A new infrared spectroscopic marker for cochleate phases in phosphatidylserine-containing model membranes

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ABSTRACT Fourier transform-infrared (IR) spectroscopic and electron microscopic studies are reported for 1,2-dimyristoylphosphatidylserine (DMPS) and for DMPS/1,2-dimyristoylphosphatidylcholine mixtures in the presence and absence of Ca^{2+} ion. The frequency of the methyl symmetric deformation mode near 1,378 cm^{-1}, previously assumed insensitive to changes in lipid morphology, has been found to respond to cochleate phase formation by undergoing an ~8 cm^{-1} increase. The new IR spectroscopic marker at 1,386 cm^{-1} has been used to identify and verify structures suggested from the phase diagram of J. R. Silvius and J. Gagné (1984. *Biochemistry.* 23:3241–3247) for this system. In addition, the ability of Mg^{2+} ion to induce cochleate formation has been demonstrated. Higher Mg^{2+} than Ca^{2+} levels are required for this process. Finally, IR spectroscopy has been used to monitor dehydration of the lipid surface through changes in the asymmetric PO_2^- stretching mode. Dehydration precedes cochleate phase formation (i.e., occurs at a lower Ca^{2+}/phosphatidylserine level).

INTRODUCTION

Studies of the interaction of Ca^{2+} with anionic phospholipids such as phosphatidylserine (PS)[1] have been widely pursued due to the possible relevance of this model system to fusion in biological membranes (1–5). As PS levels in native membranes are normally lower than the levels of zwitterionic species such as phosphatidylcholine (PC), mixtures of anionic with zwitterionic phospholipids have been extensively investigated with the battery of modern physical methods (6–9).

Required preliminary steps to vesicle fusion include charge neutralization of anionic phospholipids and dehydration of the bilayer surface to facilitate the approach of two vesicles. A local perturbation in bilayer packing also appears necessary. Ca^{2+} ion is effective in inducing fusion through the formation of a *trans* $Ca(PS)_2$ complex, which acts as a focal point between two closely apposed structures (10). In addition, Ca^{2+} effectively promotes a cochleate cylindrical phase arising from collapsed small vesicles that fuse into sheets and coil around an initial folding point (1).

Infrared (IR) spectroscopy permits ion-induced perturbations at particular phospholipid sites to be directly detected without use of a probe molecule. Investigations of PS–ion interactions have been reported by several groups. Dluhy et al. (11) noted large Ca^{2+}-induced alterations of phosphate group vibrations consistent with bidentate ligand binding. In addition, dehydration of the phosphate was identified through a characteristic increase in the asymmetric PO_2^- stretching frequency from 1,221 to 1,238 cm^{-1}. Casal et al. (12–14) have evaluated the conformation around the phosphodiester linkages in PS-cation systems. Most recently, Choi et al. (15) studied PS/Ca^{2+} systems in the presence of cholesterol.

The current work reports an IR spectroscopic study of the interaction of Ca^{2+} with 1,2-dimyristoylphosphatidylserine (DMPS) and with DMPS/1,2-dimyristoylphosphatidylcholine (DMPC) mixtures. The latter system was chosen as a suitable one from which to base correlations of spectral features with morphological structure, as a detailed phase diagram has been derived from differential scanning calorimetry (DSC) studies by Silvius and Gagné (16). It was hoped that the IR data could (after suitable control experiments with electron microscopy to detect phases) be used to develop spectroscopic markers for particular lipid morphological states. An unexpected frequency increase of ~8 cm^{-1} in the methyl symmetric deformation ("umbrella") mode at 1,378 cm^{-1}, apparently specific for cochleate phase formation, has been observed. The utility of this IR-based identification of a particular phase is demonstrated through studies of Mg^{2+} binding and lateral phase separation in mixed PS/PC systems where the acyl chains of one lipid may be selectively perdeuterated. In addition, the relationships between Ca^{2+} levels required to induce dehydration and those required for cochleate phase formation have been determined.

MATERIALS AND METHODS

Materials

1-palmitoyl,2-oleoylphosphatidylserine (POPS), DMPS, DMPC, and acyl-chain perdeuterated DMPC (DMPC-d_{54}) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Tris(hydroxymethyl)-aminomethane hydrochloride, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; 97% pure), and $MgCl_2$ (98% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). $CaCl_2 \cdot 2H_2O$ was obtained from Fisher Scientific (Fairlawn, NJ). All salts and buffers were of the highest quality commercially available and were used without further purification. All phospholipids demonstrated phase transition temperature characteristics consistent

[1] *Abbreviations used in this paper:* DMPC, 1,2-dimyristoylphosphatidylcholine; DMPS, 1,2-dimyristoylphosphatidylserine; DSC, differential scanning calorimetry; IR, infrared; PC, phosphatidylcholone; PS, phosphatidylserine.

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with literature values, as measured with a differential scanning calorimeter (model MC-1; Micro-cal Inc., Amherst, MA).

Sample preparation

Phospholipid samples were prepared from appropriate aliquots of $CHCl_3$ solutions. Bulk solvent was evaporated under a stream of dry N_2; samples were brought to dryness by placing them under vacuum (~1 torr) for 8 h. Aqueous samples were prepared with Tris buffer (10 mM Tris, 100 mM NaCl, pH 7.4) containing either 0.1 mM EGTA, or the desired levels of $CaCl_2$ or $MgCl_2$, by vortexing, heating above normal phase transition temperatures, and cooling. This cycle was repeated several times to ensure complete dispersion. Total lipid concentrations in samples prepared for FT-IR and electron microscopy experiments were ~450 and 2.5 mM, respectively. Lipid/cation molar ratios were varied from 1:0.05 to 1:3. Selected samples were also prepared in D_2O buffers exactly as described above with pD adjusted to 7.4.

FT-IR spectroscopy

Samples were transferred to AgCl windows containing a Teflon spacer of 25 μm thickness and inserted into a thermostated cell (Harrick Scientific, Ossining, NY). Temperatures were controlled with a circulating water bath and monitored with a digital thermocouple (Physitemp Instruments, Inc., Clifton, NJ) placed next to the cell window. Spectra were recorded with a spectrophotometer (either Polaris; Mattson Instruments, Madison, WI; or model FTS-40; Bio-Rad [Digilab] Cambridge, MA), each equipped with a TGS detector. 256 interferograms were collected and coadded under N_2 purge at 4 cm^{-1} resolution. The resultant interferograms were apodized with a triangular function, zero filled (one level), and Fourier transformed to yield a spectrum with data encoded every 2 cm^{-1}. Residual water vapor bands were removed with an appropriate reference spectrum, and baselines were flattened with manufacturer-supplied software. Additional spectral manipulations were performed by transferring data to a microcomputer and using software supplied by D. Moffatt of the National Research Council of Canada (Ottawa).

Electron microscopy

Formvar, carbon-coated, 200-mesh copper grids were treated with 1% BSA, after which 5 μl of dispersed lipid was applied and negatively stained with 1% uranyl acetate. Samples were examined with a transmission electron microscope (model CM10; Philips Electronic Instruments, Inc., Mahwah, NJ).

RESULTS

Effects of Ca^{2+} on PS-containing lipid systems

The effects of varying concentrations of $CaCl_2$ on the methyl umbrella region (1365–1,400 cm^{-1}) in the IR spectrum for pure DMPS membranes at room temperature are shown in Fig. 1. As the Ca^{2+}/lipid molar ratio increases, the spectrum in the vicinity of the methyl umbrella absorption band (usually 1,378 cm^{-1}) is altered from its normal appearance (17). In addition to the main band at 1,378 cm^{-1} in Ca^{2+}-free DMPS vesicles, a weak new feature is noted in the DMPS/Ca^{2+} (1:0.05) system at 1,390 cm^{-1}. At higher concentrations of Ca^{2+}, the 1,378-cm^{-1} band intensity gradually diminishes until it is barely discernible in the DMPS/Ca^{2+} (1:1) system. Concurrently, an additional feature at ~1,386 cm^{-1} increases in intensity. At the highest levels of Ca^{2+}, the two new features (1,386 and 1,390) merge into one overlapped contour with the position of maximal intensity at 1,386 cm^{-1}. Similar effects were noted for pure POPS membranes in D_2O-Tris buffer at comparable lipid/Ca^{2+} mole ratios (results not shown). The addition of Ca^{2+} is known to induce a morphological change in PS bilayers from multilamellar vesicles to cochleate lipid cylinders (2). The appearance of features at 1,390 or 1,386 cm^{-1} and the simultaneous disappearance of the 1,378-cm^{-1} methyl umbrella absorption bands thus apparently monitor the sequence of events that lead to the formation of these forms.

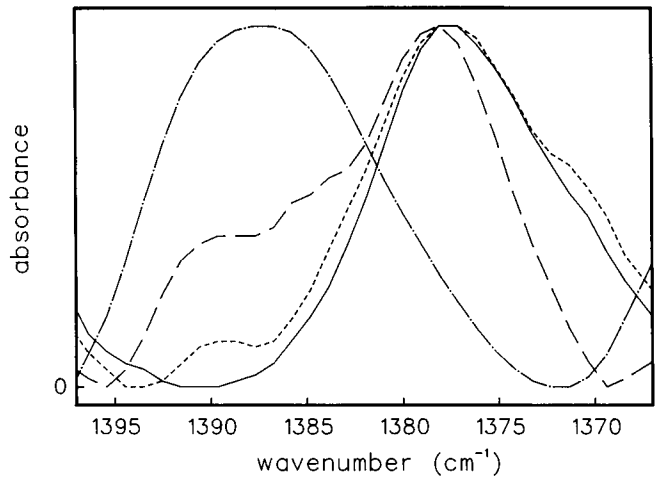

FIGURE 1 FT-IR spectra of the methyl umbrella region (1,400–1,365 cm^{-1}) for DMPS membranes as a function of calcium concentration at room temperature. The Ca^{2+}-free system is represented (——) and DMPS/Ca^{2+} molar ratios of 1:0.05 - - -, 1:0.1, ——; 1:1, —·—. Spectra have had residual water vapor bands removed and baselines flattened.

To explore the usefulness of the shift in the methyl umbrella mode as an IR indicator of cochleate phase formation, we undertook a temperature study of the DMPC/DMPS mixed lipid system. The phase behavior for this mixture in the presence and absence of Ca^{2+} has been deduced from DSC (16).

Mixtures of DMPC-d_{54}/DMPS with mole ratios 100:0, 75:25, 50:50, 20:80, and 0:100 were investigated over a temperature range of 5–50°C at ~10° intervals. In Ca^{2+}-containing samples, the total lipid-to-Ca^{2+} mole ratios were 1:3. Perdeuteration of the acyl chains of one lipid constituent enables us to separately evaluate the thermotropic properties of each component in the mixture. This procedure has been widely used in vibrational spectroscopic studies of lipid–lipid interaction (18).

Fig. 2 displays the methyl umbrella region for a 50:50 DMPC-d_{54}/DMPS mixture as a function of temperature in the absence of Ca^{2+} (Fig. 2 *a*) and with excess Ca^{2+} (Fig. 2 *b*). In Ca^{2+}-free preparations, the methyl umbrella frequency increases by ~1 cm^{-1} during the gel–liq-

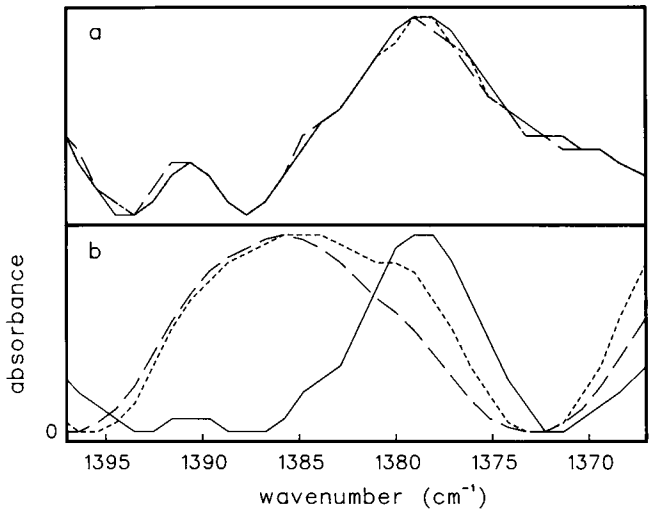

FIGURE 2 FT-IR spectra of the methyl umbrella region (1,400–1,365 cm^{-1}) for 50:50 mole ratio mixtures of DMPC-d_{54}/DMPS as a function of temperature in the absence (*a*) and presence (*b*) of excess Ca^{2+}. Temperatures were 35 (——), 43 (- - -), and 50°C (— —). Spectra have had residual water vapor bands removed and baselines flattened.

uid crystal transition. Similar results were found for all Ca^{2+}-free mixed lipid systems studied. The presence of the 1,390-cm^{-1} band in Fig. 2 *a* (bilayer phases) suggests that this spectral feature is not indicative of cochleate phase formation.

To further probe this point, pure DMPC-d_{54} and pure DMPS were also examined as a function of temperature (results not shown). In Ca^{2+}-free samples, the 1,390-cm^{-1} band is present in DMPC-d_{54} but is absent in pure DMPS, suggesting that at least some of its vibrational intensity in the binary mixture does not originate in lipid acyl chains motions but may arise from the phosphorylcholine moiety. The symmetric CH_3-bending mode of the choline headgroup is reported to absorb between 1,395 and 1,405 cm^{-1} (19), and we tentatively suggest that this vibration in the acyl-chain perdeuterated lipid actually appears at 1,390 cm^{-1} (Fig. 2 *a*).

Another source of intensity at 1,390 cm^{-1} is evident in spectra of PS (Fig. 1). At high Ca^{2+} levels, this band intensity is less than one-third the intensity of the 1,386-cm^{-1} component. However, at low Ca^{2+} levels, the 1,390-cm^{-1} band appears to the exclusion of the lower frequency component. The presence of the 1,390-cm^{-1} band at a lipid/Ca^{2+} mole ratio of 1:0.05 correlates well with a Ca^{2+}-induced increase in the asymmetric phosphate stretching frequency, which reveals dehydration of that functional group. Because (as discussed below) dehydration precedes cochleate phase formation, we suggest that some of intensity at 1,390 cm^{-1} arises from methyl umbrella modes in dehydrated but noncochleate structures. To summarize, the 1,386-cm^{-1} component of the doublet observed during the Ca^{2+}-induced structural change is characteristic of cochleate phase formation, whereas the 1,390-cm^{-1} band arises from two possible sources in binary lipid mixtures, a symmetric methyl bend of the choline moiety (PC component), overlapped with a methyl umbrella mode from dehydrated bilayers.

Further evidence for the above tentative assignments comes from the 50:50 system with excess Ca^{2+}, where the methyl umbrella mode intensity (Fig. 2 *b*) shifts from 1,378 to 1,386 cm^{-1} as the temperature increases. The main intensity redistribution occurs between 35 and 43°C. The DMPC/DMPS/Ca^{2+} phase diagram of Silvius and Gagné (16) shows that a phase change from hydrated solid bilayers to a mixture of liquid crystalline bilayers and dehydrated cochleate phases occurs exactly within this temperature interval. These observations support the current contention that the appearance of a 1,386-cm^{-1} methyl umbrella mode spectroscopically tracks cochleate phase formation and provide independent evidence for the correctness of the phases assigned by Silvius and Gagné. Shifts in the methyl umbrella band at other compositions (results not shown) were consistent with the aforementioned phase diagram. For example, the spectrum of a DMPC-d_{54}/DMPS 20:80 system with excess Ca^{2+} at 5°C shows a significant proportion of the methyl umbrella intensity at 1,386 cm^{-1}, indicating the presence of a cochleate phase.

Fig. 3 displays four electron micrographs that corroborate the results from the FT-IR experiments. Two are for the 50:50 DMPC/DMPS system, the first lacking Ca^{2+} (Fig. 3 *a*) and the second with a lipid/Ca^{2+} mole ratio of 1:3 (Fig. 3 *b*). In the absence of Ca^{2+}, only vesicles are observed, whereas with excess Ca^{2+}, larger, cylindrically shaped cochleate structures are evident. In micrographs of pure DMPS without Ca^{2+} (results not shown), vesicles of a diameter comparable to those in Fig. 3 *a* are observed. Fig. 3 *c*, DMPS/Ca^{2+} (1:0.1), displays a micrograph of a mixture of associated vesicles and cochleates. The diameter of the vesicles in this system has increased to ~11,000 Å. The observation of an increase in the diameter of DMPS vesicles upon Ca^{2+} addition agrees with the reported sequence of events, i.e., that vesicle fusion precedes cochleate formation (2). The mixture of vesicles and cochleates in Fig. 3 *c* is also consistent with the presence in the IR spectrum of bands at 1,378 and 1,386 cm^{-1} (See Fig. 1). The average dimensions of the cochleate cylinders in Fig. 3 *b* are 3,000 by 30,000 Å, which are comparable to the single large structure shown in Fig. 3 *c*.

Effect of Mg^{2+} on PS

The electron micrograph of a DMPS/Mg^{2+} system (Fig. 3 *d*) (with mole ratio of 1:3) displays a large cochleate structure. The dimensions of this structure are 12,000 by 36,000 Å, substantially larger than those for the DMPS/Ca^{2+} systems reported above. IR studies of varying DMPS/Mg^{2+} molar ratios, analogous to the DMPS:Ca^{2+} investigations, reveal similar shifts in the

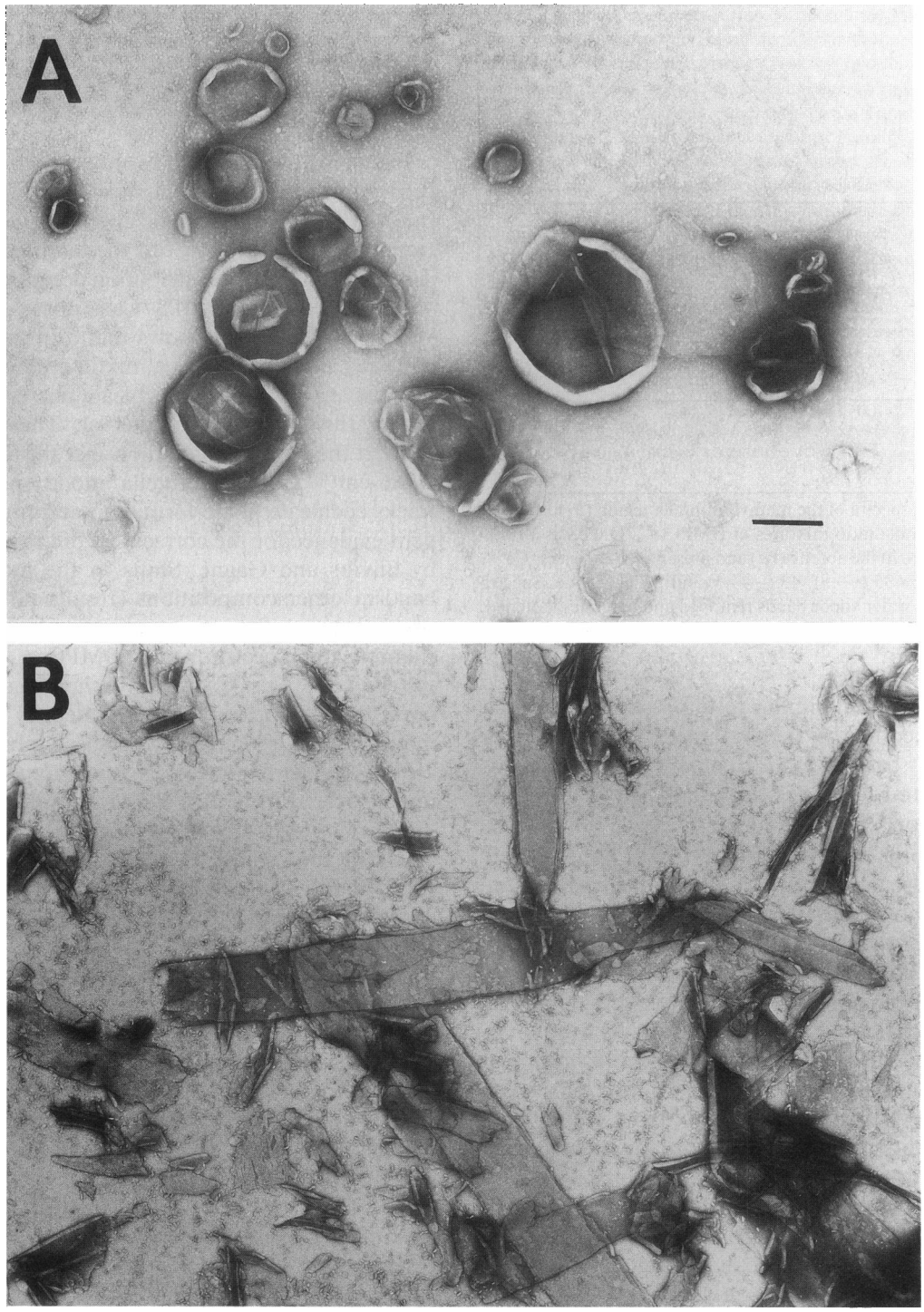

FIGURE 3 Negative stain electron micrographs of (*A*) Ca^{2+}-free DMPC/DMPS 50:50 mole ratio vesicles (bar = 3,000 Å); (*B*) 50:50 system with excess Ca^{2+} (bar = 4,300 Å); (*C*) DMPS/Ca^{2+} system with mole ratio of 1:0.1 (bar = 5,500 Å); (*D*) DMPS/Mg^{2+} mole ratio of 1:3 (bar = 3,400 Å).

methyl umbrella frequency and intensity (results not shown). However, a greater cation/lipid mole ratio in the Mg^{2+}-containing than in the Ca^{2+}-containing system is required to induce the shift from 1,378 to 1,386 cm^{-1}. The appearance of the 1,386-cm^{-1} band in the Ca^{2+} system is evident at a DMPS/Ca^{2+} mole ratio of 1:0.1 (Fig. 1), whereas in the Mg^{2+} system a comparable shift is not observed until the mole ratio is between 1:0.3 and 1:0.6.

Dehydration precedes cochleate formation

IR spectra of the asymmetric PO_2^- double bond stretching band region (1,220–1,250 cm^{-1}) are presented in

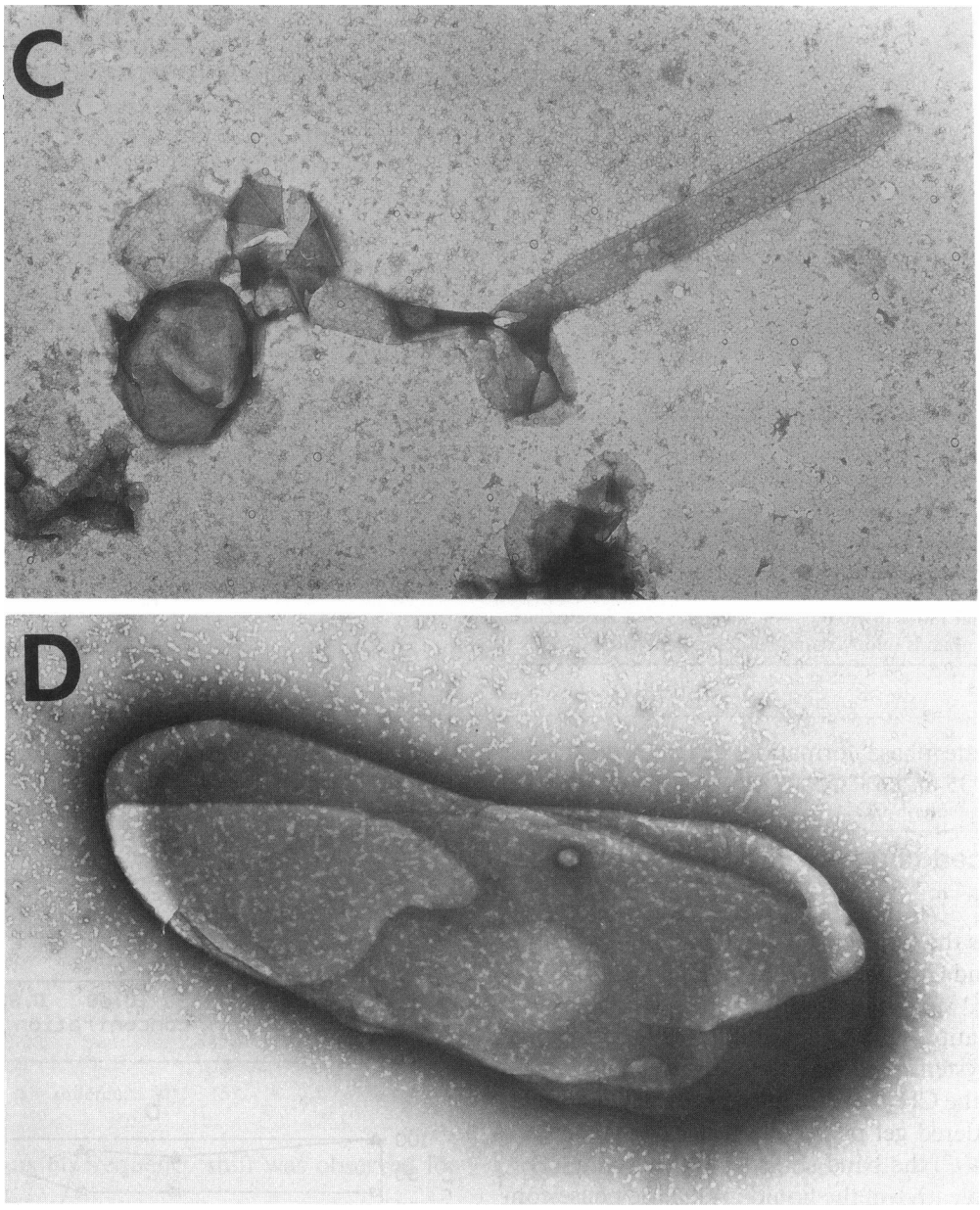

FIGURE 3 (continued)

Fig. 4 and provide information about the hydration state of the phosphate group (11). A frequency of 1,229 cm^{-1} in the Ca^{2+}-free DMPS sample is characteristic of a fully hydrated phosphate group, whereas in the DMPS/Ca^{2+} systems shown, the band observed at ~1,236 cm^{-1} reveals dehydration of this group. The PO_2^- frequency observed in the DMPS/Ca^{2+} system of 1:0.05 mole ratio is nearly identical to the frequency observed in the 1:1 system, indicating that dehydration occurs at low Ca^{2+} concentrations.

Fig. 5 *a* displays the frequency of the PO_2^- band (*right-hand ordinate*) and the relative area percentage of the 1,378- and 1,386-cm^{-1} methyl umbrella modes (*left-hand ordinate*) as a function of the Ca^{2+} concentration in pure DMPS systems. As discussed above, the decrease in the area percentage of the 1,378-cm^{-1} band and concomitant increase in the 1,386-cm^{-1} band as Ca^{2+} levels increase denotes the formation of cochleate structures. The DMPS/Ca^{2+} mole ratio at which the maximum change in area percent (cochleate formation) occurs is between 1:0.05 and 1:0.3, whereas the increase in the PO_2^- frequency has reached its maximum by 1:0.05, indicating that dehydration precedes cochleate formation. This conclusion is further strengthened by examination of the frequency of the PO_2^- asymmetric stretch in the DMPC-d_{54}/DMPS 50:50 system with Ca^{2+} as a function of temperature shown in Fig. 5 *b*. The PO_2^- band is found at a frequency indicating dehydration at all temperatures studied (including the lamellar phases predominant below 35°C), whereas the 1,386-cm^{-1} band indi-

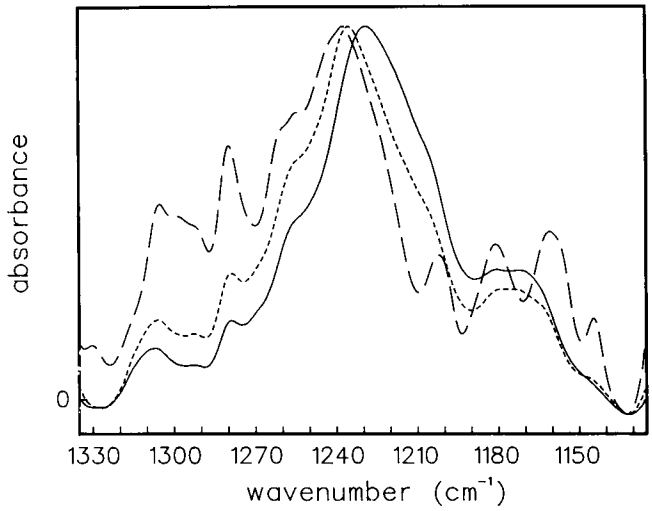

FIGURE 4 FT-IR spectra of the asymmetric PO_2^- double bond stretching region (1,250–1,220 cm^{-1}) as a function of Ca^{2+} concentration at room temperature. The Ca^{2+}-free system is represented (——) and DMPS/Ca^{2+} molar ratios of 1:0.05 - - - and 1:1 ——. Spectra have had residual water bands removed and baselines flattened.

cating cochleate phase formation becomes significant only between 35 and 43°C.

Ca^{2+}-induced changes in acyl chain ordering

Table 1 shows the temperature dependence of the symmetric CH_2 and CD_2 stretching frequencies in Ca^{2+}-free and lipid/Ca^{2+} systems with a mole ratio of 1:3 and provides information about acyl chain conformational order in the system (11). For the Ca^{2+}-free DMPS system at 15°C, the CH_2 band at 2,849.7 cm^{-1} is characteristic of an ordered gel phase (all-*trans* conformation), whereas at 50°C, the band shifts to 2,852.6 cm^{-1} (Δ = 2.9 cm^{-1}), indicative of the liquid crystalline phase containing gauche conformers in the hydrocarbon chains. In contrast, the CH_2 stretching frequency for the cochleate DMPS/Ca^{2+} system is found to be independent of the temperatures from 0 to 50°C (Δ = 0 cm^{-1}) and is furthermore indicative of an ordered phase throughout. Dluhy et al. (11) reported similar results for temperature studies of bovine PS/Ca^{2+} systems.

In DMPC-d_{54}/DMPS 50:50 mixtures, with and without Ca^{2+}, the results obtained were consistent with those observed for the fully proteated mixtures, with the added advantage that the individual components could be monitored from the C-D and C-H stretching bands, respectively. Lateral phase separation is suggested because the absence of a temperature-induced frequency shift in the CH_2 stretching band of the PS component in the Ca^{2+}-containing sample signifies that all the PS exists in a single, ordered state, whereas the change in the C-D stretching frequency shows that at least some of the DMPC-d_{54} component undergoes a transition to a disordered phase. When fully proteated DMPC/DMPS systems were studied, a temperature-dependent shift in the CH_2 frequency for the Ca^{2+}-containing sample was observed (Table 1). The magnitude of the frequency shift is less (Δ = 1.9 cm^{-1}) than that for Ca^{2+}-free samples during the gel-to-liquid crystal phase transition. This can be explained by the coexistence of PC-rich liquid crystalline and PS-rich cochleate phases, consistent with the phase diagram of Silvius and Gagné (16). Finally, the values for the CD_2 stretching mode in the 50:50 system DMPC-d_{54}/DMPS/Ca^{2+} as a function of temperature indicate that some of the PC exists in the cochleate structure. The change in frequency (Δ in Table 1) for the Ca^{2+}-containing ternary system is less than that for the gel to liquid-crystalline transition in the Ca^{2+}-free system. In contrast, at most a small fraction of the PS exists in the

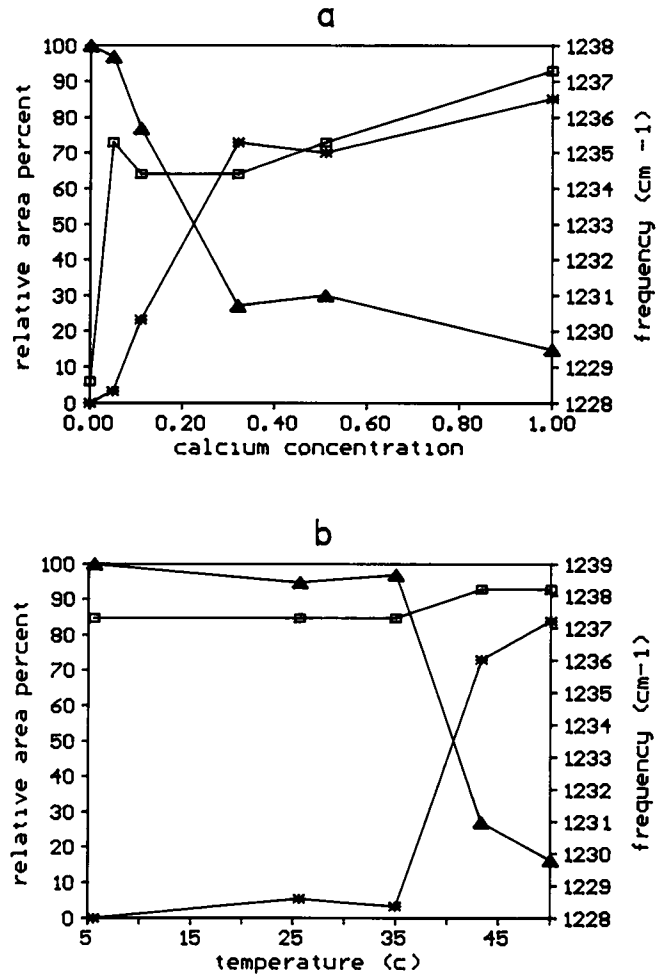

FIGURE 5 Relative area percentage of the 1,378- (▲) and 1,386- (*) cm^{-1} methyl umbrella modes (*lefthand ordinate*) and the frequency of the PO_2^- band (□, *righthand ordinate*) as a function of (*a*) Ca^{2+} concentration in pure DMPS. Ca^{2+} concentration is displayed as lipid/Ca^{2+} mole ratio where moles of lipid equals 1; and (*b*) temperature (°C) for DMPC-d_{54}/DMPS 50:50 system with excess Ca^{2+}.

TABLE 1 CH_2 and CD_2 symmetric stretching frequencies

System	CH_2 frequency			CD_2 frequency		
	15°C	50°C	Δ cm^{-1}	15°C	50°Δ	Δ cm^{-1}
DMPS	2,849.7	2,852.6	2.9	NA	NA	NA
DMPS/Ca^{2+}	2,849.7	2,849.7	0	NA	NA	NA
DMPC – d_{54}/DMPS	2,850.6	2,853.6	3.0	2,089.8	2,096.5	6.7
DMPC – d_{54}/DMPS/Ca^{2+}	2,850.6	2,850.6	0	2,088.8	2,093.6	4.8
DMPC/DMPS	2,849.7	2,852.6	2.9	NA	NA	NA
DMPC/DMPS/Ca^{2+}	2,849.7	2,851.6	1.9	NA	NA	NA

Values are in cm^{-1}. Δ = difference 50° − 15°.

lamellar phase, as no frequency shift was observed for that component.

DISCUSSION

The most important result of the current study is the observation of an IR spectroscopic marker, which appears unique for cochleate cylindrical phases. The methyl umbrella frequency increases from 1,378 to 1,386 cm^{-1} upon a Ca^{2+}-induced transition of a gel or liquid crystal phase to cochleate cylinders. This is a surprising finding, because the methyl umbrella mode has previously been assumed to be insensitive to phospholipid structural alterations. We have examined other phospholipid phases and found no similar increase. For example, the gel-liquid crystal phase transition causes at most a 1-cm^{-1} increase in this mode, whereas the L_α-H_{II} interconversion leaves the methyl umbrella mode essentially unchanged. Also, the frequency increase has not been observed in interdigitated phases or in micelles (R. Mendelsohn and C. Flach, unpublished results). The frequency increase can be mimicked easily enough by increasing the force constant for this vibration from the value given by Snyder and Schachtschneider (20, 21), but because the potential energy distribution of the mode is not 100% symmetric methyl deformation, it is possible that vibrational coupling to other internal coordinates changes upon cochleate formation, producing the observed frequency shift.

X-ray diffraction coupled with freeze-fracture electron microscopy revealed a difference in low-angle spacing between multilamellar vesicles and cochleate cylinders in systems containing varying percentages of PS and DPPC with 20 mM Ca^{2+} (8). Multilamellar vesicles yielded spacings of 69–75 Å, consistent with hydrated bilayers, whereas the cochleate cylinder lamellar repeat of 53 Å indicates tightly packed, poorly hydrated bilayers. Tight packing in cochleate cylinders presumably changes the environment of the terminal methyl groups, causing increased interaction (repulsion), which may contribute to the observed shift to higher energy of the umbrella mode.

Cochleate cylinders are reportedly formed after vesicle aggregation and fusion has occurred (reference 2, and current electron microscopy results). Thus, the observation of cochleates suggests that fusion has taken place. Fusion studies in more complex systems, perhaps involving proteins, pH gradients, or other ions, can be performed with IR spectroscopy without the extensive sample modifications required for other techniques such as electron microscopy. The current approach ought to provide a useful complement to the well-established energy transfer methods used to quantitatively assay the extent of fusion. IR data can also provide useful structural correlates of phase behavior as deduced from experiments (such as DSC) that do not yield direct molecular structure information.

The simultaneous availability of three IR markers of structural changes possibly relevant to the fusion process (i.e., the PO_2^- asymmetric stretch to monitor dehydration, the methyl umbrella mode to monitor cochleate phase formation, and the CH_2 or CD_2 stretching frequencies to monitor acyl chain order) permits us to address directly several issues relevant to fusion mechanisms. For instance, the ability of Ca^{2+} to induce the formation of a dehydrated complex has been demonstrated (4, 5, 22, 23). IR studies as reported in Fig. 5, *a* and *b*, indicate the additional power of the approach in deducing the sequence of events.

Perdeuteration of one lipid class in IR experiments involving binary lipid mixtures permits the simultaneous evaluation of chain order and phase behavior for each lipid component as well as the qualitative determination of the composition of each phase in the system. For example, the results presented in Table 1 indicate that significant levels of PC are present in the cochleate phase induced by Ca^{2+} in the ternary system, whereas little if any PS is present in the coexisting lamellar phase. Hui et al. (8) suggested that in 40/60 mixtures of bovine brain PS with PC, 30% of the PC is in the cochleate phase. In previous studies of C-H and C-D stretching modes in phospholipids, it has been determined that changes in the C-D stretching frequency are not linear functions of the extent of conversion between ordered and disordered phases (24). Therefore, quantitative com-

parisons between the current work and the studies of Hui are not appropriate until more sophisticated models for analysis of C-H (C-D) stretching data become available.

Mg^{2+}-induced fusion of PS containing membranes has been found to be dependent on vesicle size and on the degree of unsaturation in the lipid acyl chains. Limited fusion takes place in systems containing small unilamellar but not large unilamellar vesicles (25, 26). In the current work, saturated DMPS was used to produce the Mg^{2+}-induced cochleate structure shown in Fig. 3 *d*, which possessed a shifted methyl umbrella vibrational mode, implying that fusion has taken place. Mg^{2+}-induced fusion in saturated PS systems as opposed to unsaturated may be related to the molecular area at the lipid/water interface (3, 27). Casal et al. (27) report that the binding affinity of Mg^{2+} to PS decreases as the molecular area increases.

The large dimensions of the Mg^{2+}-induced cochleate may be due to incomplete dehydration leaving trapped water molecules between the bilayers. The Ca^{2+}/PS complex is reported to be more dehydrated than similar Mg^{2+} structures (28, 29). Studies of the synergistic effects of Ca^{2+} and Mg^{2+} on fusion suggest that the presence of the latter may have biological significance even for membranes with unsaturated lipids. Mg^{2+} promotes aggregation and the close apposition of membranes which lowers the Ca^{2+} threshold required for fusion (22, 30).

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