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Formation of the antiplanar-antiplanar phosphate conformation of dilauroylphosphatidylcholine bilayers

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Infrared spectroscopy was used to investigate lipid conformational changes that occur in dilauroylphosphatidylcholine ($diC_{12}PC$) bilayers with and without fatty-acid-amino-acids as guest molecules in the membrane. Incorporating 2.5 mole% *N*-decanoylglycine (decgly) into $diC_{12}PC$ liposomes caused formation of the antiplanar-antiplanar (ap-ap) phosphodiester conformation which was stable in room temperature IR spectra. Several other fatty-acid-amino-acids incorporated into $diC_{12}PC$ bilayers were found to also elicit the ap-ap phosphodiester conformation. Unlike these $diC_{12}PC$/fatty-acid-amino-acid mixed bilayers, pure $diC_{12}PC$ bilayers would form the ap-ap phosphodiester conformation only under low temperature incubation conditions. Dry $diC_{12}PC$ films incubated at 5°C for 0.5 h (brief incubation) or 16 h (prolonged incubation), and then rapidly hydrated (i.e., vortexed at 25°C in D_2O), caused the ap-ap phosphodiester conformation to persist in the $diC_{12}PC$ liposomes equilibrated to room temperature. Slow hydration for 16 h at 5°C in both buffered and non-buffered D_2O of $diC_{12}PC$ lipid films also produced the ap-ap phosphodiester conformation. In contrast, slow hydration for 16 h at 5°C in PBS/D_2O of $diC_{12}PC$/decgly mixed films caused the greatest number of ap-ap phosphodiester conformers. Using pure $diC_{12}PC$ bilayers, infrared data indicate that incubation of $diC_{12}PC$ films causes the headgroup phosphodiester conformation to change from *gauche-gauche* (g-g) conformation to the ap-ap conformation. Under all liposome formation conditions examined, no changes in hydration of either the phosphate group or the carbonyl ester group were detected and in addition, no *trans/gauche* conformational changes in the acyl chain were observed.

Introduction

Infrared (IR) spectroscopy has been previously used to examine conformational changes in phospholipids caused by low temperature incubation [1,2], ion binding to phospholipid headgroups [3–5], and guest molecules incorporated into the bilayer [6–8]. Lipid structural changes identified by IR spectroscopy include changes in the acyl chain packing [2], hydration of the glycero-backbone [2,9], hydration of the headgroup phosphate *, and conformational changes in both the lipid acyl chains and the phosphodiester group [10,11]. We used IR spectroscopy to study conformational changes in the phosphodiester group of dilauroylphosphatidylcholine ($diC_{12}PC$) bilayers with and without fatty-acid-amino-acids incorporated into the bilayers.

The phosphate conformation, determined by the torsion angles α_2 and α_3 of the O-P-O ester bonds, is usually *gauche-gauche* (g-g) not only in phospholipids but also in all other diesters of phosphoric acid [12]. From thermodynamic calculations, the phosphodiester g-g conformation is the most stable conformation relative to the *gauche*-antiplanar (g-ap) and antiplanar-antiplanar (ap-ap) forms (i.e., ΔG g-g < ΔG g-ap < ΔG ap-ap) [13,14]. However, one report demonstrated that lanthanide ions bind to hydrated PC membranes and cause the formation of the g-ap phosphodiester conformation [3], and another report demonstrated that calcium binding to hydrated phosphatidylserine (PS) bilayers caused dehydration of the interfacial phosphate with concurrent formation of the ap-ap phosphodiester conformation in the PS headgroup [4,5]. Using infrared spectroscopy we observed the ap-ap phosphodiester conformation in $diC_{12}PC$ hydrated bilayers and

* For phosphatidylcholine, complete dehydration of the phosphate group causes the ν_{as} PO_2 double-bond stretch to exist at 1262 cm^{-1}. Increasing hydration cause the band to shift to a minimum near 1222 cm^{-1} [9].

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$diC_{12}PC$/fatty acid-amino acid mixed lipid bilayers. This is the first report describing formation of the ap-ap phosphodiester conformation in a phosphatidylcholine analog.

Conformational analysis of the $diC_{12}PC$ phosphodiester conformer was based on normal coordinate analysis [11] and infrared measurements between 850 cm^{-1} and 650 cm^{-1}. The wavenumbers of the single bond O-P-O stretching and PO_2 waging modes, which occur between 850 cm^{-1} and 650 cm^{-1}, are dependent on the conformation of the phophodiester headgroup. From calculated wavenumbers of the O-P-O single bond stretching and PO_2 wagging modes, new vibrations near 757 cm^{-1} ($\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$) and 665 cm^{-1} (γ_w $(PO_2)_{ap\text{-}ap}$) occur when phosphodiester groups adopt the ap-ap conformation. Using these IR band assignments, hydration conditions required to form the ap-ap conformation in $diC_{12}PC$ bilayers were established.

Materials and Methods

Chemicals. Dilauroylphosphatidylcholine ($diC_{12}PC$) was purchased from Avanti Polar Lipids, Birmingham, AL. Decanoic acid, dicyclohexylcarbodiimide and *N*-hydroxysuccinimide were purchased from Aldrich Chemical Co., Milwaukee, WI. Potassium carbonate and phosphorous pentoxide were purchased from Mallinckrodt, Paris, KY. Glycine was purchased from Sigma Chemical Co., St. Louis, MO. D_2O 99.8% atom D was purchased from Aldrich Chemical Co., Milwaukee, WI. Calcium and magnesium free Dulbeccos phosphate-buffered saline (PBS) 10 × concentrated was purchased from Grand Island Biological Co., Grand Island, NY. After 10-fold dilution, the PBS buffer contained: KCl 2 g/l, KH_2PO_4 2 g/l, Na_2HPO_4 21.6 g/l, and NaCl 80 g/l. All other solvents were analytical grade and used as received.

Synthesis. *N*-Decanoylglycine (decgly) was synthesized as follows: decanoic acid was condensed with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide in dry ethylacetate to form the *N*-hydroxysuccinimide ester of decanoic acid [15]. Dicyclohexylurea, which precipitated out, was removed by filtration. The *N*-hydroxysuccimide ester of decanoic acid reacted readily with the sodium salt of glycine in aqueous/organic H_2O/THF (50 : 50, v/v) to form the corresponding *N*-decanoylglycine. The free acid form of decgly was obtained by lowering the pH of the reaction mixture to 2, extracting with methylene chloride (CH_2Cl_2), and recrystalizing with 50 : 50 methylene chloride/petroleum ether. Ethylacetate was dried over anhydrous potassium carbonate followed by distillation over phosphorous pentoxide.

Bilayer formation by rapid hydration. Multilamellar lipid vesicles were prepared by hydrating pre-dried lipid films with either D_2O or PBS/D_2O buffer. PBS/D_2O buffer pH 7.3 was prepared as described [16]. Lipid films were formed in a 50 ml round bottomed flask from either 60 mg of $diC_{12}PC$ or 60 mg of $diC_{12}PC$ and 0.6 mg of decgly. This corresponds to 2.5 mole% decgly in the $diC_{12}PC$ membrane. The other fatty-acid-amino-acids were also incorporated into the membrane at this molar concentration. Thus all mixed-lipid bilayers for this work used $diC_{12}PC$/ fatty-acid-amino-acid (97.5 : 2.5, mole/mole). The pre-dried lipid films were then either non-incubated, incubated at 5°C for 0.5 h (brief-film-incubation e.g., process a in Fig. 2), or incubated at 5°C for 16 h (prolonged-film-incubation e.g., process b in Fig. 2) before hydration. For incubation, the round bottom flasks were tightly sealed to prevent accumulation of atmospheric H_2O. For unknown reasons, slow hydration in PBS/D_2O caused a 10-fold increase in H_2O, contaminating the final bilayer suspension. This was the only incubation condition that caused excess H_2O in final D_2O-liposome suspension. Spectral subtraction of the aqueous buffer used to prepare the bilayer did not remove the residual HOD in the spectra. The HOD bending deformation occurs near 1460 cm^{-1}, which is similar to the IR peak position associated with the hydrocarbon bending deformation. Incubated and non-incubated lipid films were rapidly hydrated by vortexing with 0.5 ml of either buffered or non-buffered D_2O at either 25°C or 5°C. We note that $diC_{12}PC$ bilayers prepared at room temperature (25°C) from non-incubated films are considered to be in the L_α liquid-crystalline phase regardless of the aqueous dispersion media (i.e., D_2O or PBS/D_2O). The IR spectra of $diC_{12}PC$ bilayers in the L_α phase (Fig. 1) was used as a reference spectrum for IR band assignments.

Bilayer formation by slow hydration. Slow hydration of non-incubated and briefly incubated lipid films were performed by the following process: (1) an aqueous phase (0.5 ml) chilled in an ice bath was gently placed on top of the dried lipid film; (2) the tightly sealed flask was incubated for 16 h at 5°C; and (3) the dispersion was vortexed. For slow hydration of briefly incubated films, the dried lipid film was left at 5°C for 0.5 h before the addition of the 0.5 ml chilled aqueous phase.

Preparation of bilayers for infrared analysis. Bilayers were prepared for IR analysis in a microfuge. Both incubated and non-incubated bilayers were concentrated on the 0.2 μm filter of a 1.5 ml microfilterfuge tube (Rainin Instrument Co., Woburn, MA) to a final volume of 0.2 ml (300 mg lipid per ml) using a microfuge (5 min at 7000 rpm). Less than 2% of the lipid was lost through the filter as determined by IR analysis of lipid in the filtrate [17]. The bilayers were allowed to equilibrate to room temperature for a total of 30 min before IR spectra were obtained. This room temperature equilibration is process g in Fig. 2 and process f in Fig. 3. We emphasize that this 'equilibration' to room temperature is not the equilibration of the lipid to a

specific phase like the L_α or L_c. Rather, this is an equilibration of the lipid to room temperature so that the IR spectral subtraction of the aqueous phase, used to prepare the bilayers, is not complicated by temperature dependent band shifts.

Infrared spectroscopy. A Nicolet 20SXC FT-IR spectrometer utilizing a liquid nitrogen cooled mercury-cadmium-telluride (MCT-A) detector and a germanium-on-KBr beamsplitter was used to record IR spectra. All IR spectra were from 512 coadded double sided interferograms obtained at 4.0 cm^{-1} resolution, and apodized with a Happ-Genzel function before Fourier transformation. Both the background spectra and the sample spectra were obtained using a high pressure micro CIRCLE flow cell (Spectrotech Inc., Stamford, CT) [18–20] equipped with a ZnSe micro rod crystal. The air background single beam reference spectrum was obtained after the CIRCLE flow cell was throughly rinsed with D_2O and purged with nitrogen for 5 min. The flow cell was set up in a Nicolet auxiliary bench sample compartment which is a closed system under constant purge. Purge air, free of water vapor and carbon dioxide vapor, was provided by the headspace from a liquid nitrogen tank. The body of the flow cell contained inlet and outlet ports for 1 mm diameter tubing. The inlet tube was fitted with a luer adapter outside the sample compartment; sample introduction into the flow cell with a 1 cc disposable syringe thus did not require opening the sample compartment. Constant purge of the closed compartment virtually eliminated CO_2 and H_2O vapor bands in the final IR spectra. Few IR spectra showed any trace of CO_2 and H_2O vapor.

All IR spectra were obtained at room temperature on fully hydrated bilayers equilibrated to room temperature as described above in Preparation of bilayers for infrared analysis. All infrared data are shown as difference spectra, whereby the aqueous solvent used to prepare the bilayers was subtracted. The deuterated buffer and D_2O were chosen to avoid potential artifacts in the infrared region near 1640 cm^{-1} when non-deuterated aqueous solvents are subtracted [19,21]. Subtraction, of the PBS/D_2O or D_2O IR spectrum from the IR spectrum of the liposomes, used the O-D stretch band at 2400 to 2200 cm^{-1} and the O-D bending deformation at 1300 to 1100 cm^{-1} as reference bands [22,23] (a scaling factor near 1 was always used). Water vapor bands were subtracted as necessary. Subtraction of carbon dioxide vapor was performed only when the ap-ap conformation did not appear and the 670 cm^{-1} region was not critical for conformational assignment. Such subtractions were performed after subtraction of the D_2O solvent in order to more clearly see the CO_2 bands at 2350 cm^{-1} and 669 cm^{-1}. Any infrared spectra of liposomes with the ap-ap phosphodiester conformation that contained carbon dioxide vapor were remeasured thus preventing any band assignment confusion with the Q branch of the CO_2 bending vibration. All spectra were baseline corrected. Deconvolution of the carbonyl ester region 1760–1700 cm^{-1} and the phosphodiester region 900–650 cm^{-1} was performed using the DECON software available from Nicolet. Parameters for deconvolution [24,25] are given in the figure legends. The Nicolet peak pick subroutine, which utilizes a cubic spline function, was used without additional smoothing to identify IR band positions.

For Figs. 4 and 5, hydration conditions were rank ordered according to the amount of ap-ap phosphodiester conformers generated by the different hydration conditions. The number of ap-ap conformers were related to the intensity of the 759 cm^{-1} $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band, which is unique to the ap-ap conformation. The extinction coefficient for the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band has never been measured; thus direct calculation of the number of ap-ap phosphodiester conformers was not possible. Consequently the integrated intensity of the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band was used to qualitatively evaluate the number of ap-ap phosphodiester conformation. Since the intensity of the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band depends on the amount of lipid present in the specimen during IR data collection, the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band was normalized by the 720.8 cm^{-1} CH_2 rocking vibration [γ_r (CH_2)]. The γ_r (CH_2) band intensity did not change with different hydration conditions or with different conformations of the phospholipid headgroup. Thus normalizing the data involved dividing the integrated intensity of the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band by the integrated intensity of the γ_r (CH_2) band. This ratio, denoted as $I[\bar{\nu}_s\ (O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)]$, permitted comparison between different hydration conditions with regard to the formation of ap-ap phosphodiester conformers in the headgroup region. The $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band was integrated between the limits of 785 cm^{-1} and 730 cm^{-1}. The γ_r (CH_2) band was integrated between the limits of 730 cm^{-1} and 710 cm^{-1}. Band intensities were integrated using a computer program [17]. The computer program baseline corrects each peak before integration by drawing a straight line between the wavenumbers used as the integration limits.

Results

The infrared spectrum between 4000 cm^{-1} and 600 cm^{-1} of diC_{12}PC hydrated bilayers in the L_α phase is shown in Fig. 1. The IR spectrum of the phosphodiester region (top right Fig. 1) of diC_{12}PC, eliciting a *gauche-gauche* (g-g) phosphodiester headgroup conformation, shows the single bond O-P-O symmetric stretch [$\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{g\text{-}g}$] at 823.4 cm^{-1} and the single bond O-P-O asymmetric stretch ($\bar{\nu}_{as}$ $(O\text{-}P\text{-}O)_{g\text{-}g}$) at 767.2 cm^{-1} (band assignments are given in Table I). The band at 873.4 cm^{-1} to the left of the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{g\text{-}g}$ is the C-N-C symmetric stretch [$\bar{\nu}_s$ $(C\text{-}N\text{-}C)_g$] of the choline O-C-C-N

TABLE I

Calculated wavenumbers (cm^{-1}) of the single bond O-P-O stretching modes and PO_2 wagging mode of dialkyl phosphate anion [11]

g, *gauche*; ap, antiplanar.

Mode	Conformation		
	ap,ap	g,ap	g,g
$\bar{\nu}_{as}$ O-P-O	832	780	750
$\bar{\nu}_{s}$ O-P-O	757	790	819
γ_w PO_2	665	663	545

frame in the *gauche* conformation [10]. The band near 721.2 cm^{-1} to the right of the $\bar{\nu}_{as}$ $(O\text{-}P\text{-}O)_{g\text{-}g}$ is the CH_2 rock (γ_r (CH_2)) [10]. Deconvolution of this phosphodiester region (Fig. 1A, upper spectrum) revealed that the $\bar{\nu}_{s}$ $(O\text{-}P\text{-}O)_{g\text{-}g}$ band is comprised of a central peak at 824.6 cm^{-1} with shoulders at 806.3 cm^{-1} and 851.9 cm^{-1}, and that the $\bar{\nu}_{as}$ $(O\text{-}P\text{-}O)_{g\text{-}g}$ band is comprised of a peak at 768.5 cm^{-1} with a shoulder at 750.0 cm^{-1}. The phosphate group is hydrated as indicated by the position of the double-bond PO_2 asymmetric stretch ($\bar{\nu}_{as}$ (PO_2)) at 1236 cm^{-1} (Fig. 1, starred band) [9]. Deconvolution elicited two bands in the ester carbonyl region near 1740 cm^{-1} and 1725 cm^{-1} with the 1725 cm^{-1} band being the largest peak (Fig. 1, top left); this indicates that the *sn*-2 ester carbonyl (C = O) group is hydrated [9].

Fig. 2 shows the hydration conditions used to rapidly hydrate $diC_{12}PC$ films. Dry $diC_{12}PC$ films were subjected to brief-film-incubation (process a), prolonged-film-incubation (process b), or were not incubated prior to liposome formation. Liposome formation is shown as processes c, d, e, and f in Fig. 2. The IR spectra between 1900–640 cm^{-1} for $diC_{12}PC$ bilayers hydrated with and without low temperature incubation are shown in Fig. 2. The IR region from 4000 cm^{-1} to 1900 cm^{-1} was omitted in Fig. 2 because no changes in the C-H stretching region were observed, both the symmetric δ_s CH_2 and asymmetric δ_{as} CH_2 stretching bands were constant for all hydration conditions tested; δ_s CH_2 2853.2 cm^{-1} and δ_{as} $CH_2 = 2922.5$ cm^{-1}. This indicates that no change in the methylene *trans/gauche* population was detected under our incubation conditions. However, changes in the headgroup phosphate were detected as described below.

The IR spectra in Fig. 2 for $diC_{12}PC$ in the L_α phase (liquid crystalline) was obtained for non-incubated films hydrated at 25°C in both PBS/D_2O and D_2O (path c → g) and also for briefly incubated films dispersed in PBS/D_2O at 5°C and 25°C (path a → d → g). Unexpectedly, briefly incubated films dispersed in D_2O (but not PBS/D_2O) at both 5°C and 25°C showed a phosphodiester conformational change to the ap-ap conformation (path a → e → g, Fig. 2). This is evident by comparing the IR spectra in Fig. 2 in the region be-

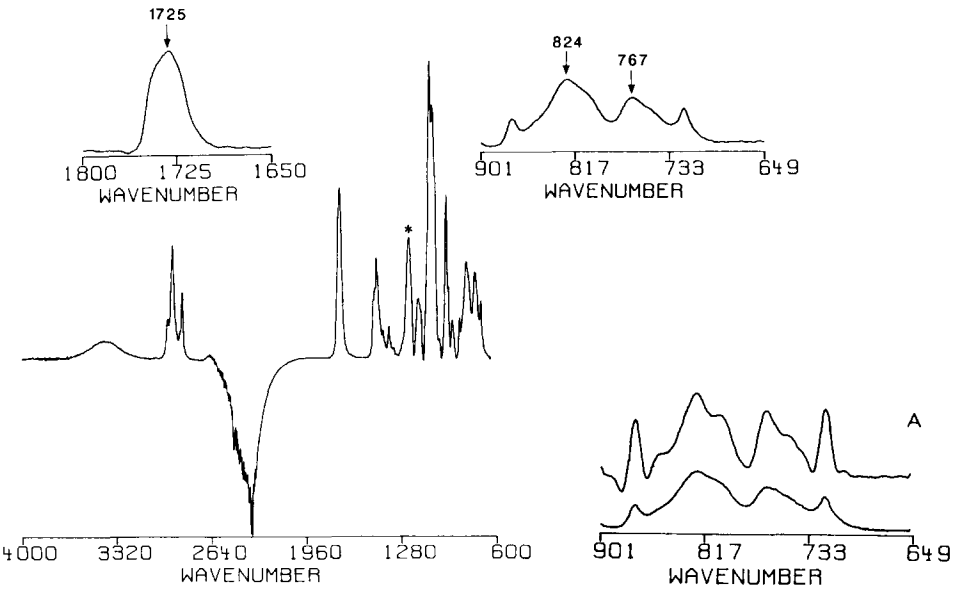

Fig. 1. Infrared spectrum from 4000 cm^{-1} to 600 cm^{-1} of hydrated $diC_{12}PC$ bilayers in the L_α liquid crystalline state (non-incubated film, room temperature formation) after PBS/D_2O subtraction. The phosphodiester region from 901 cm^{-1} to 649 cm^{-1} (top right) and the deconvolved ester carbonyl region from 1800 cm^{-1} to 1650 cm^{-1} (top left) are expanded for clarity. Deconvolution of the ester carbonyl region used a Bessel apodization function, a half-width-at-half-height of 12 cm^{-1}, and a resolution enhancement factor of 1.5. The double bond $\bar{\nu}_{as}$ PO_2 band is labelled (*).

Fig. 1A. Infrared spectrum of the phosphodiester region from 901 cm^{-1} to 649 cm^{-1} (bottom spectrum) from Fig. 1 and the same region deconvolved (upper spectrum). Deconvolution of the phosphodiester region used a Bessel apodization function, a half-width-at-half-height of 18 cm^{-1}, and a resolution enhancement factor of 1.5.

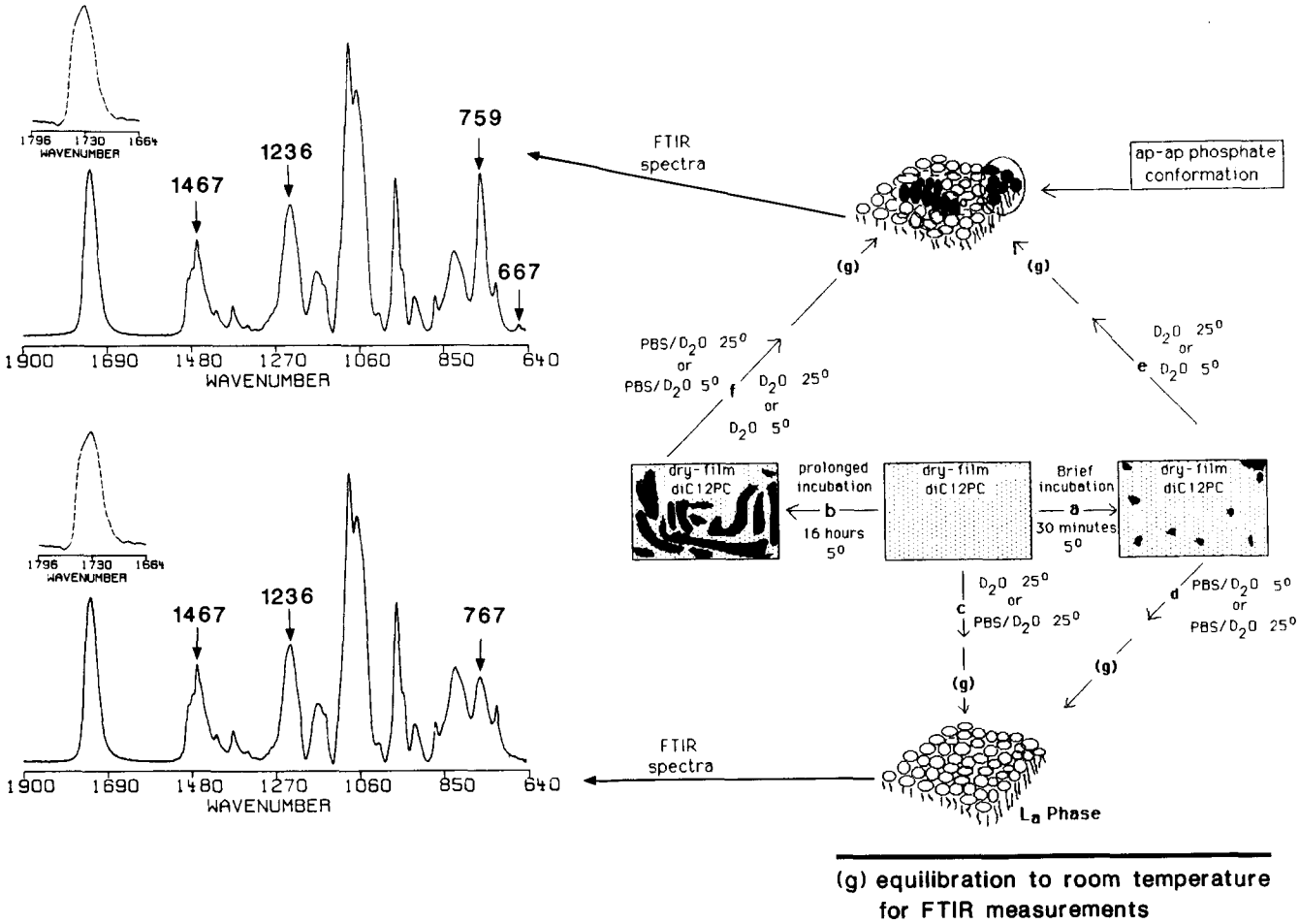

Fig. 2. Infrared spectra from 1900 cm^{-1} to 640 cm^{-1} of rapidly hydrated $diC_{12}PC$ bilayers resulting from different film incubation conditions. The three mosaic rectangles depict the dry lipid film that was non-incubated (center), briefly incubated (right), and extensively incubated (left). The darkened areas in the films represent crystalline L_c lipid patches. One-half bilayer is depicted in which the darkened headgroups represent the ap-ap phosphodiester conformation. The deconvolved ester carbonyl region (broken lines) is shown above each corresponding spectrum (see Fig. 1 legend for deconvolution parameters).

tween 850 cm^{-1} to 640 cm^{-1}. Based on dialkylphosphate anions (Table I), the extended ap-ap phosphodiester orientation is predicted to exhibit bands at 832 cm^{-1}, 757 cm^{-1}, and 665 cm^{-1}. In Fig. 2, the upper IR spectrum shows bands at 825.1 cm^{-1}, 759.3 cm^{-1}, and 667.2 cm^{-1} corresponding to the $\bar{\nu}_{as}$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ stretch, $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ stretch and γ_w $(PO_2)_{ap\text{-}ap}$ wag bands, respectively. Both the intense band at 759.3 cm^{-1} and the new band at 667.2 cm^{-1} are not present in the L_α phase (bottom spectrum of Fig. 2). Deconvolution of the phosphodiester region (Fig. 2A, upper spectrum) demonstrates that the $\bar{\nu}_{as}$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band is comprised of a central peak at 826.2 cm^{-1} with shoulders at 808.6 cm^{-1} and 853.1 cm^{-1}, whereas the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band is comprised of only one band at 759.0 cm^{-1}. The bands at 759 cm^{-1} and 667 cm^{-1} indicate a substantial population of phosphodiester ap-ap conformers. Buffer components were not present during the brief incubation of the film, or during liposome formation, and the ap-ap conformation was stable for several hours.

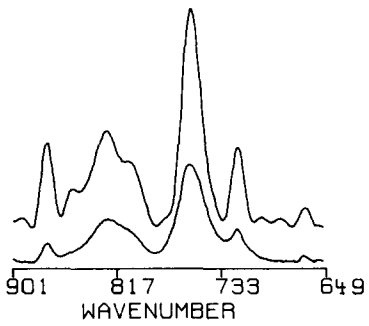

Fig. 2A. Deconvolution of the phosphodiester region shown in Fig. 2 upper spectrum. Deconvolution parameters of the phosphodiester region are given in Fig. 1A legend.

The conformational change in the headgroup phosphate (g-g to ap-ap) induced by film incubation was apparently not accompanied by dehydration of either the phosphate group or the interfacial carbonyl ester

group. Hydration of the phosphate group is indicated by the same $\bar{\nu}_{as}$ (PO_2) band position (1236 cm^{-1}) relative to the L_α phase. Hydration of the carbonyl ester group is based on deconvolution. The deconvolved ester carbonyl region for the $diC_{12}PC$ bilayers (broken lines, Fig. 2) shows the low frequency band at 1725 cm^{-1} has the largest peak intensity. Thus the extent of hydration of the *sn*-2 C = O ester carbonyl remains constant for the different film incubation conditions shown in Fig. 2.

As demonstrated above, $diC_{12}PC$ films briefly incubated (process a in Fig. 2) formed the ap-ap conformation only in the absence of buffer components. In contrast, $diC_{12}PC$ films subjected to prolonged incubation (process b, Fig. 2) formed the ap-ap conformers with or without buffered components. Thus the small patches of crystallized lipids in the briefly incubated film are destroyed when challenged with buffer (path a → d → g, Fig. 2); whereas, large crystalline lipid patches in films extensively incubated survive buffer and room temperature hydration conditions (path b → f → g, Fig. 2). Similar to brief incubation, the extent of hydration for both the phosphate group and ester carbonyl group remained constant under these conditions, as discussed in the last paragraph.

Pre-incubation of the $diC_{12}PC$ dry lipid films before rapid hydration, i.e., liposome formation, shown in Fig. 2 is different from the incubation of intact, fully hydrated, multibilayer liposome membranes which contain intralamellar water. However, our slow hydration at 5°C of predried $diC_{12}PC$ lipid films provided both hydration and intralamellar water during the process of liposome formation. Hydration conditions and IR spectra of slowly hydrated $diC_{12}PC$ films is shown in Fig. 3. Liposome formation by slow hydration at 5°C eliminates differences in the IR spectra caused by brief film incubation. For instance the slow hydration at 5°C of non-incubated dry $diC_{12}PC$ films in PBS/D_2O (path b → f, Fig. 3) generated the same infrared spectra as the slow hydration at 5°C of briefly incubated dry $diC_{12}PC$ films (path a → c → f, Fig. 3). However, there is a

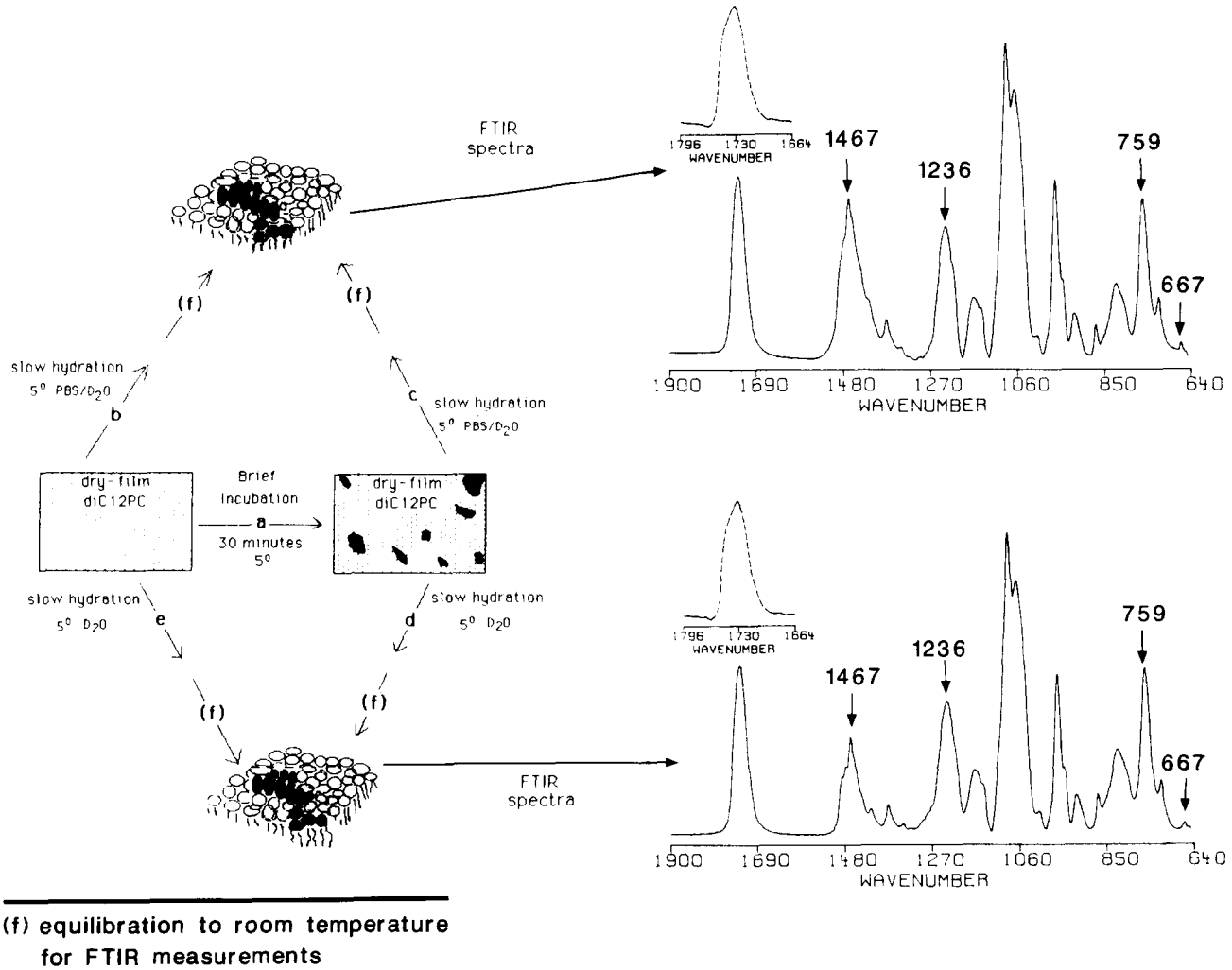

Fig. 3. Infrared spectra of $diC_{12}PC$ bilayers that were slowly hydrated for 16 h at 5°C from non-incubated and briefly incubated dry lipid films. See Fig. 2 legend for description of the model and Fig. 1 legend for ester carbonyl deconvolution parameters.

difference in the IR spectra when $diC_{12}PC$ is slowly hydrated in PBS/D_2O buffer compared to only D_2O.

Slow hydration in D_2O alone generates the ap-ap phosphodiester conformation (ap-ap phosphodiester bands $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ at 759 cm^{-1} and γ_w $(PO_2)_{ap\text{-}ap}$ at 667 cm^{-1}); whereas, slow hydration in PBS/D_2O buffer generates the ap-ap conformational change and also an increase in the band intensity under the δ (CH_2) centered at 1467 cm^{-1} in Fig. 3 (upper spectrum). In addition to the increased band intensity at 1467 cm^{-1}, there was a large increase in the O-H stretching band near 3400 cm^{-1} (not shown). This O-H stretch band area was 10 × larger than the O-H band area found in any other IR spectra. Control experiments whereby infrared spectra of a H_2O/D_2O mixture (0.5% through 2.0%, data not shown) confirmed that the increased band intensity near 1460 cm^{-1} (in Fig. 3, upper spectrum) was the O-H in plane bending deformation from HOD contamination. During slow hydration in PBS-D_2O, a few percent H_2O accumulated in the suspension and this H_2O exchanged with the D_2O buffer forming HOD.

Because of this HOD contamination, the original unsubstracted spectra from the slow hydration with PBS/D_2O was subtracted using H_2O/D_2O 1% as the reference spectrum. After subtraction, the O-H bands were no longer present (not shown), no change in the intensity of the bending deformation was found, but the intense ap-ap phosphodiester bands at 759 cm^{-1} and 667 cm^{-1} remained unchanged in Fig. 3, upper spectra. The O-H in plane bending deformation is centered at 1458 cm^{-1} and is a broad band. This also indicates that H_2O contamination was not responsible for the formation of the ap-ap phosphodiester conformation for $diC_{12}PC$ liposomes formed by slow hydration as depicted in Fig. 3. In addition the ap-ap phosphodiester conformation occurred to the same extent for all slow hydration conditions using both buffered and non-buffered D_2O solutions (compare phosphodiester regions for buffered and non-buffered D_2O in Fig. 3); this further supports the observation that H_2O contamination did not effect the formation ap-ap phosphodiester conformation.

In summary, the increased band intensity centered at 1467 cm^{-1} (Fig. 3) found in the room temperature spectra of slowly hydrated liposomes was due to uncompensated HOD and not conformational changes in the alkyl chains. This is consistent with the observation that no changes in $\bar{\nu}_s$ CH_2 or $\bar{\nu}_{as}$ CH_2 were observed. Based on low temperature incubation studies of pure $diC_{12}PC$ films, the headgroup phosphodiester undergoes a conformational change from g-g to ap-ap without an observable change in the acyl chain conformation or the degree of hydration of the phosphate group and carbonyl ester group.

The IR spectra in Figs. 2 and 3 indicate that pure $diC_{12}PC$ liposomes require low temperature incubation to form the ap-ap phosphodiester conformation. Incorporating decgly into $diC_{12}PC$ bilayers eliminated the requirement of low-temperature incubation for the ap-ap phosphodiester conformers to form. However, although some ap-ap conformers were always present, the amount of ap-ap conformers formed from $diC_{12}PC$/decgly mixed bilayers depended upon the hydration conditions. Mixed $diC_{12}PC$/decgly bilayers were hydrated by all conditions shown in Figs. 2 and 3 for pure $diC_{12}PC$ bilayers: but a model correlating lipid structural changes with the different hydration conditions could not be established. Consequently, hydration conditions were tabulated directly below the IR spectrum for diC_{12} PC/decgly mixed bilayers as shown in Figs. 4 and 5.

Hydration conditions in Fig. 4 and 5 were grouped according to the intensity ratio $I[\bar{\nu}_s\ (O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)]$. Dividing the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ by the γ_r (CH_2) rock normalized the IR data for the amount of lipid in the specimen during data collection and allowed the direct comparison of the spectra resulting from the different hydration conditions. The assumption was made that the intensity of the $\bar{\nu}_s$ $(O\text{-}P\text{-}O)_{ap\text{-}ap}$ band at 759 cm^{-1} correlates with the number of ap-ap conformers in the $diC_{12}PC$/decgly mixed bilayers. Thus Fig. 4 (top spectrum) shows hydration conditions eliciting the least number of ap-ap phosphodiester conformers (i.e., $I[\bar{\nu}_s\ (O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)] < 6$); and also hydration conditions eliciting a slightly larger number of ap-ap conformers (bottom spectrum, $6 < I[\bar{\nu}_s\ (O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)] < 10$).

The hydrocarbon stretching region in the IR spectra of mixed $diC_{12}PC$/decgly bilayers did not give any evidence of change in the alkyl chain trans/gauche ratio (i.e., the $\bar{\nu}_{as}$ CH_2 and $\bar{\nu}_s$ CH_2 band position did not change). Consequently only the phosphate region between 901 cm^{-1} and 649 cm^{-1} are shown in Fig. 4. The top spectrum in Fig. 4 shows that all hydration conditions, whereby $diC_{12}PC$/decgly mixed bilayers were rapidly hydrated, cause a small amount of ap-ap phosphodiester conformers. Deconvolution of this phosphodiester region of $diC_{12}PC$/decgly bilayers (not shown) produced spectra similar to the deconvolved spectrum in Fig. 2A. Based on Fig. 2, process b, it is likely that a significant population of ap-ap conformers form during prolonged incubation of the mixed lipid films, but vortexing (for rapid hydration) was sufficiently harsh to disrupt any crystalline patches of lipid where the ap-ap conformers exist.

Slow hydration in D_2O caused a significant population of ap-ap phosphodiester conformers (Fig. 4, bottom spectrum); whereas, slow hydration in PBS/D_2O caused the largest number of ap-ap conformers (Fig. 5). The largest value of $I[\bar{\nu}_s\ (O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)] \approx 50$ was obtained for slow hydration of $diC_{12}PC$/decgly bilayers when the films were briefly incubated before

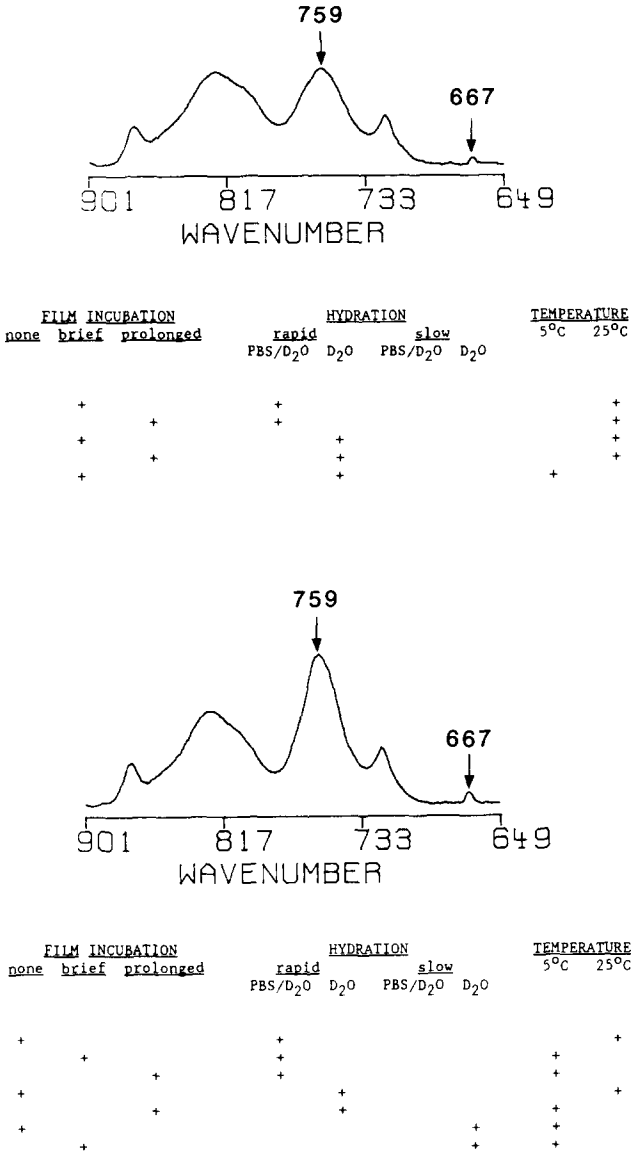

Fig. 4. Infrared spectra from 901 cm^{-1} to 649 cm^{-1} of rapidly hydrated $diC_{12}PC$/decgly bilayers resulting from different film incubation conditions. Hydration condition pathways are identical to those used in Fig. 2.

hydration (lower spectrum, Fig. 5). Slow hydration of non-film-incubated $diC_{12}PC$/decgly bilayers also elicited a large relative intensity $I[\bar{\nu}_s\ (O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)] \approx 25$ (upper spectrum, Fig. 5). Based on deconvolution of $\bar{\nu}_{as}\ PO_2$ and the carbonyl region as in the $diC_{12}PC$ bilayers alone, the headgroup phosphate conformational change (g-g to ap-ap) in $diC_{12}PC$/decgly bilayers was not accompanied by dehydration of either the phosphate group or the interfacial ester carbonyl group under both rapid and slow hydration liposome formation conditions. We note that slow hydration of pure $diC_{12}PC$ bilayers in PBS/D_2O caused a large population of ap-ap conformers to appear (i.e., $I[\bar{\nu}_s\ (O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)] \approx 12.5$). However, $diC_{12}PC$/decgly bilayers slowly hydrated in PBS/D_2O showed a greater increase in the relative number ap-ap phosphodiester conformers with a ratio $I[\bar{\nu}_s(O\text{-}P\text{-}O)_{ap\text{-}ap}]/I[\gamma_r\ (CH_2)]$ that was 2–4-times greater for $diC_{12}PC$/decgly vs. pure $diC_{12}PC$ bilayers under these hydration conditions. This indicates that the mixed lipid

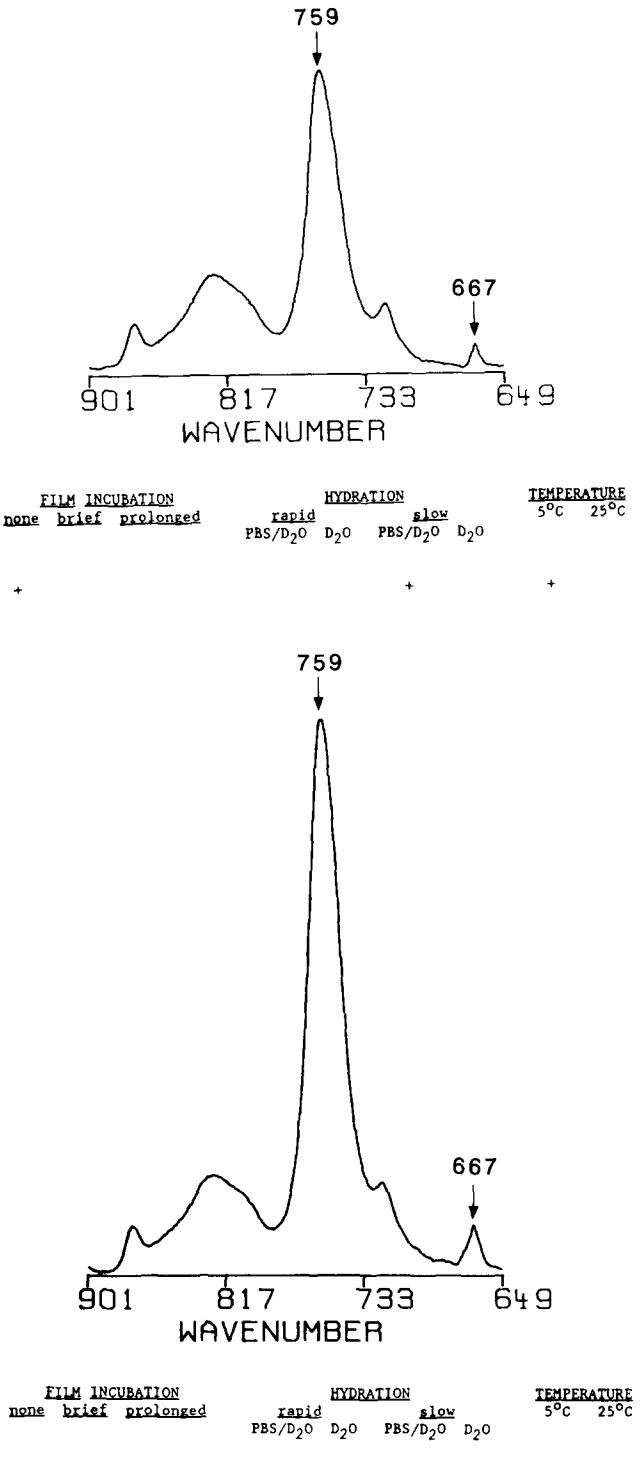

Fig. 5. Infrared spectra from 901 cm^{-1} to 649 cm^{-1} of $diC_{12}PC$/decgly bilayers that were slowly hydrated for 16 h at 5°C from non-incubated and briefly incubated dry lipid films. Hydration condition pathways are identical to those used in Fig. 3.

bilayers have 2–4-times more ap-ap phosphodiester conformers than pure $diC_{12}PC$ bilayers when the lipids are slowly hydrated in PBS/D_2O.

Although the IR spectrum of CO_2 vapor elicits an intense band near 2350 cm^{-1} another weak CO_2 fundamental which is the Q branch of the $\bar{\nu}_2$ CO_2 bending vibration is found near 667 cm^{-1}. This CO_2 band was calculated to occur at 667.3 cm^{-1} and 668.3 cm^{-1} [26], which occurs in the region of the γ_w $(PO_2)_{ap\text{-}ap}$ band at 667 cm^{-1} associated with the ap-ap phosphodiester conformation. Therefore comparing the $\bar{\nu}_2$ CO_2 bending vibration to the γ_w $(PO_2)_{ap\text{-}ap}$ band was critical for our conformational assignments [27]. Due to the experimental design of our FT-IR instrument purge system, virtually all IR spectra contained no CO_2 bands at 2350 cm^{-1}, (which is slightly to the right of the valley in the spectrum shown in Fig. 1). A few IR spectra contained the 2350 cm^{-1} CO_2 band and CO_2 vapor subtraction was performed using a CO_2 reference spectrum [28] for those spectra which did not have the ap-ap phosphodiester conformation. It is emphasized that in spectra containing the ap-ap phosphodiester conformation, any appearence of CO_2 vapor resulted in the remeasurement of the spectrum until no carbon dioxide vapor was present. However, we compared the band shapes of the $\bar{\nu}_2$ CO_2 bending vibration from our reference spectrum with the IR spectrum of $diC_{12}PC$/decgly bilayers elicting the ap-ap phosphodiester conformation. As shown in Fig. 6, the band width of the $\bar{\nu}_2$ CO_2 bending vibration is narrow compared to the γ_w $(PO_2)_{ap\text{-}ap}$ band, and the $\bar{\nu}_2$ CO_2 bending vibration is 1.5 cm^{-1} higher in peak position (668.8 cm^{-1}) than the band we report as the PO_2 wag of the ap-ap phosphodiester conformation (667.2 cm^{-1}). Although our reported value for the CO_2 vibration is approx. 1 cm^{-1} greater than the accepted frequency [26], this value was found to be reproducible for our auxiliary bench sample compartment. This discrepancy is due to several factors including the resolution at which the spectra was obtained (4 cm^{-1} with data encoded every 2 cm^{-1}), the asymmetric peak shape

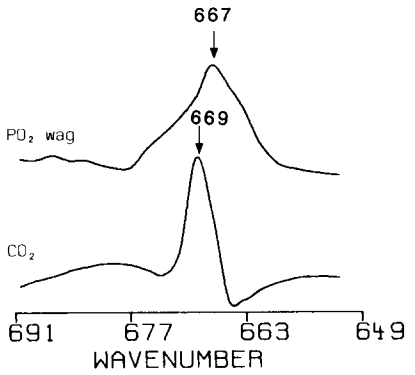

Fig. 6. Infrared spectra of the 691 cm^{-1} to 649 cm^{-1} region for CO_2 vapor reference (bottom) and $diC_{12}PC$/decgly bilayers made from the slow hydration of a briefly incubated dry lipid film.

of the $\bar{\nu}_2$ CO_2 bending vibration, how the cubic spline peak pick function fits the encoded data, and the refractive index of the ZnSe crystal of the CIRCLE microcell. Although all of our reference spectrum were taken with the CIRCLE flow cell in the spectrometer a, separate CO_2 reference spectrum obtained without the CIRCLE flow cell in the beam path had a 667.7 cm^{-1} $\bar{\nu}_2$ CO_2 bending vibration in our spectrometer (not shown).

Discussion

We examined lipid structural changes induced by low-temperature incubation of $diC_{12}PC$ and $diC_{12}PC$/decgly lipid films by IR spectroscopy. Unlike NMR, the time frame for infrared absorption is the time necessary for a molecular vibration [29]. Consequently, observing conformational changes by IR spectroscopy does not give information about the dynamics of the conformation. Thus, the dynamics of the ap-ap phosphodiester conformers elicited by different hydration conditions and mixed lipid systems cannot be evaluated by IR analysis.

Our experimental strategy was to incubate either $diC_{12}PC$ or $diC_{12}PC$/decgly (97.5 : 2.5) lipid films for different lenghts of time, form hydrated bilayers at either low temperature (5°C) or at room temperature (25°C), equilibrate to room temperature, and obtain room temperature IR spectra. Room temperature IR spectra of liposomes made from previously incubated films will identify kinetically slow lipid structural changes that form or relax slowly.

Incubation times were based on X-ray diffraction studies of $diC_{16}PC$ hydrated bilayers that identified three incubation time domains during which different structural changes in the multiple-bilayer system occurred [30]. Domain 1, incubation time from 0 to 1.5 h, caused minimal headgroup dehydration, but maximal changes in the hydrocarbon packing of the acyl chains. Domain 2, incubation time from 1.5 to 12 h, caused headgroup dehydration, interlamellar dehydration, and further changes in the hydrocarbon packing of the acyl chains. For domain 3, incubation time ≥ 12 h, no further changes in the lipid system were observable by X-ray diffraction and therefore incubation was complete. Although these time domains were established for the incubation of fully hydrated lipids, we used similar incubation time domains for pre-dried lipid films.

Low temperature incubation as descibed above, causes saturated PCs to crystallize into a metastable L_c phase denoted as a subtransition gel [2]. However, our incubation conditions did not form the expected lipid structural changes associated with the L_c phase. Characteristic of the L_c phase is a rearrangement of the acyl chain packing [2], headgroup dehydration [30,31], decreased mobility of the headgroup phosphate [32], and a conformational change in the glycerolbackbone with a

large steric barrier to rotation [1,2]. Under our incubation conditions neither rearrangement of acyl chain packing, nor dehydration of the headgroup nor changes in the glycerobackbone were apparent. However, formation of the ap-ap phosphodiester conformation undoubtably restricted the mobility of the headgroup probably similar to the L_c phase of other saturated PCs. We note the unusual phosphodiester ap-ap conformation has not been reported to occur in the L_c phase of saturated PCs.

DSC and X-ray experiments of dipalmitoylphosphatidylcholine ($diC_{16}PC$) corroborate our results [33]. $DiC_{16}PC$ lipid powder incubated under conditions similar to ours and hydrated with H_2O at 5°C resulted in the formation of new L_c phases as determined by DSC [33]. These new L_c gel phases were relatively stable for 4 h at room temperature (23°C) [33]. Structural information using X-ray diffraction indicated that the new L_c phases were different from the standard L_c phase [33].

The L_c phase of saturated PCs is metastable and, in addition, formation and relaxation of the L_c phase is kinetically slow [2,18,34]. For $diC_{12}PC$ bilayers, incubation for 5 days is required to completely convert $diC_{12}PC$ bilayers into the L_c phase [34] (i.e., after 5 days at 5°C, no further changes by DSC were observed). $DiC_{12}PC$ is the shortest chain PC that forms an L_c phase observable by DSC; shorter chain analogs require ^{31}P-NMR to observe the transition [34]. From DSC analysis, heating L_c phase $diC_{12}PC$ bilayers converts the bilayers to the $L_{\beta'}$ phase when the temperature exceeds the sub-transition temperature (T_s) [34]. Further heating converts the $L_{\beta'}$ phase to the L_α phase when the temperature exceeds the main transition temperature (T_m) [34]. Incubated $diC_{12}PC$ bilayers have a T_s at 3°C and a T_m at 4.5°C [34]. Our room temperature IR spectra of $diC_{12}PC$ bilayers demonstrate that the structural changes, induced by low temperature film incubation, persist well above the T_m and T_s of $diC_{12}PC$ bilayers in the L_c phase. The ap-ap phosphodiester conformation was stable at room temperature for at least 4 h.

The formation and persistance of the ap-ap phosphodiester conformation in room temperature bilayers was unexpected. In L_α phase bilayers, individual lipid molecules have several molecular motions including axial rotation [35], lateral diffusion [36], and even a slow flip-flop [37]. If these motions are available to the lipid molecule having the ap-ap phosphodiester conformation, relaxation of the thermodynamically unfavorable ap-ap conformation to the g-g phosphodiester conformation would occur. For this reason, patches of crystallized lipid are postulated and these patches of lipid, formed by incubating dry-lipid-films (e.g. path b → f → g in Fig. 2) survives the harsh conditions of hydration. Based on $diC_{12}PC$ films briefly incubated (Fig. 2), hydration in D_2O is more gentle than hydration in PBS/D_2O. Briefly incubated films contain crystalline patches; some of the patches survive hydration in D_2O but not hydration in PBS/D_2O.

The number of ap-ap phosphodiester conformers produced by slow hydration of pure $diC_{12}PC$ bilayers was the same for hydration in PBS/D_2O and D_2O (compare Fig. 3 top and bottom spectra). In contrast, the number of ap-ap conformers was much greater when $diC_{12}PC$/decgly bilayers were slowly hydrated in PBs/D_2O compared to slow hydration in only D_2O (compare Fig. 4 bottom spectra to the spectra in Fig. 5). The reason for this discrepancy most likely depends on the ionization of the decgly in the membrane. Decgly in the membrane will ionize when buffered water (PBS/D_2O is pH 7.3) is used as the hydration solvent whereas, in D_2O, the ionization of decgly in the membrane will depend on the pH. The pH of $diC_{12}PC$/decgly mixed bilayers hydrated in D_2O was initially 5.0, which did not change over 0.5 h, and after 16 h of slow hydration the pH was 4.7. Infrared spectra showed IR bands associated with the asymmetric carboxyl stretch ($\bar{\nu}_{as}$ COO^-) at 1600 cm^{-1} for the $diC_{12}PC$/decgly bilayers slowly hydrated in PBS/D_2O but not in D_2O. This indicates that the decgly in $diC_{12}PC$/decgly mixed bilayers hydrated in D_2O alone is in the unionized state. Although ionization of the carboxyl group of decgly correlates with the formation of significant amounts of ap-ap phosphodiester conformers, the mechanism(s) responsible for this observation is unknown. Ionized carboxyl groups may facilitate phase separation of the membrane lipids, ion-pair with the quarternary amine headgroups, and/or change the membrane surface charge. In addition, ionization may position the fatty-acid-amino-acid at a different depth in the bilayer compared to the free acid form of decgly. Further studies are necessary to elucidate the mechanism between decgly and $diC_{12}PC$ molecules in the ionized and unionized state.

Fatty acid analogs containing spin probes or fluorescent indicators have been criticized because they perturb the membrane environment they are probing [38]. Fatty-acid-amino-acids are also expected to perturb the membrane environment. However, it is exactly this membrane perturbation we wish to study because a large number of cellular and viral proteins contain a covalently bound lipid [39–41]. Included in this group are amino-terminal glycine fatty acylated proteins [39–41], therefore the observation that decgly induced the ap-ap phosphodiester conformation in phosphatidylcholine membranes may be biologically significant. Fatty acid acylation of proteins has been shown to be essential for membrane localization, cellular transformation, and viral particle assembly [42–60]. Myristoyl residues are attached co-translationally, to an N-terminal glycine residue [39–41]; whereas, palmitoyl groups are attached post-translationally to cysteine thiol res-

idues not on the N- or C-terminal of the protein [39–41]. Fatty-acid-membrane-anchors, position some of the adjacent amino acid residues of the mature proteins near the headgroup region of the membrane. Since several membrane proteins contain covalently bound fatty acids, fatty-acid-amino-acids and fatty-acid-peptides are natural probes of biologically relevant membrane proteins. We recently established a data bank of transmembrane proteins sequences with the intent of identifying biologically relevant sequences to study protein–lipid interactions at the membrane surface using infrared spectroscopy [61].

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References

1 Cameron, D.G. and Mantsch, H.H. (1982) Biophys. 38, 175–184.
2 Casal, H.L. and Mantsch, H.H. (1984) Biochim. Biophys. Acta 779, 381–401.
3 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1976) Nature 261, 390–394.
4 Casal, H.L., Mantsch, H.H., Paltauf, F. and Hauser, H. (1987) Biochim. Biophys. Acta 919, 275–286.
5 Casal, H.L., Mantsch, H.H., and Hauser, H. (1987) Biochemistry 26, 4408–4416.
6 Cortijo, M., Alonso, A., Gomez-Fernandez, J.C. and Chapman, D. (1982) J Mol. Biol. 157, 597–618.
7 Lee, D.C., Durrani, A.A., and Chapman, D. (1984) Biochim. Biophys. Acta 769, 49–56.
8 Cornel, D.G., Dluhy, R.A., Briggs, M.S., McKnight, C.J. and Gierasch, L.M. (1989) Biochemistry 28, 2789–2796.
9 Wong,P.T.T. and Mantsch, H.H. (1988) Chem. Phys. Lipids 46, 213–224.
10 Fringeli, U.P. and Günthard, H.H. (1981) in Membrane Spectroscopy (Grell, E., ed.), pp. 270–332, Springer-Verlag, New York.
11 (A) Shimanouchi, T., Tsuboi, M. and Kyogoku, Y. (1964) Adv. Chem. Phys. 7, 435–498.
(B) Kyogku, Y., Shimanouchi, T., and Tsuboi, M. (1962) Proc. Int. Mol. Struct. Spectrosc., Tokyo A106, 1–4.
12 Hauser, H., Pascher, I., Pearson, R.H. and Sundell, S. (1981) Biochim. Biophys. Acta 650, 21–51.
13 Jayaram, B., Ravishanker, G. and Beveridge, D.L. (1988) J. Phys. Chem. 92, 1032–1034.
14 Jayaram, B., Mezei, M. and Beveridge, D.L. (1988) J. Am. Chem. Soc. 110, 1691–1694.
15 Lapidot, Y., Rappoport, S. and Wolman, Y. (1967) J. Lipid Res. 8, 142–145.
16 Pidgeon, C., Hunt, A.H. and Dittrich, K. (1985) Pharm. Res. 3, 23–24.
17 Pidgeon, C., Apostol, G. and Markovich, R. (1989) Anal. Biochem. 181, 28–32.
18 Sperline, R.P., Muralidharan, S. and Freiser, H. (1986) Appl. Spectrosc. 40, 1019–1022.
19 Braue, E.H., Jr. and Pannella, M.G. (1987) Appl. Spectrosc. 41, 1057–1067.
20 Braue, E.H., Jr. and Pannella, M.G. (1987) Appl. Spectrosc. 41, 1213–1216.
21 Rothchild, K.J., Degrip, W.L., and Sanches, R. (1980) Biochim. Biophys. Acta 596, 338–351.
22 Gillette, P.C. and Koenig, J.C. (1984) Appl. Spectrosc. 38, 334–337.
23 Powell, J.R., Wasacz, F.M. and Jakobsen, R.T. (1986) Appl. Spectrosc. 40, 339–344.
24 Kauppinen, J.K., Moffatt, D.J., Mantsch, H.H. and Cameron, D.G. (1981) Appl. Spectrosc. 35, 271–276.
25 Griffiths, P.R. and Pariente, G.L. (1986) Trends Anal. Chem. 5, 209–214.
26 Kaplan, L.D. (1947) J. Chem. Phys., 15, 809–815.
27 We are grateful to the reviewer for suggesting the direct comparision between the $\bar{\nu}_2$ CO_2 Q branch and the PO_2 wag band.
28 Griffiths, P.R. and Haseth, J.A. (1986) Chemical Analysis (Elving, P.J. and Winefordner, J.D., eds.), 83, p. 172.
29 Casal, H.L. (1988) J. Am. Chem. Soc. 110, 5203–5205.
30 Ruocco, N.J. and Shipley, G.G. (1982) Biochim. Biophys. Acta 691, 309–320.
31 Ruocco, N.J. and Shipley, G.G. (1982) Biochim. Biophys. Acta 684, 59–66.
32 Fuldner, H.H. (1981) Biochemistry 20, 5707–5710.
33 Yang, C.F., Wiener, N.C. and Nagle, J.F. (1988) Biochim. Biophys. Acta 945, 101–104.
34 Lewis, R.N.A., Mak, N. and McElhaney, R.N. (1987) Biochemistry 26, 6118–6126.
35 Lee, A.G. (1975) Prog. Biophys. Mol. Biol. 29, 3.
36 Smith, B.A. and McConnell, H.M. (1978) Proc. Natl. Acad. Sci. USA 75, 2759.
37 Shaw, J.M., Hutton, W.C., Lentz, B.R. and Thompson, T.E. (1977) Biochemistry 16, 4156.
38 Muranceshi, N., Takagi, N., Muranishi, S. and Sezaki, H. (1981) Chem. Phys. Lipids 28, 269.
39 Olson, E.C. (1988) Prog. Lipid Res. 27, 177–197.
40 Schultz, A.M., Henderson, L.E. and Oroszlan, S. (1988) Annu. Rev. Cell Biol. 4, 611–647.
41 Towler, D.A., Cordan, J.I., Adams, S.P. and Glaser, L. (1988) Annu. Rev. Biochem. 57, 69–99.
42 Gheysen, D., Jacobs, E., De Foresta, F., Thiriat, M., Francotte, M., Thines, D. and Wilde, M. (1989) Cell 59, 103–112.
43 Rein, A., McClure, M.R., Rice, N.R., Luftig, R.B. and Schultz, A.M. (1986) Proc. Natl. Acad Sci USA 83, 7246–7250.
44 Rhee, S.S. and Hunter, E. (1987) J. Virol. 61, 1045–1053.
45 Chow, M., Newman, J.F.E., Filman, D., Hogle, J.M., Rowland, D.J. and Brown, E. (1987) Nature 327, 482–486.
46 Bus, J.E., Mumby, S.M., Carey, P.J., Gilman, A.C. and Sefton, B.M. (1987) Proc. Natl. Acad. USA 84, 7493–7497.
47 Ozols, J., Carr, S.A. and Strittmatter, P.J. (1984) Biol. Chem. 259, 13349–13354.
48 Paul, V.A., Schultz, A., Pincus, S.E., Oroszlan, S. and Wimmer, E. (1987) Proc. Natl. Acad Sci USA 84, 7827–7831.
49 Pershing, D.H., Varmus, H.E. and Ganem, D. (1987) J. Virol. 61, 1672–1677.
50 Streuli, C.H. and Griffin, B.E. (1987) Nature 326, 619–621.
51 Kahn, R.A., Goddard, C. and Newkirk, M. (1988) J. Biol. Chem. 263, 8282–8287.
52 Pillai, S. and Baltimore, D. (1987) Proc. Natl. Acad. Sci. USA 84, 7654–7658.
53 Schultz, A.M., Henderson, L.E., Oroszlan, S., Garber, E.A. and Hanafusa, H. (1985) Science 227, 427–439.
54 Cross, F.R., Garber, E.A., Pellman, D. and Hanafusa, H. (1984) Mol. Cell Biol. 4, 1834–1842.

55 Kamps, M.P., Buss, J.E. and Sefton, B.M. (1985) Proc. Natl. Acad. Sci. USA 82, 4625–4628.
56 Kaplan, J.M., Mardon, C., Bishop, J.N. and Varmus, H.E. (1988) Mol. Cell Biol. 8, 2435–2441.
57 Kamps, M.P., Bus, J.E. and Sefton, B.M. (1986) Cell 45, 105–112.
58 Henderson, L.E., Krutsch, H.C. and Oroszlan, S. (1983) Proc. Natl. Acad. Sci. USA 80, 339–343.
59 Trono, D., Feinberg, M.B. and Baltimore, D. (1989) Cell 59, 113–120.
60 Wills, J.W., Craven, R.C. and Achacosa, J.A. (1989) J. Virol. 63, 4331–4343.
61 Pidgeon, C., Williard, R.L. and Schroeder, S.C. (1989) Pharm. Res. 6, 779–786.