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Fourier Transform Infrared Spectroscopy of $^{13}C{=}O$-Labeled Phospholipids Hydrogen Bonding to Carbonyl Groups[†]

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ABSTRACT: Fourier transform infrared spectroscopy has been used to characterize the carbonyl stretching vibration of DMPC, DMPE, DMPG, and DMPA, all labeled with ^{13}C at the carbonyl group of the *sn*-2 chain. Due to the vibrational isotope effect, the $^{13}C{=}O$ and the $^{12}C{=}O$ vibrational bands are separated by ca. 40–43 cm^{-1}. This frequency difference does not change when the labeling is reversed with the $^{13}C{=}O$ group at the *sn*-1 chain. For lipids in organic solvents possible conformational differences between the *sn*-1 and *sn*-2 ester groups have no effect on the vibrational frequency of the C=O groups. In aqueous dispersion unlabeled phospholipids always show a superposition of two bands for the C=O vibration located at ca. 1740 and 1727 cm^{-1}. These two bands have previously been assigned to the *sn*-1 and *sn*-2 C=O groups. FT-IR spectra of ^{13}C-labeled phospholipids show that the vibrational bands of both, the *sn*-1 as well as the *sn*-2 C=O group, are clearly superpositions of at least two underlying components of different frequency and intensity. Band frequencies were determined by Fourier self-deconvolution and second-derivative spectroscopy. The difference between the component bands is ca. 11–17 cm^{-1}. Again, the conformational effect as shown by reversed labeling is negligible with only 1–2 cm^{-1}. The splitting of the C=O vibrational bands in H_2O and D_2O is caused by hydrogen bonding of water molecules to both C=O groups as shown by a comparison with spectra of model ester compounds in different solvents. To extract quantitative information about changes in hydration, band profiles were simulated with Gaussian–Lorentzian functions. The chemical nature of the head group and its electronic charge have distinctive effects on the extent of hydration of the carbonyl groups. In the gel and liquid-crystalline phase of DMPC the *sn*-2 C=O group is more hydrated than the *sn*-1 C=O. This is accord with the conformation determined by X-ray analysis. In DMPG the *sn*-1 C=O group seems to be more accessible to water, indicating a different conformation of the glycerol backbone.

In the last years Fourier transform infrared spectroscopy has been used with great success to elucidate the conformational properties of phospholipids in different lamellar phases and the interaction of phospholipids with ions, cholesterol, and membrane proteins (Cameron et al., 1979; Parker, 1983; Amey & Chapman, 1984; Casal & Mantsch, 1984; Mendelsohn & Mantsch, 1986; Mantsch et al., 1986). Most of these studies have focused on the analysis of the vibrational bands of the CH_2 groups and the change of their intensity and frequency with temperature. Other vibrational bands of phospholipids were not studied in such detail. It was noted relatively early that the C=O stretching bands of phospholipids in aqueous dispersion displayed an asymmetry which changed with the phase state of the lipid, with the degree of hydration, or with

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the addition of cholesterol (Bush et al., 1980; Umemura et al., 1980; Bicknell-Brown et al., 1980; Mantsch et al., 1981; Mushayakarara et al., 1982). A complication for the analysis of the C=O vibrational bands is the fact that phospholipids have two ester groups. Therefore it was not clear whether the two overlapping C=O bands could be assigned to the *sn*-1 and *sn*-2 C=O group, respectively. Analysis of the C=O stretching bands of diglycerides, lyso-PC, and PC in the crystalline state lead to the assumption that due to the conformational inequivalence of the two ester groups the C=O band with the lower frequency should originate from the *sn*-2 C=O group due to its closer proximity to the bilayer interface with its higher dielectric constant (Mushayakarara et al., 1982; Mushayakarara & Levin, 1982).

A way to obviate the difficulties arising from overlapping bands is the synthesis of phospholipids labeled with ^{13}C at one of the C=O groups. We have synthesized various phospholipids with myristic acid chains labeled with ^{13}C at the 1-position of the *sn*-2 chain. Due to the vibrational isotope effect the isolated ^{13}C=O stretching vibration should be shifted by ca. 40.5 cm^{-1} to lower frequency. This was indeed observed for phospholipids dissolved in organic solvents like CCl_4 or $CHCl_3$. The two observed C=O bands were separated by ca. 40–43 cm^{-1}, the exact frequency difference depending slightly on the solvent. To check whether conformational differences between the *sn*-2 and *sn*-1 ester group could account for this difference, we also synthesized the compound [*sn*-1-1-^{13}C]-DMPC.[1] The same isotope shift as before was observed, thus excluding a notable conformational effect on the frequency of the C=O vibration for lipids dissolved in organic solvents.

Aqueous dispersions of ^{13}C-labeled phospholipids also displayed two C=O vibrational bands. However, both bands are clearly comprised of at least two overlapping components of different frequency, intensity and bandwidth. We could show that the splitting of these C=O vibrational bands is caused by hydrogen bonding of water molecules to the ester carbonyl groups. A conformational effect on the C=O vibrational frequency for phospholipids in aqueous dispersion can be neglected as the compound [*sn*-1-1-^{13}C]DMPC gave the same band splittings only in reversed order. Analysis of the C=O vibrational bands of DMPC, DMPE, DMPA, and DMPG by Fourier self-deconvolution, second-derivative spectroscopy, and quantitative band-shape simulations revealed distinct differences in the extent of hydrogen bonding to the ester carbonyls depending on the chemical nature and charge of the phospholipid head group.

After completion of this study it was pointed out to use that Green et al. (1987) had used the same approach of ^{13}C labeling for an investigation of DPPC and DPPC/cholesterol bilayers. The experimental results reported by these authors are consistent with ours, but their interpretation is quite different.

MATERIALS AND METHODS

Materials. The following labeled phospholipids were synthesized: [*sn*-2-1-^{13}C]DMPC, [*sn*-2-1-^{13}C]DMPE, [*sn*-2-1-^{13}C]DMPG, [*sn*-2-1-^{13}C]DMPA, [*sn*-1-1,*sn*-2-1-$^{13}C_2$]DMPC, and [*sn*-1-1-^{13}C]DMPC. 1-^{13}C-Labeled myristic acid was prepared by established procedures starting from ω-bromotridecane (Fluka, Neu-Ulm, West Germany) and ^{13}C-labeled KCN (90% enriched, Amersham, Braunschweig, West Germany). [*sn*-2-1-^{13}C]DMPC was prepared by reacylation of myristoyllyso-PC with the fatty acid anhydride by use of *N*,*N*-dimethyl-4-aminopyridine as a catalyst (Gupta et al., 1977; DasGupta et al., 1982; Lewis et al., 1984). The reaction was carried out at room temperature with a 4-fold excess of anhydride and stopped after a reaction time of 12–16 h to reduce acyl chain migration. The reaction conditions had been tested before for the synthesis of mixed-chain phospholipids where acyl chain migration can easily be detected by gas chromatography of the fatty acid esters removed from the *sn*-2 group of the phospholipid by phospholipase A_2 (Hübner, 1983). Chain migration under our reaction conditions was between 1 and 4% in agreement with the results reported by Lewis et al. (1984). [*sn*-2-1-^{13}C]DMPE, [*sn*-2-1-^{13}C]DMPG, and [*sn*-2-1-^{13}C]DMPA were prepared from [*sn*-2-1-^{13}C]DMPC by transphosphatidylation with phospholipase D prepared from savoy cabbage (Eibl & Kovatchev, 1981). [*sn*-1-1-^{13}C]DMPC was prepared by first synthesizing [*sn*-1-1,*sn*-2-1-$^{13}C_2$]DMPC by reacylation of the $CdCl_2$ complex of glycerophosphocholine with the fatty acid anhydride, converting this to the lyso-PC with phospholipase A_2 (Sigma, Müchen) and a second reacylation with unlabeled myristic acid anhydride. D_2O for infrared spectroscopy was purchased from Aldrich (Steinheim, West Germany).

Sample Preparation. Multilamellar lipid dispersions were prepared by adding the appropriate amount of D_2O to the dry lipid. The samples were then heated to temperatures above the phase transition of the respective lipid, vortexed extensively, and sonicated shortly in a Branson bath-type sonifier. Finally, the samples were incubated at temperatures above the phase transition temperature of the lipid for at least 1 h.

FT-IR Spectroscopy. Lipid films on NaCl or KBr plates were cast from lipid solutions in absolute CCl_4 or $CHCl_3$. Lipid dispersions were filled into 25 or 50 μm thick infrared cells with CaF_2 windows. The hollow window mounts could be thermostated by an external water thermostat (Haake F3C, West Germany). Temperature was monitored by a home-built thermometer with a diode as temperature-sensing element, which was glued to the window mount. Spectra were recorded with a Bruker IFS 113v Fourier transform infrared spectrometer equipped with a DTGS detector. About 500–1000 interferograms were collected, apodized with a triangular function and Fourier transformed after one level of zero filling. Spectral resolution was 2 cm^{-1}.

Data Analysis. The frequencies, intensities, and bandwidths of the overlapping bands were evaluated in the following manner. First, the frequencies were determined by calculation of second-derivative spectra or by Fourier self-deconvolution as described by Kauppinen et al. (1981). Band shapes were then simulated by Gaussian–Lorentzian functions with 70% Gaussian proportion. This proportion for the Gaussian function gave the best fit for the band shapes of the carbonyl vibrations, independent of the solvents used. Bandwidths and band intensities were adjusted visually by an interactive procedure using software developed in this laboratory. In all cases the minimum number of bands as evaluated by Fourier self-deconvolution was used to simulate the experimental line shapes.

RESULTS

Unlabeled Phospholipids. The C=O stretching vibration of phospholipids dissolved in organic solvents is characterized by a single band at a frequency of ca. 1740 cm^{-1}. However, the exact frequency depends on the nature of the organic solvent. A solvent like $CHCl_3$ with weak proton donating capacity leads to a lower frequency and larger bandwidth of the C=O vibration. In addition the frequency depends slightly

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DMPA, dimyristoylphosphatidic acid; DMPG, dimyristoylphosphatidylglycerol.

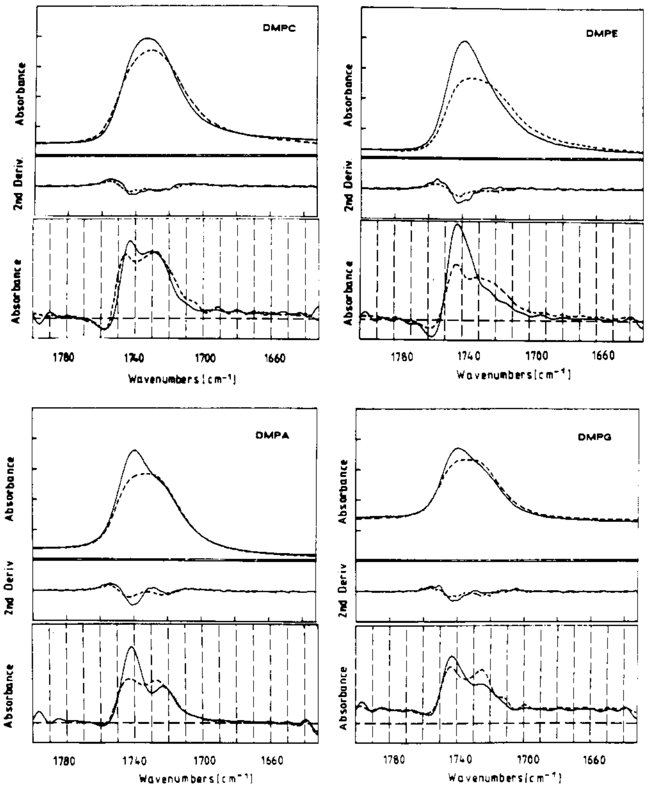

FIGURE 1: Infrared spectra of DMPC, DMPE, DMPA, and DMPG in the C=O stretching region with second-derivative (middle) and Fourier self-deconvoluted spectra (lower part). Full lines: $T < T_m$. Broken lines: $T > T_m$.

Table I: Frequencies $\tilde{\nu}$ and Full Width at Half-Height σ of the C=O Vibrational Bands of Phospholipids Dissolved in Organic Solvents, Cast as Films on KBr or NaCl Plates, and Dispersed in D_2O

phospholipid	solvent	$\tilde{\nu}$ (cm^{-1})	σ (cm^{-1})
myristoyllyso-PC	CCl_4	1736	nd[a]
	$CHCl_3$	1729	28
	D_2O	1724	36
DMPC	CCl_4	1739	25
	$CHCl_3$	1734	28
	film	1738	27
	D_2O	1743, 1727	nd
DMPE	CCl_4	1742	21
	$CHCl_3$	1737	28
	film	1742	25
	D_2O	1743, 1728	nd

[a] Not determined.

on the nature of the phospholipid; i.e., a frequency difference of ca. 2–3 cm^{-1} can be found between the C=O vibrations of DMPE and DMPC, the latter one displaying the lower frequency.

When these lipids are dispersed in water, the carbonyl stretching band is no longer symmetric but clearly shows a shoulder at lower frequencies, the height of this shoulder depending on the phase state of the lipid. Figure 1 shows the carbonyl stretching vibrations of several different phospholipids in their gel and liquid-crystalline states together with the Fourier self-deconvoluted band shapes, which clearly indicate that at least two overlapping bands of different frequency, intensity, and half-width are present, the band at lower frequency increasing in intensity, when the lipid passes into the liquid-crystalline state. Notable differences are observed between phospholipids with different head groups. Table I summarizes the frequencies and bandwidths observed for DMPC, DMPE, and palmitoyllyso-PC when dissolved in different organic solvents or in D_2O and as dry film on KBr plates.

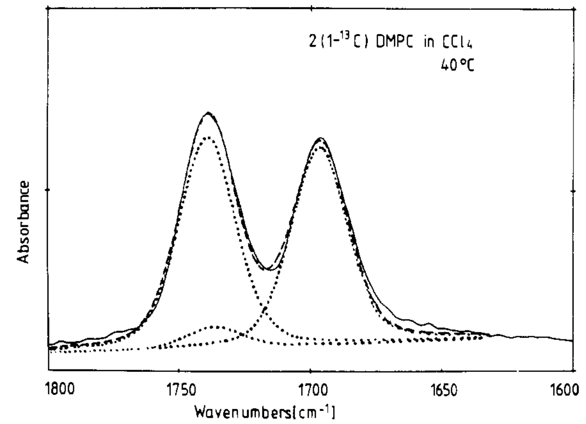

FIGURE 2: Infrared spectrum of the C=O stretching region of [*sn*-2-1-^{13}C]DMPC in CCl_4 (T = 40 °C). Full line: experimental spectrum. Dotted lines: calculated Gaussian–Lorentzian lines with a fraction of 0.7 for the Gaussian function (see text for details). Dashed line: sum of the individual calculated lines.

^{13}C-Labeled Phospholipids. Spectra of phospholipids where the carbonyl group of the *sn*-2 chain is labeled with ^{13}C show two C=O stretching vibrations due to the vibrational isotope effect. Figure 2 shows the 1600–1800-cm^{-1} region of the infrared spectrum of DMPC dissolved in CCl_4. The dashed line is a calculated line shape with a Gaussian–Lorentzian function according to

$$I(\tilde{\nu}) = (1 - P_G)f_L(\tilde{\nu}) + P_G f_G$$

with

$$f_L(\tilde{\nu}) = I_0/[1 + 4(\tilde{\nu} - \tilde{\nu}_0)^2/\sigma^2]$$

and

$$f_G(\tilde{\nu}) = I_0 \exp[-4(\ln 2)(\tilde{\nu} - \tilde{\nu}_0)^2/\sigma^2]$$

P_G is the weighting factor for the Gaussian function, I_0 the intensity at the peak maximum, σ the full width at half-height, and $\tilde{\nu}_0$ the wavenumber of the peak maximum. For the calculated curve in Figure 2 the value of σ was chosen as 25 cm^{-1} and the frequencies of the bands were set to 1739 and 1696 cm^{-1}. P_G was found to be independent of the solvent used. This was checked with the model compounds myristoyllyso-PC and ethyl acetate. The best fit could always be obtained with a weighting factor of 0.7 for the Gaussian function.

The band on the left-hand side originating from the *sn*-1 ^{12}C=O vibration is of greater intensity than that of the *sn*-2 ^{13}C=O vibration. The reason is that the ^{13}C-labeled myristic acid was enriched to only 90%. Thus 10% of the intensity of the left band is actually due to the residual ^{12}C=O vibration of the *sn*-2 ester group as indicated by the simulated curves in Figure 2. The frequency difference between the bands is 43 cm^{-1} compared to a calculated isotope shift of 40.5 cm^{-1} for an isolated C=O stretching vibration. As observed for unlabeled phospholipids, the frequencies and bandwidths of the C=O bands depend slightly on the nature of the organic solvent. In $CHCl_3$ as a solvent the band maxima are located at 1732 and 1690.5 cm^{-1}, the difference of 41.5 cm^{-1} being closer to the calculated value for the isotope shift. The width at half-height (HHW) in a weak proton donating solvent like $CHCl_3$ is somewhat larger than in a completely apolar solvent like hexane or CCl_4.

Similar results were obtained for the C=O stretching bands of [*sn*-2-1-^{13}C]DMPE in CCl_4 and $CHCl_3$, except that the absolute values of the wavenumbers were ca. 3 cm^{-1} higher

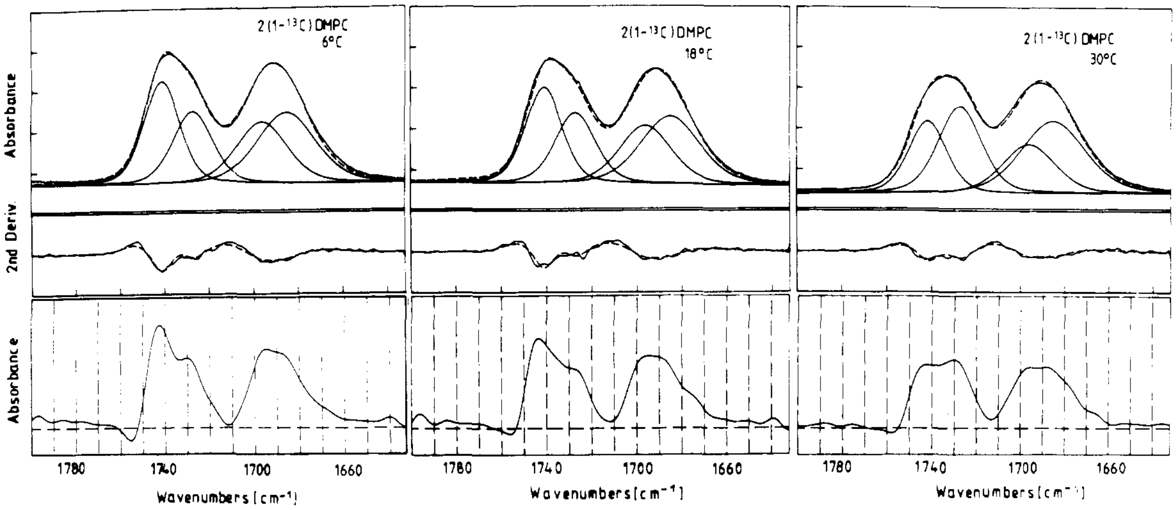

FIGURE 3: Infrared spectra of the C=O stretching region of [*sn*-2-1-^{13}C]DMPC in D_2O at three different temperatures as indicated together with the second-derivative (middle) and Fourier self-deconvoluted spectra (bottom). The dashed lines are the calculated spectra as the sum of the individual bands.

Table II: Frequencies $\bar{\nu}$ and Full Width at Half-Height σ for ^{13}C-Labeled DMPC and DMPE Dissolved in Organic Solvents and Cast as Films on KBr or NaCl Plates

		sn-1 ^{12}C=O		*sn*-2 ^{13}C=O	
phospholipid	solvent	$\bar{\nu}$ (cm^{-1})	σ (cm^{-1})	$\bar{\nu}$ (cm^{-1})	σ (cm^{-1})
[*sn*-2-1-^{13}C]DMPC	CCl_4	1739.5	25	1697	25
	$CHCl_3$	1733	26	1692	26
	film	1742	21	1695	25
[*sn*-2-1-^{13}C]DMPE	CCl_4	1741.5	21	1697	20
	$CHCl_3$	1736	27	1695	27
	film	1739	21	1697	22

compared to those of DMPC in agreement with the results obtained for unlabeled lipids. Table II shows the frequencies and half-widths of the ^{13}C=O bands for these two lipids dissolved in organic solvents and cast as films on NaCl or KBr plates.

Completely different band shapes are observed for these phospholipids when they are dispersed in D_2O. Figure 3 shows a spectrum of [*sn*-2-1-^{13}C]DMPC at different temperatures in the $L_{\beta'}$ phase below the pretransition, in the $P_{\beta'}$ phase, and in the L_α phase. Clearly the ^{12}C=O vibrational band at higher wavenumbers is a superposition of two individual overlapping bands while the ^{13}C=O vibration seems to be broadened and shifted to lower wavenumbers when compared with the spectrum of the same lipid dissolved in an organic solvent. We have used Fourier self-deconvolution and second-derivative spectroscopy to determine the frequencies of the underlying bands. While second-derivative spectroscopy clearly shows two minima for the ^{12}C=O vibration, this is less evident for the ^{13}C=O vibration at lower wavenumbers. In this case Fourier self-deconvolution is a superior procedure for resolving overlapping bands (Kauppinen et al., 1981). Figure 3 shows the band shapes of the two C=O vibrations after Fourier self-deconvolution with a Lorentzian function of 21 cm^{-1} full width at half-height using a parabolic apodization function with suitable truncation. Each of the two C=O vibrations is clearly split into at least two individual bands. The experimental spectra were simulated by superimposing four bands of different frequencies, bandwidths, and intensities with the same Gaussian–Lorentzian line-shape functions as described above. Figure 3 shows that the agreement between simulated and experimental spectrum is excellent. The frequencies used were 1741, 1727, 1696, and 1685 cm^{-1}. The lower frequency ^{13}C=O bands have a noticeably larger bandwidth compared to that of the ^{12}C=O vibration at the *sn*-1 chain (see Table II). The spectrum recorded at 6 °C indicates that possibly three bands are present for the *sn*-2 ^{13}C=O vibration. However, as Fourier self-deconvolution creates artifacts when the intensities become too low, we have decided to use only two bands for the simulation. Changing the temperature of the DMPC dispersion to 18 °C ($P_{\beta'}$ phase) and 30 °C (L_α phase) leads to changes in the intensities of the four bands. With increasing temperature the two lower frequency bands of the C=O vibrations increase in intensity, particularly when the lipid passes into the L_α phase.

To test whether the appearance of these band splittings is caused by conformational effects, we have also studied aqueous dispersions of [*sn*-1-1-^{13}C]DMPC, the isomer with the label at the *sn*-1 position. Figure 4 shows the spectra of this phospholipid at temperatures in all three different phases. The band for the *sn*-1 ^{13}C=O vibration is now on the right-hand side at lower frequency. The vibrational isotope shift for phospholipids in aqueous dispersion can now be exactly determined by comparison with the spectra shown in Figure 3. The two bands for the *sn*-1 C=O vibration previously found at 1741 and 1727 cm^{-1} have now shifted to 1699 and 1685 cm^{-1}, giving an isotope shift of 42 cm^{-1}, the same as observed before for spectra of phospholipids in organic solvents. The band intensities and their changes with temperature are similar to those observed for [*sn*-2-1-^{13}C]DMPC (see Figure 3). The nature of the head group is an important parameter in the thermotropic behavior of hydrated phospholipids. Phosphatidylethanolamines, for instance, have a smaller head group, which is dipolar like the phosphatidylcholine head group, but is also able to form intermolecular hydrogen bonds with neighboring molecules (Hitchcock et al., 1974). PE forms an L_β gel phase with untilted chains and converts directly to the L_α phase (McIntosh, 1979). Infrared spectra of [*sn*-2-1-^{13}C]DMPE at temperatures below and above the phase transition of 49 °C are shown in Figure 5. The band maxima determined by Fourier self-deconvolution are located at 1740, 1725, 1693, and 1675 cm^{-1}. The intensities of the two lower frequency bands of the two carbonyl vibrations at 1725 and 1675 cm^{-1} are noticeably smaller compared to those of DMPC (see Figure 3). The *sn*-2 ^{13}C=O vibration at lower frequency seems to be comprised of more than two components as indicated by the large bandwidth, but for the simulations only two bands were used. The major changes in the line shapes

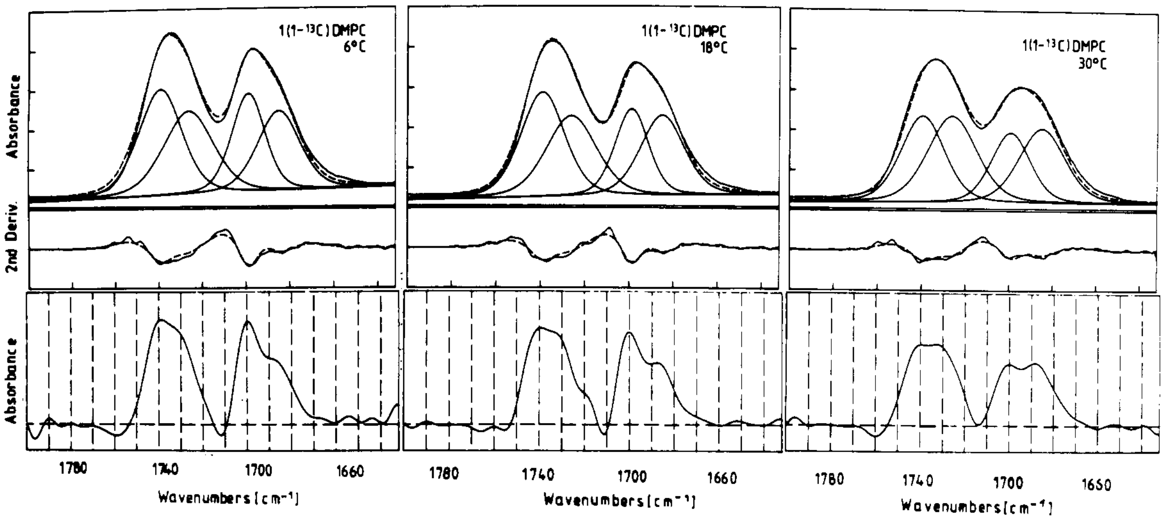

FIGURE 4: Infrared spectra of the C=O stretching region of [*sn*-1-1-^{13}C]DMPC in D_2O at three different temperatures as indicated together with the second-derivative (middle) and Fourier self-deconvoluted spectra (bottom). The dashed lines are the calculated spectra as the sum of the individual bands.

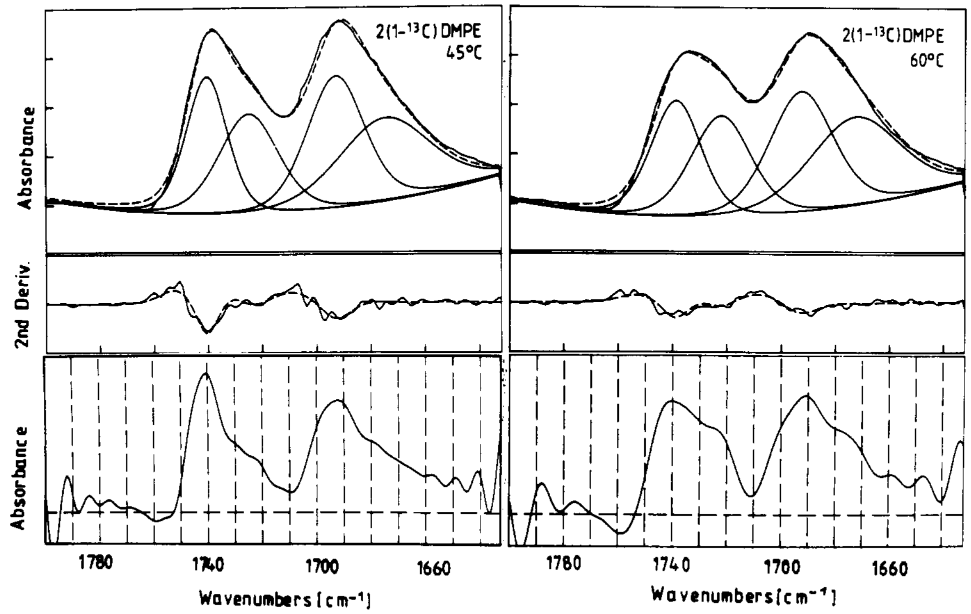

FIGURE 5: Infrared spectra of the C=O stretching region of [*sn*-2-1-^{13}C]DMPE in D_2O at temperatures in the gel and the liquid-crystalline phase together with the second-derivative (middle) and Fourier self-deconvoluted spectra (bottom). The dashed lines are the calculated spectra as the sum of the individual bands.

occur when the lipid passes into the liquid-crystalline phase. Again, particularly the intensities of the bands at 1723 and 1675 cm^{-1} increase drastically.

Dimyristoylphosphatidic acid is a phospholipid with a single negative charge at pH 7 but becomes doubly charged a pH values of ca. 12. In the gel phase at pH 7 the acyl chains are almost perpendicular to the bilayer surface, while the generation of the second charge at the head group at high pH induces a chain tilt (Eibl & Blume, 1979; Blume & Eibl, 1979; Jähnig et al., 1979). Figure 6 shows the infrared spectra in the carbonyl stretching region of [*sn*-2-1-^{13}C]DMPA at 15 and 55 °C and at pH 7 and pH 12, respectively. The large differences in the band shapes are obvious. Fourier self-deconvolution shows that each of the C=O bands is comprised of two overlapping bands with maxima at 1741.5 and 1724 cm^{-1} for the *sn*-1 $^{12}C{=}O$ and 1698 and 1681 cm^{-1} for the *sn*-2 $^{13}C{=}O$ vibration. However, the intensities are quite different. At pH 12 the lower frequency components clearly have much higher intensity. The intensity of the lower frequency bands increases further when the lipid passes into the liquid-crystalline phase (T_m = 21 °C at pH 12 and 51 °C at pH 7).

Another negatively charged phospholipid at pH 7 is dimyristoylphosphatidylglycerol (DMPG). The infrared spectrum of [*sn*-2-1-^{13}C]DMPG at pH 7 is shown in Figure 7. In this case Fourier self-deconvolution shows that the two C=O vibrations are apparently superpositions of at least three separate individual bands. Consequently, we have tried to simulate the two C=O bands with altogether six individual bands (see Figure 7). As is the case for the other phospholipids, an increase in temperature leads to band-shape changes. The lower frequency bands increase at the expense of the high-frequency components. Table III summarizes the observed frequencies and bandwidths of the $^{13}C{=}O$ and $^{12}C{=}O$ vibrational bands for phospholipids dispersed in D_2O.

DISCUSSION

Unlabeled Phospholipids

Vibrational spectra of DPPC in the dry state exhibit two major C=O vibrational bands at 1741 and 1721 cm^{-1}. In the infrared spectrum the band at higher frequency is actually split

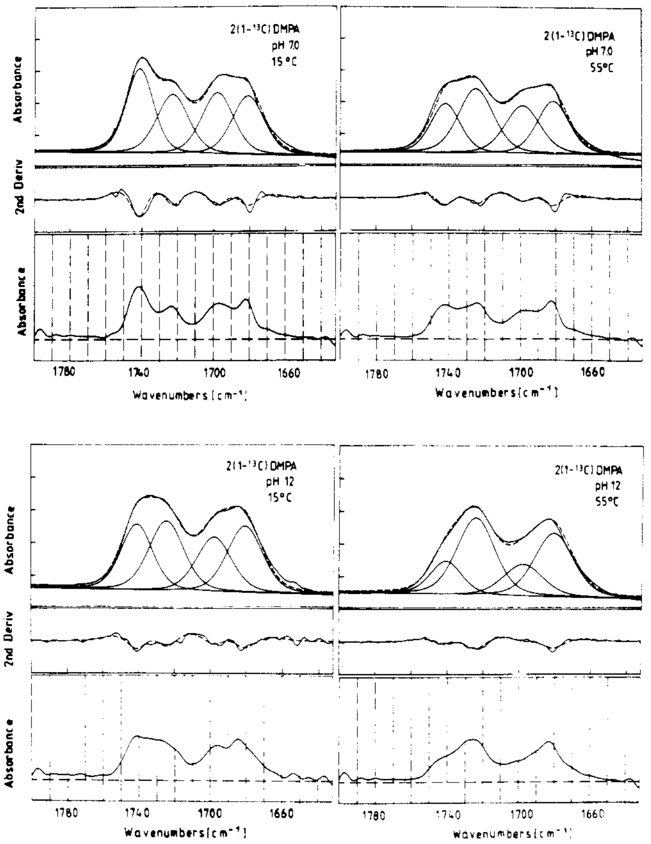

FIGURE 6: Infrared spectra of the C=O stretching region of [*sn*-2-1-^{13}C]DMPA in D_2O at pH 7 and pH 12 at temperatures in the gel and liquid-crystalline phase together with the second-derivative (middle) and the Fourier self-deconvoluted spectra (bottom). The dashed lines are the calculated spectra as the sum of the individual bands.

into a 1743- and a 1738-cm^{-1} component (Bush et al., 1980; Mushayakarara & Levin, 1982). These two major bands have been assigned to the C=O stretching vibrations of the ester groups at the *sn*-1 and *sn*-2 position, respectively. The reasons for the frequency difference were assumed to lie in the different dielectric constants of the environment of the two C=O groups, the *sn*-2 C=O group, lying closer to the bilayer plane, reflecting the more polar environment (Bush et al., 1980). Alternatively, it was suggested that the frequency difference could arise from the different conformations of the *sn*-1 and *sn*-2 chain as resolved by X-ray diffraction. As shown for DLPE and also for DMPC (Hitchcock et al., 1974; Pearson & Pasher, 1979), the *sn*-1 chain extends perpendicular to the bilayer plane with an all-trans conformation around the C–C bond of the acyl chain, while the *sn*-2 chain extends first parallel to the bilayer plane and then bends and becomes parallel to the first chain. This leads to a different conformation of the C–C bond of the *sn*-2 chain, namely, an eclipsed conformation with a torsion angle of ca. −120° (anticlinal). These different conformations were thought to produce the observed frequency differences in the C=O vibrations, the trans conformation with an torsion angle of 180° being assigned to the band with the higher frequency. The situation became more complex when it was observed that the Raman spectrum of DPPC dihydrate showed three bands in the carbonyl stretching region at 1743, 1730, and 1716 cm^{-1} (Mushayakarara et al., 1982). These three bands were thought to arise from the two structurally inequivalent molecules A and B in the unit cell of the dihydrate (Pearson & Pasher, 1979), the broader band at 1730 cm^{-1} actually being due to two bands of molecule A and B. Frequency differences were again explained by differences in dielectric constant of the surrounding.

When aqueous dispersions of phosphatidylcholines were investigated, it was always found that the addition of water caused a broadening of the bands, so that only one band contour could be observed. However, the asymmetric band shape and Fourier self-deconvolution suggested that this band was composed of two underlying components at 1741 and 1727 cm^{-1} (see Figure 1). Similar results were obtained for the isomer 1,3-DPPC and for phosphatidycholines with branched fatty acyl chains, for phosphatidylethanolamines, and phosphatidylserine (Dluhy et al., 1985; Mantsch et al., 1981, 1985; Dluhy et al., 1983).

^{13}C-Labeled Phospholipids

Phospholipids in Organic Solvents. Labeling the *sn*-2 C=O group with ^{13}C has the clear advantage of being able to distinguish between both C=O groups, because the vibrational

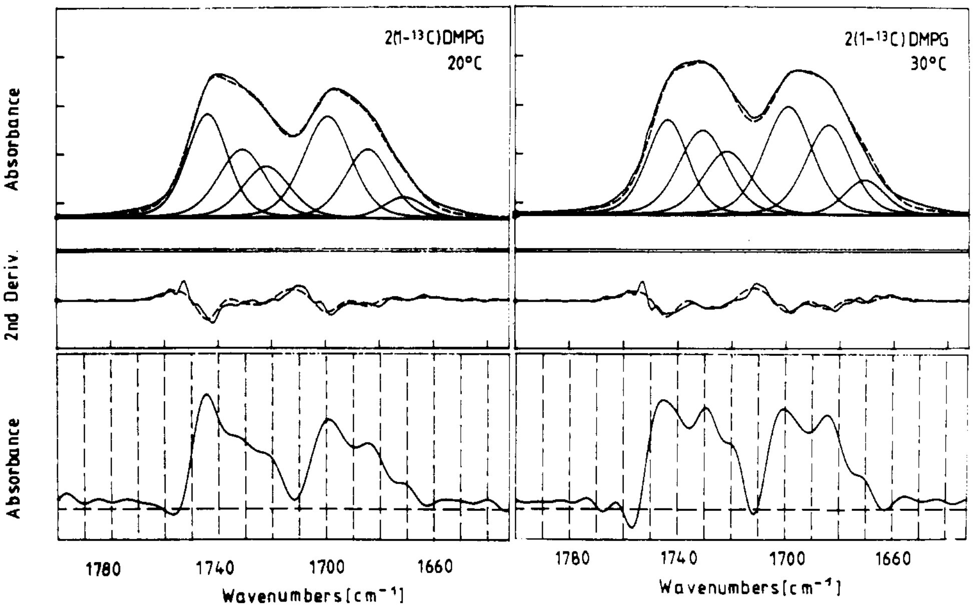

FIGURE 7: Infrared spectra of the C=O stretching region of [*sn*-2-1-^{13}C]DMPG in D_2O at temperatures in the gel and liquid-crystalline phase together with the second-derivative (middle) and Fourier self-deconvoluted spectrum (bottom). The dashed lines are the calculated spectrum as the sum of the individual bands.

Table III: Frequencies $\tilde{\nu}$ and Full Widths at Half-Height σ for the Component Bands for the $^{12}C{=}O$ and $^{13}C{=}O$ Vibration of ^{13}C-Labeled Phospholipids As Determined by Fourier Self-Deconvolution and Band-Shape Simulations with Gaussian–Lentzian Functions

	sn-1 $^{12}C{=}O$						sn-2 $^{13}C{=}O$					
	$\tilde{\nu}$ (cm^{-1})			σ (cm^{-1})			$\tilde{\nu}$ (cm^{-1})			σ (cm^{-1})		
phospholipid	$\tilde{\nu}_1$	$\tilde{\nu}_2$	$\tilde{\nu}_3$	σ_1	σ_2	σ_3	$\tilde{\nu}_4$	$\tilde{\nu}_5$	ν_6	σ_4	σ_5	σ_6
[*sn*-2-1-^{13}C]DMPC	1741	1727		19	21		1696	1685		27	31	
[*sn*-2-1-^{13}C]DMPE	1740	1725		18	26		1693	1675		25	40	
[*sn*-2-1-^{13}C]DMPA (pH 7)	1741.5	1723		18	21		1698	1681		21	21	
[*sn*-2-1-^{13}C]DMPA (pH 12)	1741	1724.5		21	23		1698	1681		23	23	
[*sn*-2-1-^{13}C]DMPG	1742	1730	1722	18	21	21	1703	1691	1679	21	20	18
[*sn*-1-1-^{13}C]DMPC[a]	1739	1726		24	27		1699	1685		18	23	

[a] For [*sn*-1-1-^{13}C]DMPC the $^{12}C{=}O$ group is at the *sn*-2 position and the $^{13}C{=}O$ group at the *sn*-1 position.

isotope effect leads to a clear separation of the bands (Green et al., 1987). The infrared spectra of ^{13}C-labeled phospholipids in organic solvents show two different vibrational bands separated by ca. 42 cm^{-1}, a value close to the calculated isotope effect of ca. 40.5 cm^{-1} for an isolated $^{13}C{=}O$ stretching vibration. Reversing the labeling, i.e., labeling the C=O group at the *sn*-1 position, has almost no effect on the frequency difference between the two bands. Thus a conformational effect on the C=O vibrational frequency for lipids dissolved in organic solvents can be excluded. However, there are slight changes in the absolute values of the frequencies when the lipids are dissolved in different organic solvents like *n*-hexane, CCl_4, or $CHCl_3$. $CHCl_3$ as a solvent shifts the bands by ca. 6 cm^{-1} to lower wavenumbers when compared with CCl_4. This shift is caused by the higher dielectric constant of $CHCl_3$ compared to those of CCl_4 and *n*-hexane and by its ability to act a a weak hydrogen-bond donor (Pimentel & McClellan, 1960). Interestingly, the frequencies observed for DMPE dissolved in CCl_4 or $CHCl_3$ are somewhat (ca. 3 cm^{-1}) higher compared to that for DMPC. In addition, the bands are narrower, ca. 21 cm^{-1} compared to 26 cm^{-1} for DMPC. Possibly different forms of aggregation, i.e., formation of inverted micelles of different form and size, are the cause for this effect.

Phospholipid Films. We have also investigated films of DMPC and DMPE deposited from absolute CCl_4 or $CHCl_3$ solutions on KBr or NaCl plates. As judged from the almost complete absence of the OH stretching band at ca. 3400 cm^{-1}, these films seemed to be nearly dry. Analysis of the C=O bands showed that for [*sn*-2-1-^{13}C]DMPE the band frequencies and bandwidths stayed almost the same compared to those of the spectrum taken in CCl_4 solution, the frequency difference between the two C=O vibrations being ca. 42 cm^{-1}. For [*sn*-2-1-^{13}C]DMPC, however, the frequency difference increased to ca. 47 cm^{-1}, and the $^{13}C{=}O$ band for the *sn*-2 chain at the lower frequency increased in width, the bandwidth of the $^{12}C{=}O$ vibration staying the same. Presumably differences in packing in the dry lamellar phase are the cause. DMPE forms gel-state bilayers with almost perpendicular chains, whereas for DMPC the chains are more tilted with respect to the bilayer normal. An additional explanation may be that in this case conformational differences are responsible. However, the crystal structures of DLPE and DMPC dihydrate reveal no major differences in the torsion angles of the glycerol backbone and the *sn*-2 chain between these two lipids (Hauser et al., 1981). Bush et al. (1980) have reported infrared spectra of dry DPPC films, where two bands were seen, one at ca. 1741 cm^{-1} and the other at 1727 cm^{-1}. Also Green et al. (1987) observed a splitting of the *sn*-2 $^{13}C{=}O$ band for DPPC deposited on CaF_2 plates. We never observed these band splittings in our preparations. At present we have no satisfactory explanation for these differences. We prepared our films on KBr or NaCl plates from absolute CCl_4 or $CHCl_3$ instead of benzene. Differences in bilayer structure of films prepared from different solvents and on different materials are conceivable.

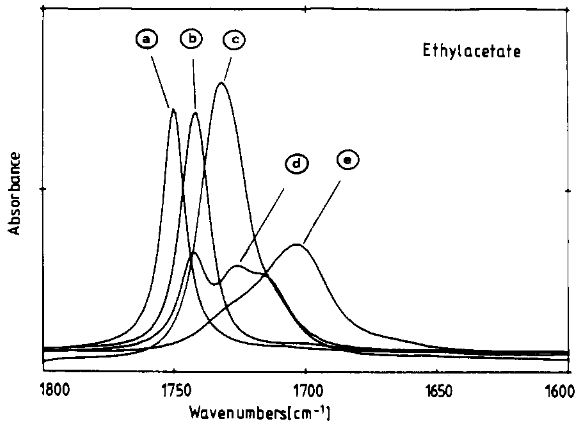

FIGURE 8: Infrared spectra of the C=O stretching region of ethyl acetate. The solvents used were (a) *n*-hexane; (b) CCl_4, (c) $CHCl_3$, (d) methanol, and (e) D_2O.

Phospholipid Dispersions. In completely hydrated phospholipid bilayers the $^{12}C{=}O$ band as well as the $^{13}C{=}O$ band is split into two or even three components. In our experiments we did not observe these splittings in dry films, nor did we see them for lipids dissolved in organic solvents. So our conclusion was that they must arise from hydration effects, i.e., hydrogen bonding of water to the carbonyl groups. The effect of hydrogen bonding to the carbonyl groups of triacetylglycerol on the frequency of the C=O vibration has been reported by Mushayakarara et al. (1986). These authors found a frequency shift to 1728 cm^{-1} upon hydrogen bonding of D_2O, exactly the same band frequency we observed for all phospholipids studied so far. Also Fringeli and Günthard (1976) using modulated excitation infrared spectroscopy have reported that the primary hydration sites of egg phosphatidylcholine are the PO_2^- and the C=O groups besides the choline head group, and studies of lipid monolayers (Smaby et al., 1983) have shown that the *sn*-1 C=O groups are in contact with water.

For a further test of this notion, we recorded infrared spectra of the model compound ethyl acetate in solvents of different dielectric constant ϵ and hydrogen-bonding capacity, namely, *n*-hexane, CCl_4, $CHCl_3$, methanol, and D_2O as shown in Figure 8. In *n*-hexane (ϵ = 1.89) or CCl_4 (ϵ = 2.24) a single band is observed at 1750 or 1742 cm^{-1}, respectively. The lower frequency for CCl_4 is clearly an effect of its higher dielectric constant. In $CHCl_3$ as a solvent (ϵ = 4.8) a further decrease in frequency to 1732 cm^{-1} occurs. In addition a drastic increase in bandwidth at half-height from 12–13 cm^{-1} in *n*-hexane or CCl_4 to 20 cm^{-1} in $CHCl_3$ is seen (See Table IV). $CHCl_3$ is known to act as a weak hydrogen-bond donor, and hydrogen bonding normally results in increased bandwidths

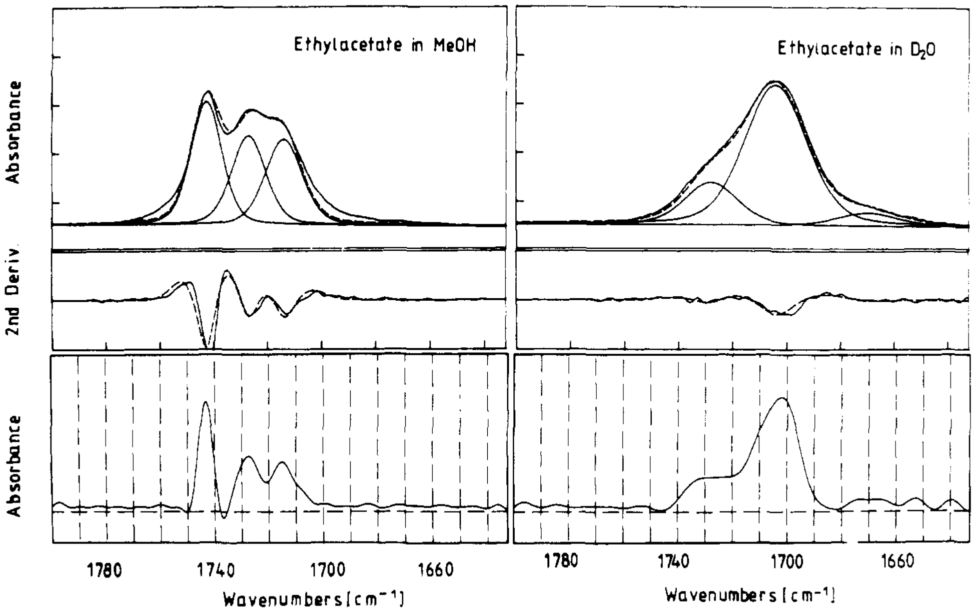

FIGURE 9: Infrared spectra of the C=O stretching region of ethyl acetate in methanol and D_2O together with the second-derivative (middle) and the Fourier self-deconvoluted spectra (bottom). The dashed lines are the calculated spectra as the sum of the individual bands.

Table IV: Frequencies $\bar{\nu}$ and Full Widths at Half-Height σ for Ethyl Acetate in Different Solvents

solvent	$\bar{\nu}_1$ (cm^{-1})	σ_1 (cm^{-1})	$\bar{\nu}_2$ (cm^{-1})	σ_2 (cm^{-1})	$\bar{\nu}_3$ (cm^{-1})	σ_3 (cm^{-1})
n-hexane	1750	11				
CCl_4	1742	13				
$CHCl_3$	1732	20				
methanol	1743	13.5	1727	15	1714	16
D_2O	1728	23	1704	27	1670	27

and lower frequencies (Pimentel & McClellan, 1960).

Completely different spectra are observed for methanol and D_2O solutions, i.e., solvents with strong hydrogen-bonding capability (see Figure 8). In these two solvents the C=O vibrations are composed of several bands (see Figure 9). In methanol three bands are observed at 1743, 1727, and 1714 cm^{-1}. In D_2O the bands are located at 1728, 1704, and 1670 cm^{-1}.

Patel et al. (1985) have recently reported a detailed IR study of the solvation of esters and carbonates in various solvents. They used methyl acetate as a model compound and found similar spectra for this ester dissolved in methanol and D_2O. Their interpretation for the observed band splittings was the notion that different solvent complexes must exist, giving different C=O vibrational frequencies. They assigned the band with the highest wavenumber to a non-hydrogen-bonded C=O vibration, while the band at 1728 cm^{-1} observed in methanol as well as in D_2O is apparently due to a C=O group with one hydrogen bond donor molecule. The bands at lower frequencies at 1714 cm^{-1} in methanol or 1704 cm^{-1} in D_2O they assigned to a disolvate and the band with even lower frequency at 1670 cm^{-1}, observed only in D_2O with very low intensity, to a trisolvate. The band fitting shows that the disolvate is clearly the dominant complex form for ethyl acetate in D_2O, while in methanol a considerable amount of nonsolvated and thus non-hydrogen-bonded ester molecules can exist. When the results obtained from these model studies are compared to the the lipid spectra, the similarities in the frequencies of the observed bands and their bandwidths are striking.

For all phospholipids we observe a high-frequency band at 1740–1742 cm^{-1}, which can be assigned to a non-hydrogen-bonded ^{12}C=O vibration. Subtracting the isotopic shift of 42 cm^{-1}, we would expect a frequency of ca. 1698–1700 cm^{-1} for the non-hydrogen-bonded ^{13}C=O. In all cases a band with this particular frequency is indeed observed. Slight differences in the absolute value between lipids with different head groups could arise from conformational effects, as the gel phases of these lipids have different structures. This can be seen by comparing DMPC labeled in the *sn*-1 or *sn*-2 position. For [*sn*-2-1-^{13}C]DMPC the *sn*-1 ^{12}C=O band is at 1741 cm^{-1} while for [*sn*-1-1-^{13}C]DMPC with reversed labeling the *sn*-2 ^{12}C=O vibration appears at 1739 cm^{-1}, indicating a conformational effect of ca. 2 cm^{-1}. Besides this high-frequency band we observe for both C=O groups in all cases at least one, sometimes two, additional vibrational band at lower frequency. For the *sn*-1 ^{12}C=O group the second band appears at ca. 1723–1728 cm^{-1}, while for the *sn*-2 ^{13}C=O group the band is located at 1675–1685 cm^{-1}. In accordance with the observations on model compounds we assign this additional band to the monohydrated C=O group.

In the case of DMPG the frequencies observed for the component bands are somewhat different. The Fourier self-deconvoluted spectrum clearly shows three bands for each of the two C=O vibrations. DMPG is a negatively charged lipid with a glycerol residue at the head group which is capable of acting as a hydrogen-bond donor. It is conceivable that inter- or intramolecular hydrogen bonds are formed between the DMPG head groups and the C=O groups or that mixed solvates with the glycerol OH groups and D_2O can form which result in different band frequencies.

We have tried to extract quantitative information on the extent of hydrogen bonding to the two ester C=O groups by simulating the observed band shapes with Gaussian–Lorentzian functions as described above and shown in the Figures. Table V shows the percentages of the integral intensities of the lower frequency bands attributed to the hydrogen-bonded C=O groups. In all phospholipids except DMPE a notable increase in intensity of the lower frequency bands occurs when the lipids pass into the liquid-crystalline phase and the lipid lattice expands. In DMPE, only a minimal change is observed. Phosphatidylethanolamines are known to be much less hydrated than phosphatidylcholines or phosphatidylglycerols, and it is also known that the hydration does not change significantly with temperature (Jendrasiak & Hasty, 1974; Seddon et al.,

Table V: Intensities (%) of the C=O Vibrational Bands Assigned to the Hydrogen-Bonded Species after Fourier Self-Deconvolution and Band-Shape Simulation

phospholipid	T (°C)	*sn*-1 ^{12}C=O	*sn*-2 ^{13}C=O
[*sn*-2-1-^{13}C]DMPC	6	44	57
	18	45	57
	30	58	64
[*sn*-2-1-^{13}C]DMPE	20	47	49
	45	51	50
	60	51	51
[*sn*-2-1-^{13}C]DMPA (pH 7)	15	45	48
	30	46	50
	55	58	54
[*sn*-2-1-^{13}C]DMPA (pH 12)	15	54	55
	30	68	61
	55	73	66
[*sn*-2-1-^{13}C]DMPG	20	52	45
	30	65	52
	40	66	53

1983; Cevc & Marsh, 1985; McIntosh & Simon, 1986). Thus the infrared spectra are a correct reflection of these previous findings.

While the intensities of the lower frequency bands attributed to the solvated C=O groups can be determined fairly accurately, they are not an exact measure for the amount of hydrogen-bonded groups. For an exact determination one would have to know the absorption coefficients for both forms. Spectra of myristoyllyso-PC dissolved in $CHCl_3$ and D_2O indicated an increase in the absorption coefficient of almost 40% when D_2O was used as a solvent. This effect is a characteristic feature for all carbonyl compounds when they act as hydrogen-bond acceptors and can be used as a diagnostic tool (Pimentel & McClellan, 1960). In the case of myristoyllyso-PC it also shows besides the band shift to lower frequency that the C=O group in the micelles formed by this lipid is indeed completely in contact with water. In bilayer-forming phospholipids the integral intensities of the lower frequency bands would have to be reduced roughly by ca. 40% to get an estimate for the total fraction of hydrogen-bonded C=O groups. With this crude approximation we can compare our infrared data with those obtained from ^{13}C NMR measurements reported by Yeagle and Martin (1976) and Schmidt et al. (1977). These authors used the solvent-dependent chemical shift of the carbonyl resonances to evaluate the amount of hydrogen bonding to the C=O groups in phospholipid vesicles. Yeagle and Martin reached the conclusion that both C=O groups in PC are hydrogen bonded, the *sn*-2 C=O groups being more exposed to water. Schmidt et al. quantified this finding reporting values of ca. 35% and 45% for the hydrogen-bonded forms of the *sn*-1 and *sn*-2 C=O groups, respectively. This compares well with our values for DMPC, keeping in mind that the values in Table V have to be reduced by ca. 40% due to the increase in absorption coefficient and that we studied multilammellar systems and not small vesicles.

Inspection of Table V shows that only for DMPC is the *sn*-2 ^{13}C=O group more hydrated than the *sn*-1 C=O. This is different for other lipids, for instance, DMPA at pH 7 and 12 in the liquid-crystalline phase. Here the *sn*-1 C=O group seems to be more hydrated. In the last years the single-crystal structures of DMPA and DMPG have been reported (Harlos et al., 1984; Pascher et al., 1987). In DMPA and DLPA (Harlos et al., 1984; Pascher & Sundell, 1986) the glycerol moiety adopts an almost layer-parallel orientation, displacing the *sn*-2 C=O group deeper down in the hydrophobic region and exposing the *sn*-1 C=O group to the interface. This conformation is quite different from that found for DLPE and DMPC. The higher fraction of hydrogen-bonded C=O groups for the *sn*-1 chain found by band-shape simulations indicates that the conformation found in the crystal may be retained in fully hydrated PA bilayers in the liquid-crystalline phase independent of the charge of the head group. This conformation might change to a layer-perpendicular orientation of the glycerol moiety when the lipid passes into the gel state, as then according to our infrared spectrum the *sn*-2 C=O group seems to be more hydrated. The generally higher proportion of hydrogen-bonded C=O groups found for doubly charged PA is obviously due to the expansion of the lipid lattice which facilitates the intrusion of water molecules between the head groups and the chains (Jähnig et al., 1979).

The crystal structure of DMPG solved recently by Pascher et al. (1987) shows two independent molecules per asymmetric unit which differ in the conformations adopted by the glycerol backbone. In molecule B the *sn*-2 C=O group is closer to the bilayer interface while for molecule A this is the *sn*-1 C=O group. For DMPG in D_2O our infrared data indicate a preferred hydration of the *sn*-1 C=O group in both phases, suggesting that in hydrated bilayers PG molecules preferentially adopt the conformation found for molecule A. However, the appearance of three vibrational bands for each of the two C=O groups indicates a more complex hydrogen bonding. It is conceivable that the glycerol OH groups of the head group can take part in the hydrogen bonding, either directly or via intercalated water molecules. In the crystal an extensive hydrogen-bonding network between the OH groups of the glycerol and the phosphate oxygens exists. In addition, intercalated sodium ions form contacts laterally and across the bilayer interface. As PG bilayers swell indefinitely, interbilayer interactions are certainly absent in our systems, but the interactions between head groups of the same bilayer via OH groups of the glycerol and/or water molecules seem to be retained. A comparison of the structure of hydrated DPPG in the gel phase (Watts et al., 1981) with the crystal structure of DMPG revealed no major differences in the bilayer thickness and the angle of tilt. The only difference occurred in the molecular area *S* which is increased on hydration of the bilayer. This can facilitate hydrogen bonding to the C=O groups as then the head groups are more separated.

Overall, the differences between the hydration of the *sn*-1 C=O and the *sn*-2 C=O groups are not very large. From the crystal structures one would expect these differences to be more pronounced. In hydrated bilayers it seems likely that due to the expansion of the lattice both C=O groups become more or less equally accessible to water, i.e., that water penetrates at least up to the *sn*-1 C=O groups. This has also been shown by neutron and X-ray diffraction and by capacity measurements (Knott & Schoenborn, 1986; Simon & McIntosh). Another possibility, namely, equilibria between different conformations of the glycerol backbone exposing both C=O groups equally, seems unlikely, as the width of the *sn*-1 C=O band is always less than that for the *sn*-2 band, indicating lower flexibility for the *sn*-1 ester group.

Green et al. (1987) have reported infrared spectra of [*sn*-2-1-^{13}C]DPPC in the dry and hydrated state. While the spectra of hydrated DPPC reported by these authors look very similar to ours, their interpretation is completely different, as no Fourier self-deconvolution was performed to reveal the underlying component bands for the *sn*-2 C=O vibration. These authors interpret the frequency shift observed upon melting with the removal of chain tilt. However, DMPE and DMPA at pH 7, which form nontilted gel phases, display similar frequency shifts for their C=O bands. We believe that also in the case of PCs increase in hydration is the cause for the observed frequency shift.

CONCLUSIONS

The labeling of phospholipids with ^{13}C at the ester carbonyl group of the *sn*-2 chain has resolved the ambiguity of the assignment of the two carbonyl bands observed before for unlabeled phospholipids. Comparison with model compounds leads to the conclusion that both carbonyl groups of hydrated phospholipids take part in hydrogen bonding to water. Lipid intermolecular hydrogen bonding, hydration of head groups, and the depth of water penetration into the hydrophobic core of the bilayer are important parameters for the structure and properties of model and biological membranes (Boggs, 1987). These parameters influence bilayer–bilayer and lipid–protein interactions and may govern the water and proton permeability of membranes, which was found to be exceptionally high (Lawaczek, 1979; Nichols & Deamer, 1980; Elamrani & Blume, 1983). The depth of water penetration can be determined by neutron diffraction (Knott & Schoenborn, 1986) or a combination of X-ray and capacitance measurements (Simon & McIntosh, 1986). Unfortunately, these measurements provide not enough resolution to determine exactly whether the carbonyl groups can be reached by water molecules or not. Infrared and NMR spectroscopy is more suited for this purpose. From ^{13}C NMR chemical shifts of the carbonyl resonances it was concluded that both carbonyl groups are in contact with water (Yeagle & Martin, 1976; Schmidt et al., 1979). The ^{13}C chemical shifts are average quantities as the water molecules exchange rapidly with bulk water on the time scale of the ^{13}C NMR experiment. Infrared spectroscopy is a method which provides an instantaneous picture of the amount of hydrogen bonding to the carbonyl groups, making it possible to see directly the various hydrogen-bonded complexes. An additional advantage of the use of ^{13}C-labeled phospholipids is the possibility of observing the C=O bands of both ester groups separately without interference. Thus, it was possible to quantify the amount of hydration of both groups and gather evidence for differences in glycerol backbone conformation between phospholipids carrying different head groups. Current work in this laboratory is adressed to the question of hydrogen bonding in lipid–cholesterol systems and will be published separately.

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Registry No. DMPC, 13699-48-4; DMPE, 20255-95-2; DMPA, 30170-00-4; DMPG, 61361-72-6.

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The Effect of Gentamicin on the Biophysical Properties of Phosphatidic Acid Liposomes Is Influenced by the O–C=O Group of the Lipid[†]

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ABSTRACT: We previously reported that gentamicin binds to liposomes composed of anionic phospholipids and depresses glycerol permeability and raises the activation energy for glycerol permeation in these liposomes. We postulated that these changes in the glycerol permeability and in the activation energy (E_a) for glycerol permeation were due to hydrogen bonding between O—C=O groups in the hydrogen belt and one or more amino groups of gentamicin. To test this hypothesis, we examined the effects of gentamicin on the membrane surface potential, the glycerol permeability coefficient ($\bar{p}$), the E_a for glycerol permeation, and the aggregation of liposomes composed of 1:1 phosphatidylcholine (PC) and phosphatidic acid with the acyl chains of phosphatidic acid in either an ester (PA) or an ether (PA*) linkage. Gentamicin depressed the membrane surface electrostatic potential, measured by the partitioning of methylene blue between the bulk solution and the liposomal membrane, to an equivalent degree in PC–PA and PC–PA* liposomes, which indicates that substitution of the ether for the ester linkage did not interfere with the electrostatic interaction between the cationic drug and the negatively charged phosphate head group. Gentamicin caused a temperature-dependent decrease of $\bar{p}$ and raised E_a for glycerol permeation from 17.7 ± 0.3 to 21.6 ± 0.4 kcal/mol in PC–PA liposomes but had little or no effect on these parameters in PC–PA* liposomes. In contrast, gentamicin induced a significantly greater degree of aggregation of PC–PA* liposomes compared to that of PC–PA liposomes. These data support the conclusion that gentamicin interacts with the carbonyl ester groups linking the acyl chains to the glycerol backbone of PA and that this interaction is entirely consistent with hydrogen bonding. These results provide new information about the molecular binding of gentamicin to anionic phospholipids and the effects of such binding on the biophysical properties of model membranes.

Aminoglycoside antibiotics are known to cause nephrotoxicity (Humes et al., 1982; Kahlmeter & Dalhlager, 1984) and ototoxicity (Brummett, 1980) in man and in experimental animals. Toxicity in the kidney is confined to proximal tubular cells that have been shown to transport and accumulate these drugs in high concentration (Kaloyanides, 1984a). Transport and accumulation of aminoglycoside antibiotics by hair cells also underlie the ototoxicity of these drugs (Tran Ba Huy et al., 1986). A growing body of evidence supports the view that the toxicity of these agents resides in their potential to bind to and alter the function of plasma and subcellular membranes (Kaloyanides, 1984b). Investigators have sought to elucidate the mechanism of binding of these drugs to membranes. Studies of biological (Kirschbaum, 1984) and model membranes (Schacht et al., 1978; Lullmann & Vollmer, 1982; Brasseur et al., 1984; Wang et al., 1984; Chung et al., 1985; Au et al., 1986, 1987) have demonstrated that these polycationic drugs bind to anionic phospholipids, especially phosphatidylinositol 4,5-bisphosphate (Schacht et al., 1978; Wang et al., 1984; Au et al., 1986, 1987), whereas little or no binding occurs to anionic nonlipid compounds such as melanin, gangliosides, or chondroitin sulfate (Williams & Schacht, 1986). This has led to the hypothesis that anionic phospholipids, especially the phosphoinositides, function as membrane receptors for these drugs (Schacht, 1979; Sastrasinh et al., 1982).

Chung et al. (1985) examined the binding of gentamicin and spermine to liposomes containing negatively charged phospholipids using the technique of microelectrophoresis. Gentamicin and spermine have similar net charges (about +3.5 at pH 7.4), and both agents depressed the membrane surface

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