and appears to be a summation of two components centered near 1742 and 1727 cm^{-1}. Thus the ester carbonyl group of the acyl-alkyl PCs appears to reside in a more polar environment than the ester carbonyl groups of their diacyl analogue. This observation implies that the polar/apolar interfaces of hydrated bilayers formed by PHPC and by HPPC are significantly different from that of DPPC and raises the question of whether the acyl-alkyl PCs are suitable models of their diacyl analogue. The absorption maximum of the $\nu_{C=O}$ band of the doubly ^{13}C=O-labeled DMPC occurs near 1691 cm^{-1} and those of its subcomponents occur near 1699 and 1685 cm^{-1}. These frequencies are consistent with a ^{12}C=O/^{13}C=O 'isotopic shift' of 42–43 cm^{-1}. *sn*1- and *sn*2-^{13}C=O-labeled DMPC each exhibit well resolved ^{12}C and ^{13}C $\nu_{C=O}$ bands with absorption maxima near 1734 and 1692 cm^{-1}, respectively. With both specifically ^{13}C=O-labeled lipids, the ^{12}C and ^{13}C $\nu_{C=O}$ bands each seem to be a summation of subcomponents with absorption maxima near 1742 and 1727 cm^{-1} (^{12}C $\nu_{C=O}$) and 1699 and 1685 cm^{-1} (^{13}C $\nu_{C=O}$), regardless of whether the ^{13}C=O-labeled fatty acyl chain is esterified at the *sn*1- or *sn*2- positions of the glycerol backbone. We conclude that in hydrated 1,2-diacyl PC bilayers, the patterns of infrared absorption exhibited by ester carbonyl groups located at the primary and secondary positions of the glycerol backbone are similar. Also, the resolvable subcomponents of their $\nu_{C=O}$ bands are each a summation of comparable contributions from both ester carbonyl groups and therefore cannot be attributed to the inequivalent locations of the two ester carbonyl groups. This result differs from that of the vibrational spectroscopic studies alluded to above and raises the question of whether data obtained in studies of dry (or poorly hydrated) lipids are applicable to fully hydrated lipid bilayers. To address questions of why the results of the two studies differ, we have also examined the $\nu_{C=O}$ bands of solid samples of DPPC, HPPC, and PHPC. We find that the $\nu_{C=O}$ bands of all solid lipids studied differ from those of the hydrated samples. Moreover, with solid lipids the $\nu_{C=O}$ bands vary with the enantiomeric configuration, enantiomeric purity and thermal thermal history as well as with the way in which the sample was prepared. Also, although the $\nu_{C=O}$ bands of solid HPPC and PHPC vary significantly with sample preparation methodology, samples of PHPC and HPPC prepared by the same method exhibit very similar $\nu_{C=O}$ absorption bands. We conclude as far as the organization of lipid polar/apolar interfaces is concerned, solid lipids are not good models of hydrated lipid bilayers and suggest that this may be largely responsible for the different conclusions drawn in this work and in previously published studies.

INTRODUCTION

The infrared spectra of diacylglycerolipids and other O-acylated lipids contain strong absorption bands arising from the stretching vibrations of their ester carbonyl groups. These $\nu_{C=O}$ absorption bands are conformationally sensitive, are responsive to changes in the polarity of their local environments, and are influenced by hydrogen bonding and other interactions with appropriate ligands. Also, for most naturally occurring O-acylated lipids and their synthetic analogues, the $\nu_{C=O}$ absorption bands occur in a region of the infrared spectrum (1650–1780 cm^{-1}), which is essentially free of significant absorptions by other infrared-active groups. These properties have proven to be very useful in IR spectroscopic studies of diacylglycerolipid bilayers because changes in the contours of the $\nu_{C=O}$ absorption bands can often be interpreted in terms of changes in the structure or hydration of the bilayer polar/apolar interface.

The $\nu_{C=O}$ absorption bands of acylated lipid bilayers usually appear to be a summation of underlying components, the properties of which tend to change with the prevailing experimental conditions (for examples see Mendelsohn and Mantsch, 1986 and references cited therein). With fully

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Abbreviations used: PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3- phosphorylcholine; PHPC, 1-palmitoyl-2-hexadecyl-*sn*-glycero-3- phosphorylcholine; HPPC, 1-hexadecyl-2-palmitoyl-*sn*-glycero-3- phosphorylcholine; IR, infrared; NMR, nuclear magnetic resonance; C=O, carbonyl; $\nu_{C=O}$, carbonyl stretching; L_β, lamellar gel phase; L_α, lamellar liquid-crystalline phase; L_c, lamellar crystalline phase.

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hydrated bilayers composed of the common diacylglycerolipids, the ester $\nu_{C=O}$ absorption bands are usually resolvable into two comparably intense components which exhibit maxima near 1743 and 1728 cm^{-1}. It was originally suggested that the underlying components of the $\nu_{C=O}$ absorption bands may arise from inequivalent infrared absorption by the stretching vibrations of the two structurally inequivalent ester carbonyl groups present in the lipid molecule. Such a possibility was experimentally supported by vibrational spectroscopic studies of solid DPPC and its acyl-alkyl analogues which suggested that the high- and low-frequency components of the $\nu_{C=O}$ absorption band arise from acyl groups esterified to the *sn*1- (i.e., primary) and *sn*2- (i.e., secondary) positions of the glycerolipid moiety, respectively (see Levin et al., 1982 and references cited therein). This assignment seemed compatible with known or generally assumed conformational inequivalence between the *sn*1 and *sn*2 ester groups of phospholipid bilayers (Hitchcock et al., 1974; Pearson et al., 1979; Hauser et al., 1981, 1988) and constituted the basis for interpretation of the $\nu_{C=O}$ bands observed in IR spectroscopic studies of hydrated lipid bilayers for some time. However, such an assignment has been seriously questioned in recent IR spectroscopic studies of fully hydrated, specifically $^{13}C{=}O$-labeled phospholipid bilayers (Blume et al., 1988; Lewis and McElhaney, 1992). Using hydrated bilayers composed of *sn*2-$^{13}C{=}O$-labeled DMPC, it was demonstrated that after appropriate correction for the isotopic shift, the maxima of the *sn*1- and *sn*2- $\nu_{C=O}$ absorption bands should differ by no more than 4 cm^{-1}. Moreover, the *sn*1- and *sn*2- ester $\nu_{C=O}$ absorption bands are themselves each resolvable into components which, when appropriately corrected for the isotopic shift, should exhibit their maxima near 1743 and 1728 cm^{-1}. These workers thus concluded that the underlying components normally resolved in the $\nu_{C=O}$ absorption bands of common diacylglycerolipids are the summation of comparable contributions from both of the ester carbonyl groups rather than arising from the stretching vibrations of the individual *sn*1- and *sn*2 ester carbonyl groups, as originally proposed. These authors further suggested that, at least within the context of hydrated diacylglycerolipid bilayers, the high- and low-frequency bands that are normally observed may be attributable to subpopulations of free and hydrogen-bonded ester carbonyl groups, respectively.

A determination of the molecular basis for the multicomponent nature of the $\nu_{C=O}$ absorption bands of hydrated diacylglycerolipid bilayers is fundamental to the structural interpretation of the data obtained in IR and other vibrational spectroscopic studies of these systems. Since the opinion on this subject is divided, we have reexamined the original experimental work in this area to gain some insight into the reasons for the discrepancies in the literature. Possible causes of the differences in the conclusions reached stem from the fact that the two sets of experiments were performed with different types of lipid molecules and from the fact that the measurements were not made under comparable conditions. Specifically, the work by Levin et al. (1982) was performed with unhydrated solid samples, whereas the work of Blume et al. (1988) was performed with hydrated lipid bilayers. Given this situation we have recorded and reanalyzed the IR spectra of the lipid molecules used in those studies under strictly comparable conditions, both as dried solids and as hydrated lipid bilayers. Our results indicate that at least within the specific context of fully hydrated phosphatidylcholine bilayers, the high- and low-frequency components of the $\nu_{C=O}$ bands cannot be assigned to the individual ester carbonyl groups of acyl chains esterified at the *sn*1- and *sn*2-positions of the glycerol backbone.

MATERIALS AND METHODS

Unlabeled samples of DMPC and DPPC, as well as doubly $^{13}C{=}O$-labeled samples of DMPC, were synthesized and purified from appropriate fatty acids by the methods described by Lewis et al. (1985). The *sn*1-$^{13}C{=}O$- and *sn*2-$^{13}C{=}O$-labeled samples of DMPC were synthesized as described by Lewis and McElhaney (1992). Samples of *d*-, *l*-, and *dl*-DPPC obtained from the Sigma Chemical Co. (St. Louis, MO) were judged to be chromatographically pure and used without further purification. PHPC and HPPC were obtained from Biochemisches Labor (Bern, Switzerland) and purified as described by Lewis and McElhaney (1985). Diffuse reflectance infrared spectra of dried crystalline samples were recorded with the aid of a Spectra-Tech diffuse reflectance accessory. In the case of dried films, transmission IR spectra were obtained from samples that were deposited onto the surface of a NaCl window by the evaporation of the solvent and subsequently dried in vacuo for several hours. Hydrated lipid dispersions were prepared for IR spectroscopy as follows. 3–4 mg of dry lipid were hydrated by the addition of 50 μl of D_2O followed by vigorous vortexing at temperatures near 70°C. This dispersion was then squeezed between the CaF_2 windows of a heatable liquid cell (equipped with a Teflon spacer) to form a 10-μm film. Once mounted in the sample holder of the instrument, the sample temperature could be controlled (between −20° and 90°C) by an external, computer-controlled circulating water bath. Infrared spectra were recorded with a Digilab FTS-40 infrared spectrometer (Digilab, Cambridge, MA) using the acquisition parameters previously described by Mantsch et al. (1985). The spectra obtained were analyzed and plotted with software supplied by Digilab and other computer programs obtained from the National Research Council of Canada and Microcal Software Inc. (Northampton, MA). In the analysis of the contours of the C=O stretching bands of these lipids, Fourier self-deconvolution was used to obtain accurate estimates of the peak frequencies of the component bands. Typically, the C=O stretching bands observed in the gel and liquid-crystalline states were deconvolved using band width parameters (18 and 20, respectively) and band narrowing factors (1.8–2) as defined by the software package supplied with the Digilab FTS-40 instrument. Under the conditions of these experiments, band narrowing factors of up to 2.2 could be employed without causing significant distortions of the deconvolved spectra. Subsequently, curve-fitting procedures were used to obtain estimates of the widths and integrated areas of the component bands by reconstructing the contours of the original absorption band. This was achieved by a linear combination of component bands with the aid of standard nonlinear least squares minimization procedures. The peak frequencies returned by Fourier self-deconvolution were used as starting estimates and each band was simulated by a Gaussian-Lorentzian function, for which best fit estimates of band shape was achieved with ~70% Gaussian contribution. The ^{13}C-NMR spectra of the labeled lipids were recorded in deuterated chloroform solution with a Varian Unity-300 spectrometer operating at 75.42 MHz for ^{13}C. Chemical shifts are reported downfield from dioxane, the external reference.

RESULTS

Fig. 1 shows the $\nu_{C=O}$ regions of the IR spectra of hydrated DPPC bilayers at temperatures above and below the gel/liquid-crystalline phase transition of the lipid. The spectra

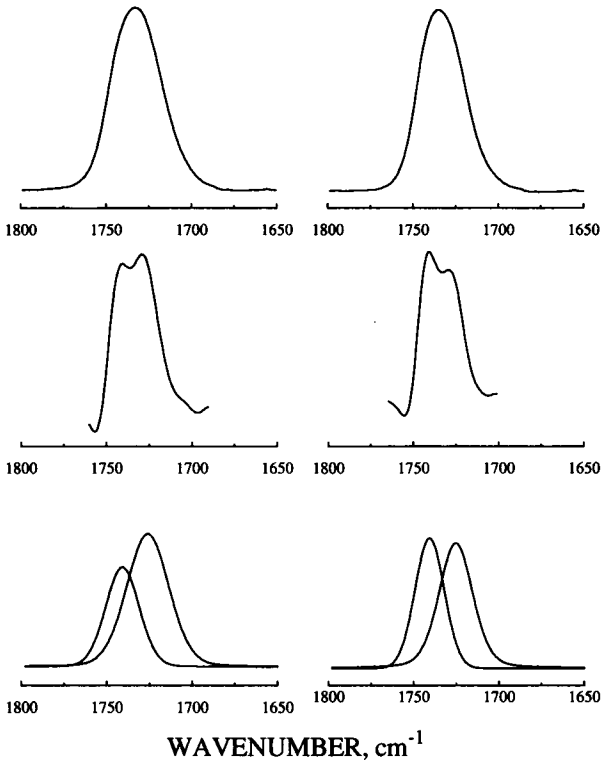

FIGURE 1 The $\nu_{C=O}$ absorption bands of aqueous dispersions of DPPC at temperatures above (*left*, 50°C) and below (*right*, 20°C) its gel/liquid-crystalline phase transition. (*Top*) Contours of the absorbance spectra actually observed. (*Middle*) Spectra obtained after band narrowing by Fourier deconvolution. (*Bottom*) Our estimates of the contours of the component bands derived by a combination of Fourier deconvolution and curve fitting.

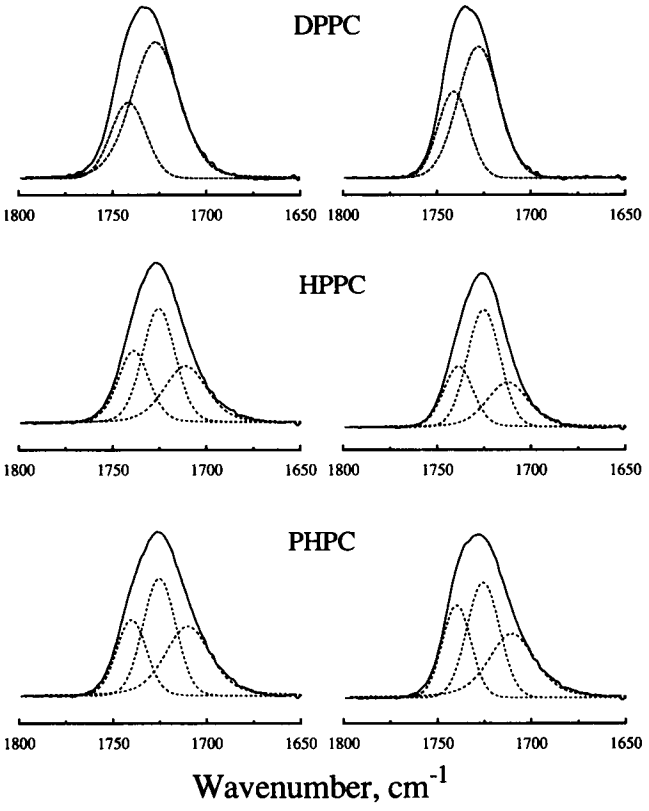

FIGURE 2 The $\nu_{C=O}$ region of the infrared spectra of aqueous dispersions of DPPC (*top*), HPPC (*middle*), and PHPC (*bottom*). Absorbance spectra are shown for both the gel (*right*) and liquid-crystalline (*left*) phases. The solid lines describe the observed original spectra and the dashed lines represent our estimates of the contours of the subcomponents.

shown in Fig. 1 typify those exhibited by the L_β and L_α phases of most of the common diacylglycerolipids that have been studied so far. These spectra usually exhibit their maxima near 1733 cm^{-1} and appear to be a summation of component bands centered near 1742 and 1727 cm^{-1}. With most lipids, the relative intensities of the subcomponents of their $\nu_{C=O}$ absorption bands are both phase-state and temperature dependent. Our approach to an evaluation of the physical basis of these subcomponents involves a comparison of the spectroscopic data presented in Fig. 1 with comparable data obtained from the IR spectra of appropriate 1-acyl, 2-alkyl and 1-alkyl, 2-acyl lipid analogues, as well as a comparison of the spectra of an unlabeled diacylglycerolipid with those of analogues which are specifically $^{13}C{=}O$-labeled at the 1- and 2-positions. The results of these experiments are presented below.

The $\nu_{C=O}$ absorption bands of hydrated bilayers composed of DPPC, HPPC, and PHPC are shown in Fig. 2, and a summary of the observed band maxima and our estimates of the absorption maxima of their component bands are listed in Table 1. The data indicate that neither the absorption maximum ($\cong$1726 cm^{-1}) nor the contours of the $\nu_{C=O}$ band of PHPC differs radically from that of the reversed alkylated isomer, HPPC. Thus, with these particular lipids, the location of the acyl group at the primary or at the secondary positions of the glycerol backbone does not cause any appreciable difference in the infrared absorption properties of the ester carbonyl group. This result differs from that of previous studies in which the $\nu_{C=O}$ absorption maxima for PHPC and HPPC were reported to occur at frequencies near 1737 cm^{-1} and 1716 cm^{-1}, respectively (Levin et al., 1982). However, an obvious difference between this work and the vibrational spectroscopic studies of Levin et al. (1982) is the fact that the present studies were performed with fully hydrated samples, whereas the earlier study was performed with dried lipid films and with lyophilized solid samples. The possible effects of the differences between the two experimental protocols will be examined later.

TABLE 1 Infrared absorption maxima* of the $\nu_{C=O}$ bands of hydrated diacyl and acyl-alkyl phosphatidylcholines

Sample	Observed (cm^{-1})	Subcomponents (cm^{-1})
DPPC	1733	1742, 1727
PHPC	1726	1740, 1725, 1710
HPPC	1726	1739, 1725, 1712
Unlabeled DMPC	1733	1742, 1727
$^{13}C{=}O$ DMPC	1691	1699, 1685
*sn*1-$^{13}C{=}O$ DMPC	1734, 1692	1741, 1727, 1699, 1684
*sn*2-$^{13}C{=}O$ DMPC	1734, 1692	1742, 1727, 1698, 1685

*Data were obtained from spectra recorded in the L_α phase of the given lipids and have been rounded to the nearest wave number.

Another significant feature of the results summarized in Fig. 2 and Table 1 is that the $\nu_{C=O}$ absorption maxima of the mono-alkylated analogues of DPPC occur some 7–9 cm^{-1} below that commonly observed for the gel and liquid-crystalline states of DPPC and, indeed, the majority of 1,2-diacylglycerolipids that have been studied so far (Mendelsohn and Mantsch, 1985). It should also be noted that the $\nu_{C=O}$ absorption bands of the mono-alkylated analogues of DPPC both appear to be summations of at least three overlapping components with maxima at 1740, 1725, and 1711 cm^{-1} (Fig. 2, Table 1). The marked difference between the $\nu_{C=O}$ absorption bands of hydrated DPPC bilayers and those of its acyl-alkyl derivatives (HPPC and PHPC) strongly suggests that the structural features of the polar/apolar interfaces of hydrated diacylglycerolipids differ significantly from those of either of its acyl-alkyl analogues. This finding brings into question the issue of whether these acyl-alkyl analogues are appropriate models for assigning the physical basis of the subcomponents of the $\nu_{C=O}$ absorption bands of fully hydrated DPPC bilayers. Such considerations and other aspects of the data presented above will be explored further in the Discussion.

A second approach to an evaluation of the origin of the subcomponents of the $\nu_{C=O}$ bands of 1,2-diacylglycerolipids involved a comparison of the IR spectra of unlabeled and specifically $^{13}C{=}O$-labeled diacyl phospholipids (Blume et al., 1988). In this study, experiments were performed with samples in which the level of $^{13}C{=}O$ enrichment was only 90% and some concerns were also expressed about the positional specificity of the $^{13}C{=}O$ labeling. We have therefore repeated those studies with samples which are highly enriched in $^{13}C{=}O$ (≅99%) and have also evaluated the positional specificity of the isotopic labeling by ^{13}C-NMR spectroscopy. Fig. 3 shows the carbonyl regions of ^{13}C-NMR spectra of the three $^{13}C{=}O$-labeled DMPC samples used in this study. The doubly $^{13}C{=}O$-labeled sample shows two well-resolved downfield resonances of comparable integrated intensity near 173.5 and 173.2 ppm. These resonances originate from the *sn*1- and *sn*2-ester carbonyl groups, respectively (Braach-Maksvytis and Cornell, 1988). The carbonyl regions of the ^{13}C-NMR spectra of the *sn*1- and *sn*2-$^{13}C{=}O$-labeled samples are each dominated by one downfield resonance, the frequency of which corresponds with that assigned to the *sn*1- and *sn*2-resonances of the ^{13}C-NMR spectrum of the doubly labeled sample (Fig. 3). The ^{13}C-NMR spectra of the *sn*1- and the *sn*2-$^{13}C{=}O$-labeled isomers each exhibits another low-intensity resonance attributable to the very low levels of $^{13}C{=}O$ groups at their *sn*2- and *sn*1-positions, respectively. Our analyses of the integrated intensities of these low-intensity peaks indicate that the degree of $^{13}C{=}O$ enrichment of the nominally unlabeled carbonyl groups of these lipids amounts to no more than a threefold enrichment over natural abundance levels. This result indicates that the $^{13}C{=}O$ labeling of the material used for this study is highly specific and effectively eliminates the possibility of artifacts arising from significant contamination of our samples by the reverse-labeled isomer.

Illustrated in Fig. 4 are the $\nu_{C=O}$ regions of the infrared spectra of unlabeled, doubly $^{13}C{=}O$-labeled, and the specifically $^{13}C{=}O$-labeled samples of DMPC used in this

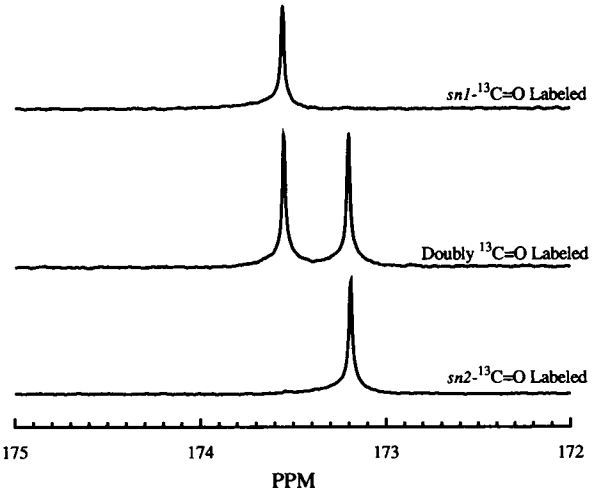

FIGURE 3 ^{13}C-NMR spectra of chloroform ($CDCl_3$) solutions of *sn*1-$^{13}C{=}O$-labeled (*top*), doubly $^{13}C{=}O$-labeled (*middle*), and *sn*2-$^{13}C{=}O$-labeled (*bottom*) samples of DMPC.

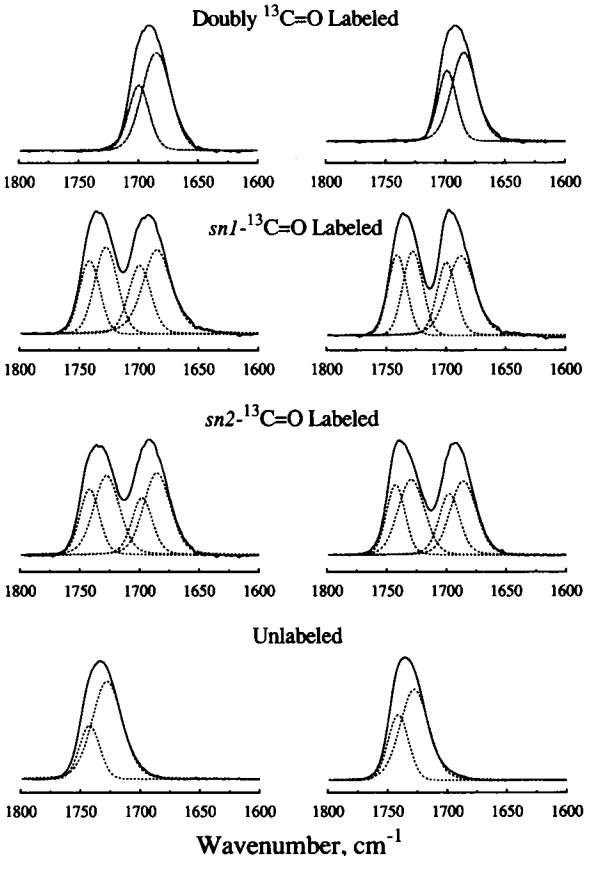

FIGURE 4 The $\nu_{C=O}$ region of the infrared spectra of aqueous dispersions of unlabeled and $^{13}C{=}O$-labeled DMPC. Absorbance spectra are shown for both the gel (*right*) and liquid-crystalline (*left*) phases. The solid lines describe the observed original spectra and the dashed lines represent our estimates of the contours of the subcomponents.

study. The general properties of the $\nu_{C=O}$ absorption band of unlabeled DMPC are similar to those described above for hydrated DPPC bilayers. The overall band maximum occurs near 1733 cm^{-1} and the broad band seems to be a summation of subcomponents with maxima near 1742 and 1727 cm^{-1} (Table 1). The properties of the $\nu_{C=O}$ band of doubly ^{13}C=O-labeled DMPC are also similar to those of the unlabeled samples of DMPC and DPPC. However, because of the greater reduced mass of the ^{13}C=O oscillator, the observed band maximum occurs near 1691 cm^{-1} and those of its subcomponents near 1699 and 1685 cm^{-1}. These results are consistent with a ^{12}C=O/^{13}C=O isotopic shift of 42–43 cm^{-1}, similar to those observed in previous studies of hydrated, specifically ^{13}C=O-labeled diacylglycerolipid bilayers (Blume et al., 1988; Hübner et al., 1990; Hübner and Mantsch, 1991; Lewis and McElhaney, 1992, 1993b). With both the *sn*1- and *sn*2-^{13}C=O-labeled samples, discrete absorption maxima arising from the stretching vibrations of their respective ^{12}C and ^{13}C ester carbonyl groups are observed near 1734 and 1692 cm^{-1}. The data indicate that the ^{12}C=O/^{13}C=O isotopic shifts exhibited by the *sn*1- and *sn*2-ester carbonyl groups (≅42–43 cm^{-1}) are essentially the same as that estimated from the band maxima of the unlabeled and doubly ^{13}C=O-labeled samples (Table 1). Thus, the observable ^{12}C=O/^{13}C=O isotopic shift is independent of whether the ester carbonyl group is located at the *sn*1 or *sn*2 position of the glycerol backbone. The data also indicate that the ^{12}C=O and ^{13}C=O absorption bands of the specifically labeled lipids each appear to be a summation of underlying components. Our analyses of the ^{12}C=O and ^{13}C=O bands of the two specifically labeled lipids (Fig. 4, Table 1) suggest that regardless of whether the ^{12}C=O or ^{13}C=O absorption band originates from the *sn*1- or *sn*2- ester C=O group of the lipid, they appear to be a summation of subcomponents with maxima near 1741 and 1727 cm^{-1} (^{12}C $\nu_{C=O}$ band) and 1699 and 1685 cm^{-1} (^{13}C $\nu_{C=O}$ band). From the above, it is evident that after suitable correction for the ^{12}C=O/^{13}C=O isotopic shift, the observed absorption maxima attributable to the $\nu_{C=O}$ vibrations of the *sn*1 and *sn*2 ester C=O groups are virtually identical and the same as the observed band maximum of the unlabeled lipid. Moreover, an examination of the band maxima of the resolvable subcomponents of the *sn*1 and *sn*2 $\nu_{C=O}$ bands of the two singly labeled lipids studied supports the same conclusion. These results are clearly consistent with the thesis that when fully hydrated, the subcomponents in the $\nu_{C=O}$ band of these diacylglycerolipid bilayers are not simply a reflection of the inequivalent locations of the two ester C=O groups on the glycerol backbone. A similar conclusion was reached in the studies of Blume et al. (1988).

The results described above clearly indicate that the subcomponents of the $\nu_{C=O}$ absorption band of fully hydrated diacyl PC bilayers are not due to the inequivalent location of the two ester carbonyl groups on the PC glycerol backbone. Clearly, this picture is at variance with that originating from earlier assignments based on studies of dried lipid samples. One should note, however, that apart from the obvious differences in sample hydration, there are other aspects of the methodology used in the earlier studies which complicate the process of comparing this work with previously published data. For example, in the earlier experiments the spectra of dried solid samples of PHPC and HPPC were not recorded under comparable conditions (i.e., a lyophilized sample of HPPC vs. a solvent cast of PHPC). Also, most of the earlier experiments in which two components of the $\nu_{C=O}$ absorption bands of DPPC were identified were performed with solid samples of *dl*-DPPC (i.e., the racemic mixture), and not the more commonly used 1,2-*sn*- enantiomer (Bicknell-Brown et al., 1980; Bicknell-Brown and Brown, 1984; Bush et al., 1980a, b). To specifically address the issue of the possible effects of differences in the materials and experimental conditions used in the previous studies, we have examined the infrared spectra of dried solid samples of dl-DPPC and its two optically active enantiomers, as well as those of dried solid samples of HPPC and PHPC.

Illustrated in Figs. 5 and 6 are some of the spectra obtained in our studies of the $\nu_{C=O}$ absorption bands of various dried solid samples of DPPC. The observed absorption maxima and our estimates of the absorption maxima of their component bands are summarized in Table 2. A number of

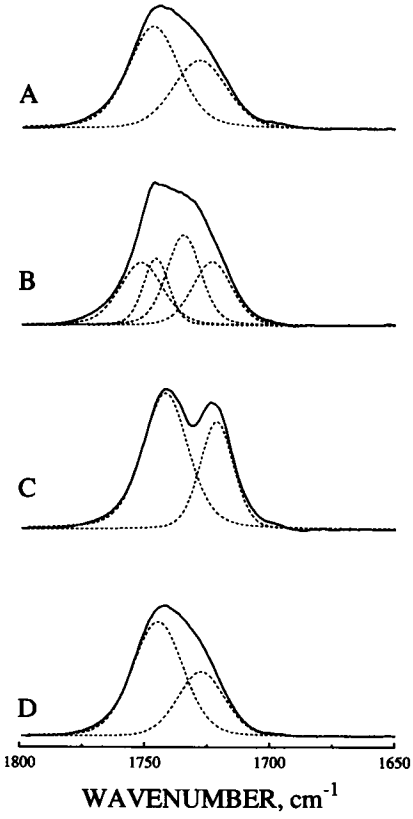

FIGURE 5 The $\nu_{C=O}$ region of the infrared spectra of dried samples of the *d*-, *l*-, and *dl*-DPPC obtained from the Sigma Chemical Company. Absorbance spectra shown with the solid lines representing the observed spectra and the dashed lines representing our estimates of the contours of the subcomponents. Data are presented for vacuum dried crystalline DL-DPPC (*A*); vacuum dried crystalline L-DPPC (*B*); vacuum dried crystalline D-DPPC (*C*); Sigma DL-DPPC after lyophilization from benzene (*D*).

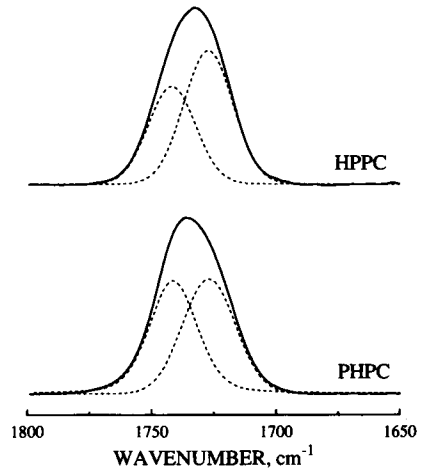

FIGURE 6 The $\nu_{C=O}$ region of the infrared spectra of lyophilized solid samples of HPPC (*top*) and PHPC (*bottom*). Absorbance spectra shown with solid lines representing the observed original spectra and the dashed lines representing our estimates of the contours of the subcomponents.

TABLE 2 Infrared absorption maxima of the $\nu_{C=O}$ bands of dried solid samples of diacyl and acyl-alkyl phosphatidylcholines

Sample	Observed (cm^{-1})	Subcomponents (cm^{-1})
D-DPPC (Sigma)*	1745	1747, 1728
L-DPPC (Sigma)*	1746	1751, 1746, 1734, 1723
DL-DPPC (Sigma)*	1742, 1721	1742, 1721
DL-DPPC (Fluka)*	1743	1745, 1724
DL-DPPC (lyophylized)‡	1742	1742, 1721
L-DPPC (lyophylized)§	1739	1742, 1726
PHPC (lyophylized)§	1735	1741, 1727
HPPC (lyophylized)§	1732	1741, 1727
PHPC ($CHCl_3$)¶	1731	1741, 1727
HPPC ($CHCl_3$)¶	1730	1741, 1727
PHPC ($CHCl_3$:MeOH)¶	1737	1741, 1727
HPPC ($CHCl_3$:MeOH)¶	1735	1741, 1727

*Commercially supplied crystalline samples dried in vacuo.
‡Commercially supplied sample (Sigma) relyophylized from benzene.
§Repurified and lyophylized from benzene.
¶Dried films prepared by evaporation of the solvent indicated.

relevant observations emerge from our experiments. First, the contours of the $\nu_{C=O}$ bands exhibited by solid samples of nominally pure DPPC obtained from different commercial sources, or different batches from the same commercial source, vary considerably. Thus, for example, the spectrum B in Fig. 5 typifies that exhibited by *l*-DPPC obtained from the Sigma Chemical Company, whereas solid samples of *l*-DPPC obtained from Avanti Polar Lipids (Alabaster, AL) or synthesized by us exhibit $\nu_{C=O}$ absorption bands similar those illustrated in spectra A and D in Fig. 5. Since all of the samples examined were judged to be chromatographically pure, it is unlikely that the variable spectroscopic properties observed are a reflection of variations in sample purity. We did find, however, if the various samples were redissolved in a common solvent and relyophilized, they all tend to exhibit similar spectroscopic properties. Under our conditions, for example, nominally pure DPPC samples that were lyophilized from benzene exhibit $\nu_{C=O}$ bands similar to those shown in Fig. 5, spectrum D, regardless of the source from which they were obtained. The latter observations suggest that the variability noted above may be the result of variations in the solvent systems from which the various DPPC samples were prepared. Since lipids are known to form different polymorphic forms when crystallized from different solvents (Chapman et al., 1966; Mantsch et al., 1983; Small, 1986), it is possible that the observed variations in the substructure of the $\nu_{C=O}$ bands exhibited by the different solid samples of DPPC are the result of the formation of different crystalline polymorphic forms of this lipid. This possibility raises fundamental questions about the suitability of extrapolating data obtained from studies of solid lipids to the fully hydrated situation, issues which will be further explored in the Discussion.

Second, we also found that the contours of the $\nu_{C=O}$ bands of *d*-, *l*-, and *dl*-DPPC samples obtained from the same commercial source (Sigma) themselves vary significantly (Fig. 5, spectra A, B and C), despite the fact that the samples were nominally prepared under the same conditions (personal communication form Sigma Chemical Company). This observation could also be a reflection of differences in the types of crystalline form which the various optical isomers of DPPC may adopt under the conditions where the samples were manufactured. Interestingly, we did find that after the same samples of Sigma *l*-, *d*-, and *dl*-DPPC were redissolved and relyophilized from benzene, their spectroscopic features were all similar to that illustrated in Fig. 5 *D*. Thus, in addition to effects attributable to the chirality of the glycerol backbone of DPPC, the variable nature of the $\nu_{C=O}$ bands observed may also be a function of the solvent from which the samples were crystallized. The possibility that the nature of the crystallizing solvent may be a significant determinant of the unusual $\nu_{C=O}$ band contours of Sigma dl-DPPC has been suggested in previous studies (Wong and Mantsch, 1988). However, a very important feature of our observations is that the crystalline samples of dl-DPPC obtained from Sigma exhibits two sharp and fairly well resolved $\nu_{C=O}$ bands ($\cong$1742 cm^{-1} and 1721 cm^{-1}, respectively) which, to our knowledge, have not been observed with *dl*-DPPC samples from other commercial sources. It is therefore possible that the unusual spectroscopic properties of Sigma *dl*-DPPC may arise from its forming a distinctive polymorphic form under the crystallization conditions used in its preparation. Such a possibility is particularly relevant to the issues raised here because a survey of the literature indicates that the early studies in which two subcomponents were identified in the $\nu_{C=O}$ absorption bands of DPPC (Bicknell-Brown et al., 1980; Bicknell-Brown and Brown, 1984; Bush et al., 1980a, b) were all performed with Sigma *dl*-DPPC.

Representative $\nu_{C=O}$ absorption bands of dried lyophilized samples of PHPC and HPPC are shown in Fig. 6 and the results of our analyses of the substructure of these bands are also summarized in Table 2. The data shown therein reveal a number of interesting features. First, an examination of the maxima and the substructures of the $\nu_{C=O}$ bands of the lyophilized solids indicates that $\nu_{C=O}$ bands of solid samples

of both acyl-alkyl lipids absorb IR radiation over a higher range of frequencies than do the those of the hydrated samples (Fig. 6, Table 2; compare with data shown in Fig. 2, Table 1). This observation suggests that the ester carbonyl groups of dried solid samples of these acyl-alkyl lipids reside in less polar environments than do those of hydrated dispersions of the same lipids and raises the issue of whether data obtained from studies of dried lipid films are applicable to hydrated lipid bilayers. Second, for the most part the contours of the $\nu_{C=O}$ absorption bands of HPPC and PHPC seem to be similar and are each resolvable into components near 1741 and 1727 cm^{-1}. However, the two isomers differ with respect to the contribution of these subcomponents to the total integrated intensity of the $\nu_{C=O}$ band, and as a result the observed overall absorption maximum of HPPC ($\cong$1732 cm^{-1}) occurs at a slightly lower frequency than does that of PHPC ($\cong$1735 cm^{-1}). The overall similarity of the $\nu_{C=O}$ absorption bands of PHPC and HPPC found here differs from the results of previously published vibrational spectroscopic studies of these lipids (Levin et al., 1982). Third, our studies of dried films of these particular lipids also indicate that the contours of their $\nu_{C=O}$ absorption bands can change with changes in the conditions under which the samples were prepared. Thus, for example, with films prepared from chloroform solutions, band maxima near 1730 cm^{-1} were typically observed, whereas with samples dried from chloroform: methanol (1:1) the observed band maxima could be as high as 1737 cm^{-1} (Table 2). Despite this variability, however, we consistently find that differences between the $\nu_{C=O}$ band contours of dried films of HPPC and PHPC prepared from the same solvent are relatively small. This observation is significant because it indicates that variations in the methodology used to prepare solid samples of these acyl-alkyl lipids can result in larger variations in the contours of their $\nu_{C=O}$ bands than can be unambiguously attributed to the difference in the chemical structures of the two lipids. Since in the previously published study, spectra of PHPC and HPPC were not recorded from similarly prepared samples (Levin et al., 1982), it is therefore possible that the large difference in the reported absorption maxima of PHPC and HPPC could be attributed to differences in sample preparation.

DISCUSSION

This work addresses a number of issues relevant to the molecular basis of the existence of subcomponents in the $\nu_{C=O}$ absorption bands of hydrated diacylglycerolipid bilayers. Our studies indicate that when dispersed in excess water, PHPC and HPPC exhibit fairly complex $\nu_{C=O}$ absorption bands that are each resolvable into subcomponents with similar maxima and integrated intensities. Our observation that the contours of the $\nu_{C=O}$ absorption bands of HPPC and PHPC are virtually identical indicates that their patterns of infrared absorption in the carbonyl stretching region of the spectrum are independent of whether the fatty acyl chain is esterified to the *sn*1- or the *sn*2- position of the lipid glycerol backbone. Also, our experiments with ^{13}C=O-labeled DMPC indicate that when dispersed in excess water, the subcomponents of $\nu_{C=O}$ absorption bands exhibited by the unlabeled and doubly ^{13}C=O-labeled lipids are each a summation of comparable contributions arising from the stretching vibrations of the *sn*1- and *sn*2-ester carbonyl groups of the lipid. Similar results have been reported in other studies of fully hydrated, ^{13}C=O-labeled 1,2-diacyl phospholipids (Blume et al., 1988; Hübner and Mantsch; 1990; Lewis and McElhaney, 1992, 1993b). Such studies provide strong evidence that the subcomponents resolved in the $\nu_{C=O}$ bands of each of the fully hydrated 1,2-diacylglycerolipids studied are not the result of differential patterns of infrared absorption by ester carbonyl groups located at the primary and secondary positions of the lipid glycerol backbone. This conclusion differs markedly from those Levin et al. (1982). The latter authors concluded that the *sn*1- and *sn*2-ester carbonyl groups of diacylglycerolipids exhibit different patterns of infrared absorption and they suggested that such differences arise because of the known (or generally assumed) conformational differences between the *sn*1- and *sn*2-ester carbonyl groups of the lipid.

Our examination of possible reasons why this work supports different conclusions from those drawn from earlier vibrational spectroscopic studies raises the issue of the suitability of various experimental approaches that have been used to address the question. The experimental work using the acyl-alkyl DPPC analogues (PHPC and HPPC) is based on the assumption that replacement of any one of the two ester-linked hydrocarbon chains with an appropriate ether-linked chain does not significantly alter the pattern of infrared absorption by the remaining ester carbonyl group. This assumption would only hold if the properties of the lipid polar/apolar interface are not radically changed by the replacement of one of the ester-linked hydrocarbon chains with its ether-linked counterpart. To our knowledge, this assumption has never been experimentally verified. Our experiments suggest that such an assumption may not hold for aqueous lipid dispersions. Interestingly, our data also indicate that the $\nu_{C=O}$ bands of the acyl-alkyl PC analogues exhibit their maxima at lower frequencies than DPPC and that the absorption bands extend to a lower frequency range than in the corresponding diacylglycerolipid. These results suggest that ester carbonyl groups of fully hydrated bilayers composed the acyl-alkyl PC analogues reside in a more polar environment than do those of hydrated bilayers composed of the diacyl PC. This conclusion seems counterintuitive when one considers that the nominal polarity of the hydrophobic/hydrophilic interfaces of the diacylglycerolipid is greater than that of the acyl-alkyl analogue. It is difficult to envisage how the hydrophilic/hydrophobic interfaces of hydrated acyl-alkyl PC bilayers could be more polar than those the hydrated diacyl PC bilayers if the glycerol backbones of the acyl-alkyl PC analogues retain a similar conformation to that deduced from NMR and single-crystal x-ray diffraction studies of 1,2-diacylglycerolipids (Hitchcock et al., 1974; Pearson and Pascher, 1979; Hauser et al., 1981, 1988). To our knowledge, the conformation of the glycerol backbones of

the lipid molecules in hydrated bilayers composed of the acyl-alkyl PC analogues has not been determined and, indeed, the possibility that the replacement of an ester-linked hydrocarbon chain of a 1,2-diacylglycerolipid with an appropriate ether-linked counterpart might change the conformation of the lipid glycerol backbone has not been examined in studies of such lipids. Our evidence that the structure of the polar/apolar interfaces of the acyl-alkyl glycerolipids may differ from that of the diacyl counterpart suggests that such possibilities need to be considered. If these two classes of phospholipids are not conformationally equivalent in hydrated bilayers, then the carbonyl absorption bands of the two acyl-alkyl analogues of DPPC may not be valid models for assigning the infrared absorption properties of *sn*1- and *sn*2-C=O groups of DPPC itself.

One should note that the use of ^{13}C=O-labeled material effectively eliminates all complications arising from potential differences in the conformation of the lipid glycerol backbone. However, the use of ^{13}C=O-labeled lipids to aid the assignment of the molecular basis of the subcomponents of the $\nu_{C=O}$ bands of 1,2-diacylglycerolipids is itself based on the assumption that the observed $\nu_{C=O}$ band contour can be described in terms of a linear combination of absorption bands attributable to the unperturbed stretching vibrations of the two ester carbonyl groups of the lipid. Although the latter assumption has not yet been rigorously tested with a wide range of diacylglycerolipids, previous studies indicate that such assumptions hold true in the L_α and L_β phases of all specifically ^{13}C=O-labeled lipids studied so far (Blume et al., 1988; Hübner and Mantsch 1989, 1990; Lewis and McElhaney, 1992, 1993b). Moreover, such assumptions even seem to hold true for the quasi-crystalline L_c phases of DPPC and the longer chain *n*-saturated diacyl phosphatidylcholines (Lewis and McElhaney, 1992). To date, the assumption that contours of the $\nu_{C=O}$ band of 1,2-diacylglycerolipids is describable in terms of a linear combination of bands arising from the unperturbed stretching vibrations of the lipid ester carbonyl groups has only been shown to be invalid in the case of the quasi-crystalline L_c phase of DMPC (Lewis and McElhaney, 1992). In the latter case, there is evidence that the $\nu_{C=O}$ absorption bands of the lipid are perturbed by close-contact interactions between subpopulations of *sn*1- and *sn*2- ester carbonyl groups.

Another general question raised here is whether $\nu_{C=O}$ assignments based on a spectroscopic examination of dried lipid material are applicable to hydrated lipid bilayers. The ester carbonyl groups of diacylglycerolipids reside in the polar/apolar interfacial region of the lipid bilayers where they are potential recipients of hydrogen-bonding interactions with interfacial water or with other polar moieties of the lipid. Given that the $\nu_{C=O}$ absorption band will be influenced by the local environment of the ester carbonyl group, it seems unlikely that the behavior of the ester carbonyl group in the presence of excess water can be identical to that of dried lipid films. Another important consideration stems from the fact that the infrared spectra of dried materials are known to vary with the microcrystalline structure of the sample under observation. However, amphiphilic materials such as glycerolipids can crystallize into many polymorphic forms, the formation of which varies with factors such as the solvent from which the lipid was crystallized, the temperature, the thermal history of the sample, the number of waters of crystallization, etc. Consequently, the infrared spectra of dried glycerolipid samples may vary considerably with the conditions under which the sample was prepared (Chapman et al., 1966; Mantsch et al., 1983; Wong and Mantsch, 1988; Lewis and McElhaney, 1993a). Such factors were evident in this study and may well be responsible for the considerable variations which were observed in our examination of the $\nu_{C=O}$ band contours of crystalline and lyophilized samples of DPPC. It should be noted, however, that these concerns do not apply to the L_α and L_β phases of hydrated glycerolipid bilayers because the lipid headgroups and polar/apolar interfaces usually remain fully hydrated under those conditions. For the L_α and L_β phases of diacylglycerolipids relatively small changes are observed in the contours of the $\nu_{C=O}$ band and these changes are largely determined by the chemical structure of the lipid and by phase state of the lipid assembly. However, large variations in the IR spectra of hydrated lipid bilayers can be observed under conditions favoring the formation of the quasi-crystalline L_c phases of these lipids, especially when significant changes in headgroup and interfacial hydration occur. For these reasons it seems unlikely that the spectroscopic behavior of dried lipid samples will reliably reflect that the L_β and L_α phases of fully hydrated glycerolipid bilayers. On the other hand, it is possible that the spectroscopic behavior of dried lipid films may be more closely related to that exhibited by the partially dehydrated, quasi-crystalline L_c phases which are usually formed when the L_β phases of some lipids are incubated at low temperatures (for an example, see Lewis and McElhaney, 1993a).

Although conformational differences between the *sn*1- and *sn*2- ester carbonyl groups of hydrated 1,2-diacylglycerolipids do not seem to be significant determinants of the substructure of the $\nu_{C=O}$ absorption bands observed when the lipids are fully hydrated, such conformational inequivalence could be a significant determinant of the substructure of the lipid $\nu_{C=O}$ absorption bands of crystalline lipids. However, our results indicate that even in such systems, the interpretation of spectroscopic data should proceed with considerable caution because in crystalline lipids there are many other factors which can influence the vibrational environment of the ester carbonyl groups. Finally, one should note that our data still leaves open the question of the molecular basis of the substructure of the $\nu_{C=O}$ bands of fully hydrated diacylglycerolipid bilayers. Blume et al. (1988) suggested that the resolvable subcomponents of the $\nu_{C=O}$ bands of diacyl phospholipids may be a reflection of subpopulations of hydrogen-bonded and non-hydrogen-bonded ester carbonyl groups. This suggestion has been adopted in the interpretation of the $\nu_{C=O}$ bands in several studies of diacylglycerolipid bilayers (Lewis and McElhaney, 1990, 1992, 1993a, b). Currently, there is insufficient data for one to make an unequivocal judgment

on this subject. However, data presented by Blume et al. (1988) and others (Mushayakarara et al., 1986) show that the frequency shifts attributable to the hydrogen-bonding of ester carbonyl groups are comparable to those observed here and elsewhere (Mendelsohn and Mantsch, 1986) and suggest that the interpretation proposed by Blume et al. (1988) is quite plausible.

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REFERENCES

Bicknell-Brown, E., and K. G. Brown. 1984. Raman temperature study of conformational changes in anhydrous dipalmitoylphosphatidylcholine. *Biochim. Biophys. Acta.* 778:317–323.

Bicknell-Brown, E., K. G. Brown, and W. B. Person. 1980. Configuration-dependent Raman bands of phospholipid surfaces. I. Carbonyl stretching modes at the bilayer interface. *J. Am. Chem. Soc.* 102:5486–5491.

Blume, A., W. Hübner, and G. Messner. 1988. Fourier transform infrared spectroscopy of $^{13}C{=}O$ labeled phospholipids. Hydrogen bonding to carbonyl groups. *Biochemistry.* 27:8239–8249.

Braach-Maksvytis, V. L. B., and B. A. Cornell. 1988. Chemical shift anisotropies obtained from aligned egg yolk phosphatidylcholine by solid state 13C nuclear magnetic resonance. *Biophys. J.* 53:839–843.

Bush, S. F., R. G. Adams, and I. W. Levin. 1980. Structural reorganization in lipid bilayer systems: effect of hydration and sterol addition on Raman spectra of dipalmitoyl phosphatidylcholine multilayers. *Biochemistry.* 19: 4429–4436.

Bush, S. F., H. Levin, and I. W. Levin. 1980. Cholesterol-lipid interactions: an infrared and Raman spectroscopic study of the carbonyl stretching mode region of 1,2 dipalmitoyl phosphatidylcholine bilayers. *Chem. Phys. Lipids.* 27:101–111.

Chapman, D., P. Byrne, and G. G. Shipley. 1966. The physical properties of phospholipids I. Solid state and mesomorphic properties of some 2,3-diacyl-dl-phosphatidylethanolamines. *Proc. R. Soc. Lond.* A290: 115–142.

Hauser, H., I. Pascher, R. H. Pearson, and S. Sundell. 1981. Preferred conformation and molecular packing of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta.* 650:21–51.

Hauser, H., I. Pascher, and S. Sundell. 1988. Preferred conformation and dynamics of the glycerol backbone in phospholipids. An NMR and x-ray single crystal analysis. *Biochemistry.* 27:9166–9174.

Hitchcock, P. B., R. Mason, K. M. Thomas, and G. G. Shipley. 1974. Structural chemistry of 1,2 dilauroly-dl-phosphatidylethanolamine: molecular conformation and intermolecular packing of phospholipids. *Proc. Natl. Acad. Sci. USA.* 71:3036–3040.

Hübner, W., and H. H. Mantsch. 1991. Orientation of specifically 13C=O labeled phosphatidylcholine multilayers from polarized attenuated total reflectance FT-IR spectroscopy. *Biophys. J.* 59:1261–1272.

Hübner, W., H. H. Mantsch, and H. L. Casal. 1990. Beware of frequency shifts. *Appl. Spectrosc.* 44:732–734.

Levin, I. W., E. Mushayakarara, and R. Bittman. 1982. Vibrational assignment of the sn1 and sn2 carbonyl stretching modes of membrane phospholipids. *J. Raman Spectrosc.* 13:231–234.

Lewis, R. N. A. H., and R. N. McElhaney. 1985. Thermotropic phase behavior of model membranes composed of phosphatidylcholines containing isobranched fatty acids. 1. Differential scanning calorimetric studies. *Biochemistry.* 24:2431–2439.

Lewis, R. N. A. H., and R. N. McElhaney. 1992. Structures of the subgel phases of n-saturated diacyl phosphatidylcholine bilayers. FTIR spectroscopic studies of 13C=O and ^{2}H labeled lipids. *Biophys. J.* 61:63–77.

Lewis, R. N. A. H., and R. N. McElhaney. 1993a. Calorimetric and spectroscopic studies of the polymorphic phase behavior of the homologous series of n-saturated 1,2-diacyl phosphatidylethanolamines. *Biophys. J.* 64:1081–1096.

Lewis, R. N. A. H., and R. N. McElhaney. 1993b. Studies of mixed-chain diacyl phosphatidylcholines with highly asymmetric acyl chains. A Fourier transform infrared spectroscopic study of interfacial hydration and hydrocarbon chain packing in the mixed interdigitated gel phase. *Biophys. J.* 65:1866–1877.

Mantsch, H. H., S. C. Hsi, K. W. Butler, and D. G. Cameron. 1983. Studies on the thermotropic behavior of aqueous phosphatidylethanolamines. *Biochim. Biophys. Acta.* 728:325–330.

Mantsch, H. H., C. Madec, R. N. A. H. Lewis, and R. N. McElhaney. 1985. Thermotropic phase behavior of model membranes composed of phosphatidylcholines containing isobranched fatty acids. 2. Infrared and ^{31}P-NMR spectroscopic studies. *Biochemistry.* 24:2440–2446.

Mendelsohn, R., and H. H. Mantsch. 1986. Fourier transform infrared studies of lipid-protein interaction. *In* Progress in Lipid Protein Interactions 2. A. Watts and J. J. H. H. M. De Pont, editors. Elsevier, Amsterdam. 103–146.

Mushayakarara, E. C., P. T. T. Wong, and H. H. Mantsch. 1986. Detection by high pressure infrared spectroscopy of hydrogen bonding between water and triacetyl glycerol. *Biochem. Biophsy. Res. Commun.* 134:140–145.

Pearson, R. H., and I. Pascher. 1979. The molecular structure of lecithin dihydrate. *Nature.* 281:499–501.

Small D. M. 1986. Handbook of Lipid Research. D. J. Hanahan, editor. Plenum Press, New York.

Wong, P. T. T., and H. H. Mantsch. 1988. High pressure infrared spectroscopic evidence of water binding sites in 1,2 diacyl phospholipids. *Chem. Phys. Lipids.* 46:213–224.