Fourier Transform Infrared Spectroscopy as a Probe for the Study of the Hydration of Lipid Self-Assemblies. II. Water Binding Versus Phase Transitions

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ABSTRACT: The gradual hydration of phospholipid films can be effectively probed by Fourier transform infrared (FTIR) spectroscopy (cf. part I of this series). The hydration-induced changes observed for lipid IR-absorption bands are probably composed of contributions arising from the effects of both the direct binding of water molecules and the thereby caused conformational changes and phase transitions in the lipid molecules and assemblies, respectively. In this article, an attempt is made to attribute some of the more indicative spectroscopic results to these molecular and supermolecular processes with a view to separating their individual contributions to the relevant spectroscopic data. This is done by considering a series of suitable PLs consisting of the palmitoyl and oleoyl lecithins, DPPC, DOPC, POPC, and OPPC, and one cephalin, DOPE. This choice of PCs and DOPE means that at room temperature and different degrees of hydration, several phase states including lamellar gel and liquid crystalline as well as certain nonlamellar phases are covered. The separation of the water-binding and phase-transition contributions to the FTIR-spectroscopic data, we believe, is clearly demonstrated by interpreting the hydration-dependent wavenumber shifts of the νC=O band of the PCs. Carbonyl groups are affected to a more significant degree for lipids arrayed in the $L_a$ phase than in the gel phase. A number of spectral features reveal the lyotropically triggered chain-melting transition as well as other structural rearrangements of PCs. This is discussed in detail and demonstrates the excellent sensitivity of the FTIR methodology for the study of such systems. © 1998 John Wiley & Sons, Inc. Biospectroscopy 4: 281–294, 1998

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INTRODUCTION

For phospholipids, the presence of water leads to an enhanced diversity of the manifestations of their intrinsic amphiphilicity resulting in a greater variety of the specific supermolecular structural organizations or phases, which distinguish this important class of chemical compounds. Currently, there is a great deal of research interest in the factors, which control and influence the lipid hydration process. In the Introduction of part I of this series,1 an overview of the most common general approaches and physicochemical methods used to study phospholipid hydration is given and fundamental questions were addressed.

So far, Fourier transform infrared (FTIR) spec-
FTIR spectroscopy has been extensively used to characterize either anhydrous or fully hydrated lipids. Our efforts were aimed at developing an algorithm suitable for studying the gradual hydration of PLs in model-membrane systems within a wide range of water activities by this method. The preceding article focused on some general results obtained for a series of systematically related PCs comprising all possible compounds with palmitoyl and oleoyl fatty-acid chains, namely DPPC, POPC, OPPC, and DOPC. For the series of PCs selected, \( L_0 \) and \( L_a \), the two principal lamellar phases possible for lipid systems, are present in the films under the conditions chosen, that is, room temperature. Of particular interest is the fact that depending on the hydration levels, mixed-chain PCs can adopt both these phases, \( L_0 \) and \( L_a \), within the same sample. In the present article (part II of the series), results for the cephalin DOPE, some of which have already been published, are included in order to further enhance the possibility for cross-correlations to be made. Thus, another structural variation concerning the chemical nature of the headgroup was introduced into the PL set. In this article, using FTIR data obtained by methods described in part I of this two-part series, a more sophisticated interpretation than was possible in part I will be given concentrating on a more specific differentiation of selected spectroscopic data with respect to their possible inherent characteristics and origin.

The experimental procedures used to obtain these data were based on FTIR spectroscopic measurements of differently (via RH) hydrated lipid films and were described in detail in part I.

**RESULTS AND DISCUSSION**

**General Remarks**

With respect to the influence of hydration on the nature of the change in the IR bands of PLs, two principal possibilities can be identified: the character of the change in a spectral parameter such as a wavenumber can be either smoothly continuous or abruptly discontinuous. The former, the more usual case, indicates gradual changes in the relevant properties of a chemical bond, such as, electron density; the latter, however, which is less often observed, suggests the occurrence of more inherent structural or conformational changes. For lipids, abrupt changes in spectral band parameters, especially if found in a concerted or correlated way involving several vibrational modes, can be taken as revealing more significant rearrangements most probably on a supermolecular scale or, in other words, phase transitions. The next question might be what are the boundaries between these two cases. In this article an attempt will be undertaken to provide answers to this question (see “Comparative Analyses of the \( \nu C=O \) Vibration Mode”).

The phenomenological difference between these two possibilities is illustrated in Figure 1 which shows a juxtaposition of hydration-induced wavenumber shifts for a characteristic infrared band, namely that due to the symmetric PO\(_2\) stretch of DPPC and DOPE. The shapes of the curves reflect exemplarily the dependencies of the wavenumber on the degree of hydration that comprise the continuous and abrupt changes typically obtained for DPPC and DOPE, respectively.

In a pseudo-3-D representation, with \( A_{wr} \) (as previously defined in part I) which is the normalized absorbance of the \( \nu_1,3OH \) band of water and is proportional to the lipid film water content, as the third axis, an even more revealing impression can be gained. A respective example was given by the survey on the headgroup core IR region at 900–1300 cm\(^{-1}\) of DOPE shown in Figure 4 of a very recent article. This figure demonstrated the simultaneous, that is, “isohumidic” appearance of a number of abrupt spectral changes at an \( A_{wr} \) value of about 0.2, thus emphatically suggesting the occurrence of a phase transition. In a sense, as discussed above, these findings have been at-
tributed to a lyotropic phase transition undergone between two nonlamellar, two-dimensional phases, that is, the inverse hexagonal \( (H_{II}) \) and the fluid inverse ribbon (denominated as \( P_a \)) phase.3

In a corresponding 3-D plot for DPPC (not shown), on the other hand, the gross wavenumber and intensity changes found are largely continuous and uncorrelated, differing in the loci on the hydration axis for several of the pertinent bands. This can be understood as reflecting gradual structural variations in the headgroup region of DPPC that might be induced, for instance, by a successive binding of water molecules to phosphate groups rather than by substantial conformational and/or phase changes. This behavior is in agreement with the idea of an overall lamellar gel phase adopted by DPPC for all hydration levels.1

Spectroscopic Features Mirroring the Isothermal Lipid Main Transition

Another example of evidence of a lyotropic phase transition at room temperature was the detection of the chain-melting/freezing (main) transition for the mixed-chain PCs, POPC, and OPPC. In Figure 6 of the preceding article1 that phenomenon was demonstrated by a steep wavenumber change in the bands due to the chain methylene stretching vibration. This provides a heuristically very valuable case for attributing significant wavenumber shifts exclusively to the influence of conformational changes (here between the all-trans form existing in the gel phase and a disordered form with a substantial amount of gauche conformers in the liquid-crystalline phase) since direct water binding to the methylene groups (together with its presumable effects on the \( CH_2 \) wavenumbers) can undoubtedly be ruled out. In Figure 2, wavenumber shifts from nonstationary dehydration and rehydration scans of POPC are depicted involving the symmetric stretching and bending scissoring modes. Two findings are noteworthy, on the one hand, the existence of pronounced hystereses for both bands and on the other hand their correlated appearance at strictly the same \( A_{wr} \) values. Since in isopiestic experiments no such hysteresis was observed, the first finding is ascribed to the nonequilibrium conditions applied in these “dynamical” measurements. An experimental setup of this type of study might be considered similar to the regime used in calorimetric scans of lipids where very often heating/cooling hystereses can also be observed. The correlation involved in the second finding testifies that the wavenumber not only of the \( \nu CH_2 \) bands but also of the scissoring-vibration band (\( \delta CH_2 \)) is indicative of the lyotropic main transition albeit the latter band to a much less degree: the wavenumber shift is not more than 0.5–0.6 cm\(^{-1}\) and, in contrast to \( \nu CH_2 \), is directed downwards. To the best of our knowledge, this is the first evidence for such a propensity of the \( \delta CH_2 \) band to serve as a main-transition marker. An abrupt wavenumber displacement of this band at \( T_m \) has so
lipids, the corresponding spectroscopic evidence was much more pronounced.\textsuperscript{4,5} Accompanying the lyotropic main transition in mixed-chain PCs, an increase in band halfwidth of the CH\textsubscript{2} rocking-band vibration near 720 cm\textsuperscript{-1} is also observed. In contrast to the scissoring-vibration band [see above, Fig. 2(b)], the wavenumber of the rocking band behaves almost invariantly with hydration (data not shown).

Parallel to the appearance of a slight overall hydration-induced $\ddot{\nu}_{\text{CH}_{2}}$ increase for POPC and OPPC\textsuperscript{1} [see Fig. 2(a)] the band halfwidths characterizing the gel-phase and liquid-crystalline phase states of the mixed-chain PCs are generally not constant. Rather, they frequently display a systematic increase with hydration beyond the very "transition range." The explanation is, as before,\textsuperscript{1} that the state of order may vary slightly when the lipid remains within the same phase whether it be $L_{a}$ or $L_{b}$. Accordingly, hydration is thought to lead to very small increments of an intraphase chain disordering. Figure 4 shows the dependence of the total integral absorbance of the bands due to the CH stretching modes of POPC. The pattern found deviates qualitatively from the behavior exhibited by the wavenumbers and band halfwidths of the CH\textsubscript{2} stretching bands [Figs. 2(a) and 3(a)], but resembles that obtained for

![Figure 3](image-url)  
**Figure 3.** Band halfwidths of the symmetric stretching (a) and scissoring (b) vibration bands of the chain-methylene groups of POPC versus $A_{wr}$. Data are from a rehydration scan.

far never been reported. Instead, the $\ddot{\nu}_{\text{CH}_{2}}$ band has been used to indicate the main transition via a strong increase in its band halfwidth\textsuperscript{4}; this is observed also for the lyotropic main transitions in POPC as shown in Figure 3(b) and in OPPC (data not shown). A further spectral feature accompanying the lyotropic main transition of POPC and OPPC is the increase in line widths at half height of the $\nu_{\text{CH}_{2}}$ symmetric and antisymmetric bands. The $\ddot{\nu}_{\text{CH}_{2}}$ data are depicted in Figure 3(a); the latter are not shown. A line-width change of about 10\% although not high is still significant. For the temperature-induced main transitions of various

![Figure 4](image-url)  
**Figure 4.** The dependence of the overall integral absorbance of the C–H stretching-vibration bands on the water content of POPC films for dehydration (\textdegree) and rehydration (\textdegree) scans.
the CH₂ scissoring-vibration band [Figs. 2(b) and 3(b)]. In these latter cases, the ordinate values for the gel phase are generally constant or change only very slightly. After the abrupt changes in the range of the chain melting/freezing transition, they still vary substantially within the span of hydration levels where the $L_a$ phase is shown to exist. This may indicate that the parameters $A_{lt}$, $\nu(\delta CH_2)$ and band halfwidth of $\delta CH_2$ are especially sensitive to certain molecular-physical bond-parameter changes of the acyl chains in mixed-chain PCs but only when they have adopted the $L_a$ phase. On the other hand, it should be noted that the large variability found for $A_{lt}$ demands serious caution in determining $A_{wr}$, the parameter defined and used to quantify hydration, in the case of lipids where lyotropic main transitions are involved, such as for POPC and OPPC. Though DOPC is not supposed to undergo a lyotropic chain melting/freezing transition at room temperature, or even because of this fact, it is interesting to compare, for this particular lipid, some of the parameters considered above for the mixed-chain PCs. In Figure 5, data for DOPC is collected for the wavenumbers of the $\nu CH_2$ and $\delta CH_2$ vibrational bands as well as for the parameter $A_{lt}$. Considering the $\nu CH_2$ dependence on hydration pictured in Figure 6 of part I, reveals a slight decline at $A_{wr} < 0.5$ and a small minimum near 2 $A_{wr}$ units. Interestingly, the other parameters displayed $A_{lt}$ and $\nu(\delta CH_2)$, each demonstrate a pronounced maximum at exactly the same $A_{wr}$ value for DOPC and so far behave once more in a manner identical to the case of the mixed-chain PCs discussed above. The band halfwidths of the $\nu CH_2$ and $\delta CH_2$ vibration bands, on the other hand, do not exhibit any striking variations (data not shown). Altogether, the distinct changes in spectral features arising from bands attributable to groups in the apolar region of DOPC reveal that, at a water activity in the film corresponding to $A_{wr}$ of ca. 1.9, some structural or conformational rearrangement appears to occur in this lipid although, on balance, it seems to maintain the $L_a$ form all the time. Considering the present state of knowledge, however, it would be rather speculative to interpret this structural variation in terms of its nature in more detail.

**Comparative Analyses of the $\nu C=\!-\!O$ Vibration Mode**

This mode is seen as the most pronounced IR absorption band of a chemical group belonging to the molecular region at or around the polar/apolar lipid interface; it is considered of particular importance because it is, in a structural sense, the coupling (or hinge) between the hydrocarbon chains and headgroups. Thus, representing the so-called hydrophobic/hydrophilic boundary, it is, moreover, connected with the penetration depth of water molecules into the lipid bilayers. Data from recent investigations of a set of lipids with systematic structural modifications near the glycerol backbone have confirmed the particular significance of this interfacial region. Therefore, the $\nu C=\!-\!O$ band belongs to that category of IR-marker bands of central interest which, up to now, have been analyzed most carefully. In particular, band-separation as well as isotope-exchange techniques have been applied frequently to this mode and considerably improved.
A more precise examination of the PC curves in Figure 6 shows that the $\bar{v}_{C=O}$ data points are systematically ordered according to the sequence: DPPC $>$ POPC, OPPC $>$ DOPC at least for any $A_{wr}$ value higher than 1.5. This sequence agrees with the oppositely directed order of the water-uptake capacities of these PCs determined from our knowledge about the biophysical background governing its spectral behavior. Our efforts aim at examining whether or not and, if yes, how $\bar{v}_{C=O}$ is affected by well-defined molecular–physical events. Such events could consist of solvent interaction or structural reorganization up to phase transitions involving a major participation of either the polar or apolar molecular region of the amphiphile. The actual discovery of some relevant influence could then encourage speculation of some “mediator function” of the interface region for propagating structural effects along the whole lipid molecule.

Figure 6 gives an overview of the $\bar{v}_{C=O}$ dependencies on $A_{wr}$ for all five PLs studied. Table I presents relevant values drawn from the spectroscopic data. In each case hydration causes $\bar{v}_{C=O}$ to decrease. An overall $\bar{v}_{C=O}$ decrease in terms of hydration \cite{11,13,14} or temperature \cite{12,15} has previously been found and has been unanimously discussed as indicating a marked, that is, measurable increase in carbonyls influenced by hydration. Semiempirical AM1 calculations performed for a suitable model fragment have been shown to be in accordance with the idea that hydrogen-bonded water molecules can induce a downwards shift of $\bar{v}_{C=O}$.\cite{16} Correspondingly, a decrease for $\bar{v}_{C=O}$ can be interpreted as reflecting the influence of directly bound water molecules. However, the appreciable influence of long-range effects of the water globally imbibed by a lipid as well as of conformational changes in the lipid backbone region possibly accompanying phase transitions cannot be excluded as contributing to this spectral finding.

In addition, Figure 6 provides the impression that the curves for the four PCs lie essentially one upon another. Only the DOPC plot deviates clearly in so far as the wavenumbers are generally distinctly higher than for the PCs. The latter finding is explained in terms of a significantly different, that is, less hydrated or less polar environment for the carbonyls in DOPC. A similarly high $\bar{v}_{C=O}$ value of 1742 cm$^{-1}$ has also been found for DPPE,\cite{16} and such figures seem to reflect a situation typical for the structural and/or conformational peculiarities in PEs.\cite{16}

Figure 6. Wavenumber shifts for the carbonyl stretching-vibration bands of DPPC (□), DOPE (○), DOPC (○), POPC (▲) and OPPC (▲) obtained in terms of $A_{wr}$; data are from dehydration scans.

The gradual diminution of $\bar{v}_{C=O}$ found for DPPC can be—in view of the absence of phase transitions in this case—attributed exclusively to the effects of water binding.\cite{1} In contrast, for DOPC, two plateaus are found; these are connected by a small wavenumber drop at $A_{wr} = 0.2$. This is obviously correlated with other spectroscopic features indicating that the particular lyotropic nonlamellar phase transition of DOPC discussed above occurs at just the same water activity.\cite{9} Because of this correlation and in view of the steepness of the slope due to the $\bar{v}_{C=O}$ decrease, the latter can be clearly attributed to that phase transition, especially as the small water content (about 0.5 water molecules per DOPC) and the relatively low magnitude of the wavenumber shift (about 0.8 cm$^{-1}$, see Table I and below) disfavor the idea that this shift would be evoked by direct water binding. Thus, the DPPC and DOPC examples phenomenologically mark the patterns of how $\bar{v}_{C=O}$-versus-$A_{wr}$ curves will look if the un-
Table I. Comparison of Hydration-Dependent Spectroscopic Data Obtained for the Carbonyl Stretching-Vibration Band Together with Relevant Interpretations for the PLs Studied in Films at Room Temperature

<table>
<thead>
<tr>
<th></th>
<th>~ Onset of (ΔνC＝O) (Å_wr units)</th>
<th>~ End of (ΔνC＝O) (Å_wr units)</th>
<th>~ Extent (ΔνC＝O) (cm⁻¹)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>1.5–2</td>
<td>3.1</td>
<td>1.6</td>
<td>w. b.</td>
</tr>
<tr>
<td>DOPE</td>
<td>0.15</td>
<td>0.25</td>
<td>0.8</td>
<td>p. t. H_II － P_a</td>
</tr>
<tr>
<td>DOPC</td>
<td>1. 0</td>
<td>0.4</td>
<td>1.3</td>
<td>p. t. ?</td>
</tr>
<tr>
<td></td>
<td>2. 2.0</td>
<td>4.3</td>
<td>3.7</td>
<td>w. b.</td>
</tr>
<tr>
<td></td>
<td>1. + 2.</td>
<td></td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>POPC</td>
<td>rehy^b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. 1.1</td>
<td>1.4</td>
<td>1.2</td>
<td>p. t. chain melting</td>
</tr>
<tr>
<td></td>
<td>2. 1.1 (?)-1.8</td>
<td>3.3</td>
<td>2.4</td>
<td>w. b.</td>
</tr>
<tr>
<td></td>
<td>1. + 2.</td>
<td></td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>dehy^b</td>
<td>1. 1.2</td>
<td>1.5</td>
<td>1.3</td>
<td>p. t. chain freezing</td>
</tr>
<tr>
<td></td>
<td>2. 1.2 (?)-1.8</td>
<td>4.0</td>
<td>2.6</td>
<td>w. b. (desorption)</td>
</tr>
<tr>
<td></td>
<td>1. + 2.</td>
<td></td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>OPPC</td>
<td>1. 1.0</td>
<td>1.3</td>
<td>1.0</td>
<td>p. t. chain fr./melt.</td>
</tr>
<tr>
<td></td>
<td>2. 1.0 (?)-1.8</td>
<td>3.8</td>
<td>2.6</td>
<td>w. b.</td>
</tr>
<tr>
<td></td>
<td>1. + 2.</td>
<td></td>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

^a w. b. is for water binding, p. t. for phase transition.

^b rehy and dehy mean that the data are from rehydration and dehydration scans, respectively.

derlying mechanisms are given by the effects of direct water binding and a lyotropic phase transition, respectively.

A striking and unique criterion of the DOPC dependency is a steep fall of νC＝O by ca. 1 cm⁻¹ in the range of lowest water activity. This appears to be correlated with a number of further sharp wavenumber shifts concerning mainly the bands at about 2853 [Fig. 5(a)], 1240 [Fig. 9(b), below], 1070 (not shown) and 930 cm⁻¹ [Fig. 10(b), below] in the very low-hydration range (at Å_wr < ca. 0.4). Altogether, these discontinuities seem to indicate the occurrence of some conformational (or phase) transition leading to a particular structural constellation in the largely water-depleted DOPC which is, in terms of IR markers, significantly different from that in the “canonical” L_a phase existing in the range Å_wr > ca. 0.4 (if one desists from another irregularity at Å_wr near 2, see Fig. 5). It might be difficult to determine the exact nature of this conformational or a phase transition by IR-spectroscopic means alone (as it is in principle^2), but one can state that the presumed structural change is, on balance, not very demanding in affecting IR-spectral marker bands if compared to DOPE.^3 Possibly, a state in water-depleted DOPC is formed, where, as in the P_a phase postulated as existing in dry DOPE, lipid–water interactions are substituted by lipid–lipid interactions in the polar region when a critical threshold is surpassed on dehydration. Investigations using other methods are necessary to address the question of which peculiar structure or superstructure may be eventually formed at very low water contents in DOPC films apart from the “regular” L_a phase existing for most of the hydration levels studied. Apart from this discontinuity, the wavenumber decrease of the DOPC νC＝O band starts seriously at about 2 Å_wr units and its fairly smooth. Since the lipid can be imputed to adopt the lamellar liquid-crystalline phase at least at Å_wr values above ca. 0.4, the νC＝O decrease observed at higher Å_wr values near 2 should be provoked most favorably by the results of direct water binding. Interestingly, its value of nearly 4 cm⁻¹ is significantly larger than in the case of DPPC (see Table I). This finding is once more in agreement with the fact discussed in part I (see Fig. 4) that DOPC is able of imbibing much more water than DPPC.^1 Thus, hydration effects can be expected to be considerably stronger especially in the critical regions near the hydrophilic/hydrophobic boundary as presently indicated by comparing the νC＝O probe for DOPC adopting the L_a phase and DPPC adopting the gel phase (at room temperature in each case). The question may arise why water binding to the carbonyl groups of DOPC starts only at Å_wr values as high as 2 if one takes into account that the L_a phase also exists already at smaller water content (most
probably at least above 0.4 $A_{\text{wr}}$ units), and the onset of a $\nu\text{C}=\text{O}$ decrease in the presumably not so hydrophilic gel phase is located at smaller water content according to $A_{\text{wr}} \approx 1.5$ (in DPPC). Possibly, the high onset of the $\nu\text{C}=\text{O}$ decrease induced by direct water binding can be associated with the conformational changes believed to occur near $A_{\text{wr}} = 1.9$ in the apolar region of DOPC (see “Spectroscopy Features Mirroring the Isothermal Lipid Main Transition,” results in Fig. 5).

In Figure 7, a comparison of the hydration-driven behaviors of $\nu\text{C}=\text{O}$ and $\nu_{\text{s}}\text{PO}_2$ is shown for POPC. This corresponds to the results presented in Figure 8 of part I of this two-article series. The results for OPPC in terms of $\nu\text{C}=\text{O}$, are practically the same [see Fig. 8(e) below]. The principal conclusion is the same as for DPPC.\textsuperscript{1} Since $\nu\text{C}=\text{O}$ starts to significantly diminish only at $A_{\text{wr}} > 1$, where $\nu_{\text{s}}\text{PO}_2$ has already substantially shifted (i.e., has already substantially responded to hydration), the sequence of covering the potential water-binding sites should be again phosphate prior to carbonyl. This is plausible for PLs in general, since the phosphate groups are situated in a more exposed position at the hydrophilic outer sphere than carbonyls. This is in accord with other literature data presenting phosphate groups as primary water-binding sites for PLs\textsuperscript{17,18} as well as in nucleic acids.\textsuperscript{19} The POPC data [similar to those for OPPC, see Fig. 8(e)] must be considered, however, with some caution, since complications may arise from the existence of hydration-triggered chain-melting transition at a water content corresponding to an $A_{\text{wr}}$ value around 1.3, see above and Ref. 1. Indeed, the first onset of a $\nu\text{C}=\text{O}$ decrease in POPC is fairly coincident with that particularly critical $A_{\text{wr}}$ figure where the lyotropic main transition gets underway. Therefore, it seems obvious that the first step of the $\nu\text{C}=\text{O}$ decrease in POPC can be attributed to this phase transition, particularly as its slope is in the first-instance rather steep. It has been known for some time that the thermally provoked main transition is accompanied by such a wavenumber shift of the $\nu\text{C}=\text{O}$ band.\textsuperscript{15}

In order to further support this interpretation, some of the $\Delta\nu = f(A_{\text{wr}})$ functions have been plotted together with their first derivatives. The results are shown in Figure 8. In both the mixed-chain PCs exhibiting the isothermal main transition, the striking extreme values found in the first derivatives of the functions including $\nu\text{C}=\text{O}$ and $\nu_{\text{s}}\text{PO}_2$ (as minima and maxima, respectively) in the critical $A_{\text{wr}}$ region are clearly correlated with each other [see Fig. 8(a,d) and Fig. 8(b,e)]. Thus, the assignment of the first onset of the $\nu\text{C}=\text{O}$ decrease to the lyotropic main transition appears to be justified. On the other hand, no significant sharp extremum emerges in the case of DOPC in this range of $A_{\text{wr}}$ values (between 1 and 1.5) in accordance with the expectations that result from the absence of any phase transition at this hydration level [Fig. 8(c, f)]. Instead, in the first-derivative plot formed for the $\Delta\nu = f(A_{\text{wr}})$ function of DOPC, two minima appear. These correspond to the two steps of the total $\nu\text{C}=\text{O}$ decrease found experimentally: first a sharp minimum at $A_{\text{wr}} = \text{ca}. 0.2$, then a very broad one at about 3 $A_{\text{wr}}$ units that may represent the predicted conformational (or phase) transition at very low hydration as discussed above and the broad effects of gradual water binding, respectively. It seems highly speculative to conclude from the mere appearance of an extremum arising in a first-derivative plot of the particular type discussed here as necessarily being attributable to the existence of a phase transition. Acceptance of such an extremum as actually marking a phase transition would rather demand the satisfying of certain quantitative criteria with respect to the height and the width of the first-derivative band formed. Moreover, this would depend on the nature of the system under study. In a qualitative discussion such as is presently possible, one could determine that the higher and the narrower the first-derivative band in question, the greater the probability that it
Figure 8. Dependence of the wavenumber of the bands due to the symmetric chain-methylene stretching (a–c) and carbonyl stretching vibrations (d–f) on the water content in POPC (a,d), OPPC (b,e) and DOPC (c,f) films together with their first derivatives. Differentiation procedures (smoothing: 20%) of the relevant functions were performed by the program TABLE CURVE for WINDOWS.

does in fact indicate a conformational or phase transition. The shapes of the first-derivative bands formed for POPC and OPPC (see Fig. 8(a,b,d,e)) can be taken as typical examples of phase- or conformational-transition markers. This is also true for the low-hydration $\tilde{\nu}C=O$ decrease in DOPC (Fig. 8(f)). The minimum of the first-derivative band of DOPC obtained at higher $A_{wr}$ values (Fig. 8(f)), however, is considered too broad to indicate a phase transition in a single-lipid model-membrane system such as exists in DOPC films. In more complex systems, such as found in stratum corneum lipids representing multicompartment mixtures, the first-derivative bands undoubtedly indicate that the thermally induced $L_d - L_a$ transition is actually relatively broad (C. Selle et al., unpublished data).

The total wavenumber shift of 3.5–4 cm$^{-1}$ found for POPC and OPPC is, a bit smaller than the one evaluated for DOPC (counted only at $A_{wr}$ above ca. 0.4), but clearly larger than for DPPC. This once more underlines the mean position of the mixed-chain PCs. A more detailed inspection of the curves for POPC and OPPC suggests that the overall wavenumber shift can be separated into two steps. The first onset of a $\tilde{\nu}C=O$ decrease at about 1.2–1.3 $A_{wr}$ units has already been assigned to the occurrence of the main transition. This demonstrates that the interfacial region of the PCs is connected “tightly” to the acyl chains in such a way as to be influenced by the effects of the main transition which itself is a structural event proceeding primarily in the apolar hydrocarbon–chain region. In each case as for DOPC the second onset starts somewhere towards $A_{wr}$ $\approx$ 2. It is difficult to define the exact position of this second step, since it may be obscured by the right-hand shoulder of the first wavenumber decrease characteristic for the phase transition (see also below). This uncertainty is indicated in Table I by the question marks set in the first data column. Because of local similarities, the conclusion reached is that the same factor is responsible for the appearance of the second wavenumber decrease for the mixed-chain PCs and DOPC, and that is, for all these lipids existing in the $L_a$ phase,
direct water binding to the C=O groups. However, it cannot be excluded that during the course of the \( L_a - L_n \) transition some water binding to the carbonyl groups occurs concomitantly as has been discussed previously.\(^{15}\) A possible contribution of such direct water–carbonyl interactions to the \( \nu C=O \) shift at \( A_{\text{wr}} \) values near 1.2 such as those found for POPC and OPPC, can be a priori estimated as not very high in view of the extent of wavenumber shifts when compared to the corresponding magnitudes for \( \nu C=O \) shifts at higher \( A_{\text{wr}} \) values which fairly unambiguously account for water binding to the liquid-crystalline lipids.

A criterion for what might be the final limit of the very phase-transition effects on \( \nu C=O \) could be determined by the disappearance of the hystereses found for the main transition when comparing the results of the nonstationary dehydration and rehydration scans. Figures 2 and 4 reveal that all hystereses expire at \( A_{\text{wr}} \) values of around 1.8–2, thus confirming the validity of the above discrimination. In conclusion, it could be established that in mixed-chain PCs, two different processes are occurring during the course of hydration and these can be simultaneously detected with the help of IR spectroscopy, even using the same diagnostic marker, \( \nu C=O \). These two events are a lyotropic phase transition, in this case chain melting, followed by water binding to the polar region of the lipids existing in the more hydrophilic \( L_a \) phase.

The relevant interpretations assigned to the carbonyl stretching mode behaviors for the PLs under consideration are summarized in the last column of Table I. First, all the lipids respond to hydration. Moreover, all PLs except DPPC reflect influences of phase or conformational transitions, and all except DOPE exhibit effects of the direct interaction of water molecules with the C=O groups. In that connection, lipids adopting the \( L_a \) phase are more strongly affected by water molecules, but apparently there is also some accessibility of carbonyl groups for water molecules in lipids existing in the gel phase as exemplified by DPPC.

The total extent of the \( \nu C=O \) shifts reproduces the known order\(^{1,20}\) of decreasing hydrophilicity: DOPC > OPPC, POPC > DPPC > DOPE (cf. also above, discussion of Fig. 6). As to phase or conformational transitions, it can be stated that structural events preferentially proceeding in either the apolar acyl-chain or the polar headgroup region are still measurable using the \( \nu C=O \) vibrational mode, since they obviously influence the PL interface region. This was revealed by the \( \nu C=O \) response observed for both the mixed-chain PCs studied here and DOPE. Independent of the nature of the transition, typical \( \nu C=O \) shifts for a phase or conformational transition have been found to consistently amount to about 1 cm\(^{-1}\).

**Headgroup Region**

Finally, some findings arising from an examination of the influence hydration exerts on the IR bands of the phosphate and choline moieties are presented.

In part I in this series, it was shown in some detail that the stretching bands due to the \( PO_2^\equiv \) moiety display the strongest response to the lipid hydration process. This is especially true for the antisymmetric mode. Among the PL subfragments, phosphate groups are sterically, as well as electronically,\(^{18,19}\) most exposed to interaction with hydrogen-bond water molecules. The former is naturally determined by their location in the headgroup region. This is above all reflected in the strong wavenumber decrease in the low-hydration range exhibited by both \( \nu PO_2^\equiv \) vibrations of the hydrophilic PCs.\(^1\) The question arises whether or not the chain-melting transition, as a structural process involving primarily the acyl-chain region, is still “felt” by or involves feedback to the phosphate groups. In Figure 9, the \( \nu_a PO_2^\equiv - f(A_{\text{wr}}) \) functions for POPC and DOPC are shown together with the corresponding first-derivatives. At \( A_{\text{wr}} \) values of around 1.2, that is, the locus where the main transition appears to take place in POPC (indicated by well-defined markers, see above), no significant minimum which might be considered as marking a phase transition can be found. On the other hand, minima appear at smaller \( A_{\text{wr}} \) values which are, however, most probably associated either with the existence of the \( CH_2 \) wagging–progression bands typical of lipids adopting the gel phase (as for POPC) or with suspected conformational (or phase) transition occurring at very low water contents such as occurring in DOPC (see “Comparative Analyses of the \( \nu C=O \) Vibration Mode”).

In contrast to the main transition, the phase transition between the interdigitated gel phase and the “normal” gel phase as occurs in temperature runs of dinhexadecylphosphatidylethanoline is clearly indicated by a distinct increase for \( \nu_a PO_2^\equiv \). This may be understood as an indication
mational changes undergone in the choline moiety. As stated before, a wavenumber decrease such as that found in all the PCs used in this study, is indicative of a diminution of the antiplanar rotamer population for the benefit of conformers with an anticlinal or synclinal conformation about the N—C—C—O and C—C—O—P torsional angles. It is surprising that the hydration-induced conformational changes of the choline group indicated by the $\tilde{v}_s$CCN$^+$ decrease occur, in each case, at very small water contents, and that they are qualitatively similar independent of whether or not the lipids have adopted a fluid (DOPC) or rigid (dry POPC) phase. An examination of the minima of the first-derivative bands reveals a lower position on the $A_{wr}$ axis for DOPC compared to POPC and the other PCs (data

![Figure 9](image1)

**Figure 9.** Wavenumber displacement of the antisymmetric stretching-PO$_2$ vibration band of POPC (a) and DOPC (b) in dependence on $A_{wr}$ together with their first derivatives.

that the phase transition between the interdigitated gel phase and the “normal” gel phase, in terms of its overall structural implications in the headgroup region, is more demanding than the main transition.

Figure 10 shows the response of the symmetric CCN$^+$ stretching vibration band to hydration for POPC and DOPC. In principle, their dependence looks quite similar to both the other PCs (data not shown). First, there is a drastic decrease in the band wavenumber followed by a segment of $A_{wr}$ values with nearly constant (for POPC) or slightly increasing (for DOPC) $\tilde{v}_s$CCN$^+$ values. The wavenumber of the symmetric CCN$^+$ stretching vibration is correlated with the relevant torsional angles, and is sensitive primarily to confor-

![Figure 10](image2)

**Figure 10.** Wavenumber shifts of the POPC (a) and DOPC (b) symmetric stretching CCN$^+$ vibration band in terms of $A_{wr}$ together with their first derivatives.
not shown). This difference may arise from the fact that, in DOPEC, most probably the $\tilde{\nu}_{CCN}^+$ decrease is associated with the postulated (see data above) conformational or phase transition [see Figs. 2(b) and 6].

CONCLUSIONS

FTIR-spectroscopic investigations of the hydration of a series of systematically varied glycerol PLs resulted in two different principal response patterns as reflected in the sensitive parameters of the absorption bands. These patterns are characterized by predominantly either continuous or sharp changes of spectral features, mainly wavenumbers, in terms of the hydration increment. They are believed to account for the effects of either quantitative (such as the successive hydrogen bonding of water molecules) or qualitative (lyotropic phase transitions and conformational changes) structural processes. Relevant indications of the former are the gradual “intraphase” wavenumber shifts of the $\nu C=O$ and $\nu PO_2^-$ bands found in the course of the hydration of PCs. The latter processes are revealed by the abrupt distinct wavenumber and band halfwidth changes observed for mixed-chain (oleoyl/palmitoyl) PCs and for DOPE at particular water activities which are indicative of $L_\alpha - L_\beta$ and $H_{II} - P_a$ transitions, respectively. And so to say “intermediate” situation is that of an intraphase conformational transition occurring in the choline moiety of PCs as indicated by a steep wavenumber decrease for the $\nu_s CCN^+$ band.

With respect to the wavenumber of their carbonyl stretching-vibration bands, all the PCs with oleoyl chains studied exhibit hydration effects in a complex manner. This spectral behavior is resolvable, in each case, into two separate steps. First a rather sharp drop occurs at lower water contents due to some phase transition, which is followed by a second more continuous decrease most probably indicating direct water binding to the C==O groups. DPPC and DOPE are exceptional in showing only monocausal effects originating in water binding and in the lyotropic $H_{II} - P_a$ transition, respectively. DOPEC displays two aberrations, presumably correlated with conformational changes. At very low water activities, this concerns primarily the polar region including the choline unity (see above), and at medium water contents preferentially the hydrocarbon chains. Unraveling the nature of the biophysical events behind these discontinuities exhibited by DOPEC is a challenge, which will require interpretation help from physicochemical methods other than FTIR spectroscopy.

Apart from proven parameters, such as the wavenumbers of the $\nu CH_2$ bands, a number of further spectral features have been checked whether or not they are indicative of the lyotropic main transition. Such a diagnostic approach was realized for the wavenumbers of the CH$_2$ scissoring-vibration band as well as for the band half-widths of the CH$_2$ stretching-, scissoring-, and rocking-vibration bands and the pattern of how such markers behavior differs from that found for the thermotropic main transition.

There is IR-spectroscopic evidence that the lyotropic chain-melting transition does indeed also significantly affect the interface regions but not the polar headgroup region of the lipids studied (although water binding as the causal driving force of main transition occurs primarily in that region). Similarly, the presumably headgroup-interdigitating $H_{II} - P_a$ transition in DOPE, has detachable consequences also only in the interface, but not in the acyl-chain region (see also Ref. 3).

Altogether, the results obtained highlight FTIR spectroscopy as a methodology well-suited to studying the structural implications of an important biomedical/biophysical process such as lipid hydration, in a sometimes uniquely detailed manner hardly to achieve by other physicochemical methods. First-derivatives of functions describing the dependencies of wavenumbers on water content in the lipid films have been found to be a useful data-processing technique profitable in the discrimination of both assignments and explanations.

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ABBREVIATIONS AND SYMBOLS

$A_{Hr}$ absorbance of the totality of C—H stretching-vibration bands

$A_{nr}$ normalized absorbance of the $\tilde{\nu}OH$ band of water (in a lipid film)
DOPC  1,2-dioleoyl-sn-glycero-3-phosphocholine or dioleoylphosphatidylcholine
DOPE  dioleoylphosphatidylethanolamine
DPPC  dipalmitoylphosphatidylcholine
DPPE  dipalmitoylphosphatidylethanolamine
\( \delta \text{CH}_2 \)  scissoring (bending) vibration of methylene CH bonds

\( H_\text{II} \)  inverse hexagonal phase
\( L_\alpha \)  liquid-crystalline phase
\( L_\beta \)  gel phase
\( \bar{\nu} \)  wavenumber
\( \nu \text{C}==\text{O} \)  carbonyl stretching vibration
\( \nu_\text{sPO}_2 \)  antisymmetric stretching vibration of the \( \text{PO}_2 \) moiety
\( \nu_\text{CCN}^+ \)  symmetric stretching vibration of \( \text{CCN}^+ \) bonds
\( \nu_\text{sCH}_2 \)  symmetric stretching vibration of methylene CH bonds
\( \nu_\text{sPO}_2 \)  symmetric stretching vibration of the \( \text{PO}_2 \) moiety
\( \nu_1 \text{OH} \)  O–H stretching vibrations of water
OPPC  1-oleoyl, 2-palmitoylphosphatidylcholine

\( P_\alpha \)  inverse fluid ribbon phase
PC  phosphatidylcholine
PE  phosphatidylethanolamine
PL  phospholipid
POPC  1-palmitoyl, 2-oleoylphosphatidylcholine

\( \text{RH} \)  relative humidity

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