Fourier Transform Infrared Spectroscopic Studies of the Effect of Calcium Ions on Phosphatidylserine†

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ABSTRACT: Fourier transform infrared (FT-IR) spectroscopy is used to investigate the complex conformational changes that occur as phosphatidylserine (PS) binds to calcium. The spectra confirm the isothermal crystallization of the hydrocarbon chains in the PS–Ca^{2+} complex. However, in contrast to differential scanning calorimetry, which detects no phase transitions under 100 °C in PS–Ca^{2+} complexes, several FT-IR parameters detect structural changes at 30–40 °C in these complexes analogous to those observed in solid–solid phase transitions of alkanes. Site symmetry splitting observed in the PO_2^- bands suggests that Ca^{2+} binds to the PS phosphate as a bidentate ligand; in addition, Ca^{2+} causes a dehydration of the phosphate ester. No evidence is found for the specific chelation of Ca^{2+} by the ionized carboxylate group or the dehydration of this group; instead, the carboxylate exists in an immobilized conformation in the presence of Ca^{2+}. Splitting of the degenerate vibrations of the carbonyl group at the interfacial region suggests different rotational chain isomers in the Ca^{2+} complex and the possibility of hydrogen bonding with trapped interstitial water.

The molecular mechanism by which biological membranes fuse has received extensive investigation in recent years. Due to the complexity of natural membranes, the large majority of these studies have used pure or binary lipid mixtures as membrane models. The acidic phospholipid phosphatidylserine has been the subject of much of the study in this area.

Phosphatidylserine (PS)[1] has previously been shown to strongly bind certain divalent cations to its head-group area (Poste & Allison, 1973). The calcium ion, in particular, induces a large structural reorganization which seems to be the equivalent of an isothermal phase transiton from the liquid-crystalline to the crystalline state (Papahadjopoulos et al., 1977). In addition, it has been proposed that the effect of Ca^{2+} is to induce a close approach of two apposing PS vesicles by displacement of water from the interbilayer space and the formation of an anhydrous, trans, PS–Ca^{2+} complex spanning the interbilayer space (Portis et al., 1979). These events are postulated to occur as precursors to true membrane fusion. Several proposals relating these observations to the mechanism of membrane fusion have appeared (Wilschut et al., 1981; Ohki, 1982).

As a means of studying model membranes, spectroscopic methods have had considerable success in elucidating the conformational behavior of phospholipids. A wide variety of techniques such as NMR, ESR, fluorescence depolarization, infrared spectroscopy and Raman spectroscopy have provided detailed molecular information on lipid structure [see Grell (1982) and references cited therein]. However, compared with the extensive literature on phosphatidylcholines and phosphatidylethanolamines, relatively little spectroscopic work has been done on phosphatidylserines. Browning & Seelig (1980) studied selectively deuterated saturated PS's and showed distinct motional differences in the head-group region between PS's and PC's or PE's. Kurland et al. (1979) used ^{31}P NMR to show a rigid conformation for the phosphate ester when Ca^{2+} is bound to PS, consistent with the X-ray and NMR results of Hauser et al. (1975, 1977). ^{31}P NMR was also used by Cullis & Verkleij (1979) to detect the phase separation of PS–PE mixtures upon addition of Ca^{2+}.

Several studies employing Raman spectroscopy have appeared which focused on the structure of the acyl chains in PS–Ca^{2+} complexes (Hark & Ho, 1979, 1980). The data showed that the addition of Ca^{2+} caused highly rigid acyl chains. In addition, mixed vesicles of PS and PC showed evidence of lateral phase separation in the presence of Ca^{2+}.

The work described here uses Fourier transform infrared spectroscopy to investigate the complex conformational changes that occur as PS binds to Ca^{2+}. It is shown that in contrast to DSC, which detects no phase transition under 100 °C in PS–Ca^{2+} complexes, infrared spectroscopy detects subtle structural changes at 30–40 °C in these complexes analogous to those observed in solid–solid phase transitions of alkanes or fatty acids. In addition, spectral changes observed in the head-group region are consistent with the bidentate binding of Ca^{2+} to the phosphate ester, leading to a rigid, immobilized, partially hydrated structure for the head-group region.

Materials and Methods

Materials. Bovine brain phosphatidylserine was purchased from Avanti Biochemicals (Birmingham, AL) and stored in chloroform solution under nitrogen at −40 °C. Lipid concentrations were determined by phosphorus assay (Ames & Dubin, 1960). All lipids gave a single spot on TLC plates.

Sample Preparation. Multilamellar lipid vesicles were prepared as follows. Aliquots of the lipid chloroform solution containing the desired amounts of lipid were placed in a test tube, and the solvent was removed by N_2 drying followed by overnight evacuation. The lipid film was rehydrated in a buffer of 100 mM NaCl, 2 mM Tes, 2 mM His, and 0.1 mM EDTA, pH 7.4, by vortexing. The lipid multilayers were heated above the phase transition and cooled to ensure complete dispersion. This cycle was repeated several times. Calcium was added

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[1] Abbreviations: FT-IR, Fourier transform infrared; PS, bovine brain phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; NMR, nuclear magnetic resonance; ESR, electron spin resonance; DSC, differential scanning calorimetry; Tes, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; His, L-histidine; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; DPPC, dipalmitoylphosphatidylcholine.

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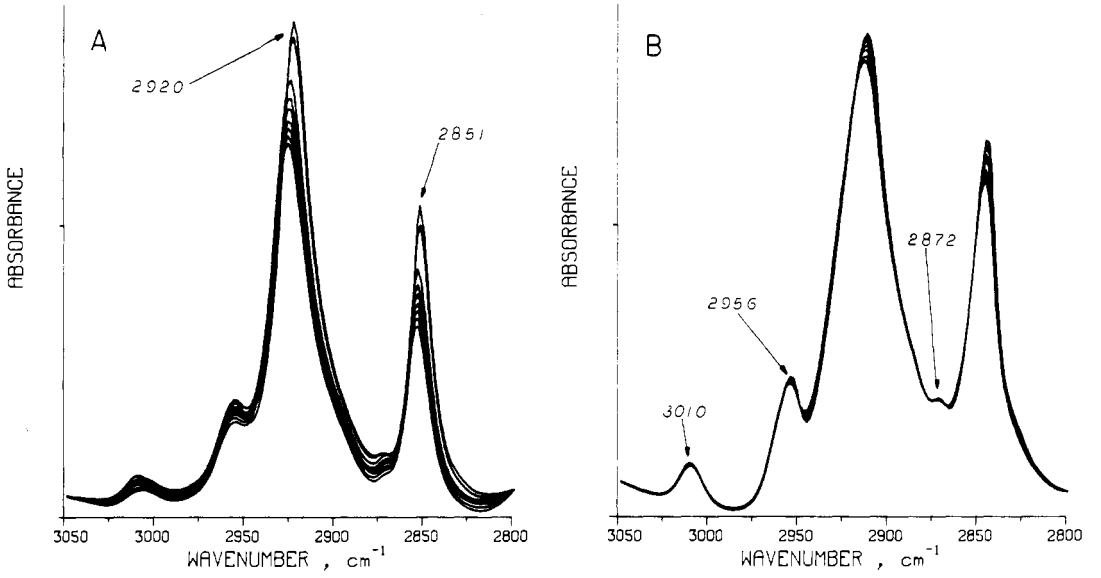

FIGURE 1: Temperature dependence of the infrared spectra of the C–H stretching region for (A) pure PS multilayers and (B) a PS–Ca^{2+} complex. Spectra decrease in peak height with increasing temperature and are plotted in intervals of ~4 °C over the range 5–50 °C. For the purpose of display, a linear base line extending from 3050 to 2800 cm^{-1} has been subtracted. The assignment of the bands to particular vibrational modes is given in the text.

by mixing the PS multilayer suspension with an equal volume of 100 mM NaCl, 2 mM Tes, 2 mM His, and 100 mM $CaCl_2$, pH 7.4, at room temperature. Typically, the final concentration of both PS and Ca^{2+} in the complex was 50 mM. Due to the interfering infrared absorption of H_2O, samples were also made up in D_2O buffers. In these cases, the samples were prepared exactly as described above with the exception that the buffers were adjusted to pD 7.4 in D_2O.

Fourier Transform Infrared Spectroscopy. Samples were prepared for infrared spectroscopic analysis in 50 μM thick cells with CaF_2 windows. Spectra were recorded on a Digilab FTS-11 Fourier transform infrared spectrometer equipped with a HgCdTe detector. Six hundred interferograms, collected with an optical velocity of 1.26 cm s^{-1} and a maximum optical retardation of 0.25 cm, were coadded, apodized with a triangular function, and Fourier transformed with one level of zero filling to yield a resolution of 2 cm^{-1} and data encoded every 1 cm^{-1}.

Temperatures were controlled by a thermostated EtOH–H_2O mixture flowing through a hollow cell mount and monitored by a copper–constantan thermocouple placed against the edge of the cell window (Cameron & Jones, 1981).

Frequencies were determined with an uncertainty of less than ±0.05 cm^{-1} by using a center of gravity algorithm (Cameron et al., 1982). Since the maximum uncertainty in bandwidth values is a function of the signal to noise ratio of the spectra (Cameron et al., 1982), given the high signal to noise ratio of the spectra presented here we expect errors in the bandwidth values to be of the same magnitude as those of the frequencies.

Results

Acyl Chain Modes: C–H Stretching Region. The temperature dependence of the infrared spectra of the C–H stretching region for pure PS and the PS–Ca^{2+} complex is shown in Figure 1. The observed spectral features have been previously assigned to (a) the asymmetric and symmetric C–H stretching modes of the acyl chain methylene groups at 2920 and 2850 cm^{-1}, respectively, (b) the asymmetric and symmetric C–H stretching modes of the terminal methyl groups at 2956 and 2872 cm^{-1}, respectively (Snyder et al., 1982), and (c) the C–H stretching mode of alkenic hydrogens at 3010 cm^{-1}

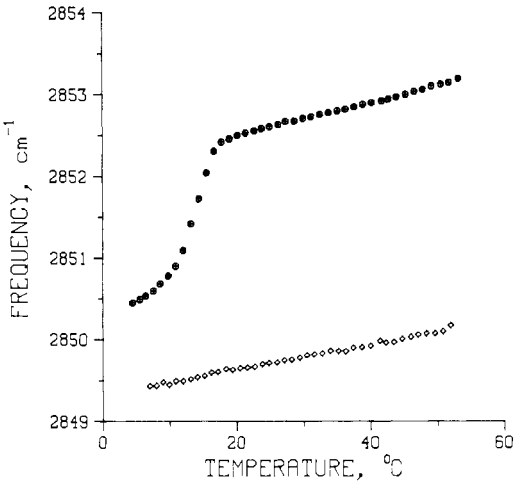

FIGURE 2: Plots of frequency vs. temperature for the symmetric CH_2 stretching band at 2850 cm^{-1} in pure PS multilayers (⊕) and a PS–Ca^{2+} complex (◊).

(Bellamy, 1975). The latter mode results from the high degree of unsaturation of the bovine PS chains (Papahadjopoulos & Miller, 1967).

The infrared spectral parameters normally used to characterize lipid phase transitions in the hydrocarbon region are the frequencies and bandwidths of the C–H methylene stretching bands (Cameron et al., 1980a). Each of these parameters is sensitive to various aspects of lipid conformation and mobility. The CH_2 stretching frequencies respond primarily to conformational disorder and increase with the introduction of gauche bonds while the lines broaden as a result of the increasing motional freedom of the methylene groups (Snyder et al., 1978).

The temperature dependence of the frequency and half-width of the 2850-cm^{-1} band for pure PS and the PS–Ca^{2+} complex are shown in Figures 2 and 3, respectively. Both the frequency and half-width parameter for pure PS multilayers reflect, at approximately 10–16 °C, the phase transition of pure PS as seen by DSC (Jacobson & Papahadjopoulos, 1975). The phase transition temperature of native, bovine PS as seen in the infrared melting curves occurs over a much wider temperature interval than those of synthetic, saturated lipids,

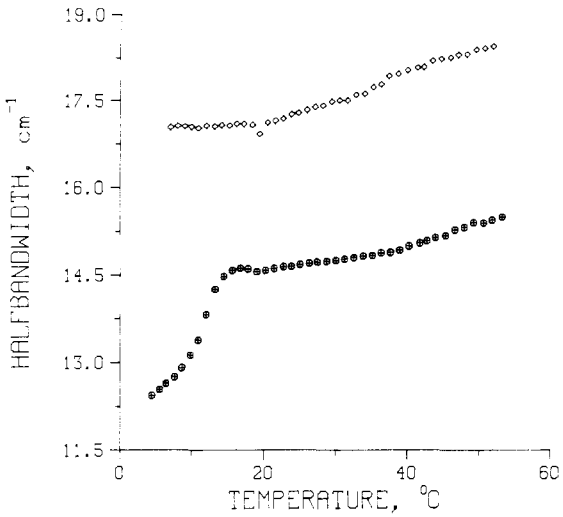

FIGURE 3: Plots of the full width at half-maximum peak height vs. temperature for the symmetric CH_2 stretching band at 2850 cm^{-1} in pure PS multilayers (⊕) and a PS–Ca^{2+} complex (◊).

reflecting the heterogeneity of the PS acyl chains.

With the addition of Ca^{2+} to the PS multilayers, the spectra change dramatically. As seen in Figure 2, the frequency of the symmetric CH_2 stretching mode has been reduced from 2850.5 to 2849.4 cm^{-1} in the gel phase of the PS–Ca^{2+} complex and remains essentially invariant with temperature. This low frequency is indicative of highly ordered, all-trans hydrocarbon chains. Figure 3 shows the half-width of the same band in the spectrum of the PS–Ca^{2+} complex as a function of temperature. In this case, the half-width at low temperature has increased from 12 to 17 cm^{-1} and does not significantly change with temperature. However, we note that in the spectrum of the PS–Ca^{2+} complex both CH_2 stretching bands are much more asymmetric than in the spectra of the pure PS complex, with the bands being broader on the high-frequency side (Figure 1B). We would expect absorptions in the region, i.e., at frequencies slightly higher than that of extended all-trans methylene segments, from both (a) methylenes adjacent to HC=CH groups and (b) regions where the packing is slightly less rigid due to perturbations introduced by the HC=CH groups. Consequently, we ascribe the broadening to the heterogeneity of the sample, such broadening being emphasized when the packing is more rigid and hence most intolerant of perturbing factors.

CH_2 Scissoring Modes. The acyl CH_2 scissoring bands are located in the region 1450–1480 cm^{-1}. The intense, narrow absorption at 1468 cm^{-1} results from the out of plane CH_2 scissoring mode of the fully extended acyl chain while the underlying weak bands arise from the deformation modes of the glycero and head-group methylene groups (Cameron et al., 1980b).

Figure 4 shows the temperature dependence of the bandwidth of the CH_2 scissoring band in the spectra of PS in the presence (Figure 4B) and absence (Figure 4A) of Ca^{2+}. Clearly, the spectra of pure PS reflect the phase transition at 10–16 °C that was evident in the spectra of the C–H stretching region (see above). However, unlike the C–H stretching region, the behavior of the CH_2 scissoring band in the PS–Ca^{2+} complex also indicates a transition occurring at approximately 30 °C.

The CH_2 scissoring region of the infrared spectra of hydrocarbon chain assemblies is known to be sensitive to structural differences in hydrocarbon chain packing (Stein & Sutherland, 1953, 1954). For lipids, factor-group splitting and specific frequencies of the CH_2 scissoring band have been used

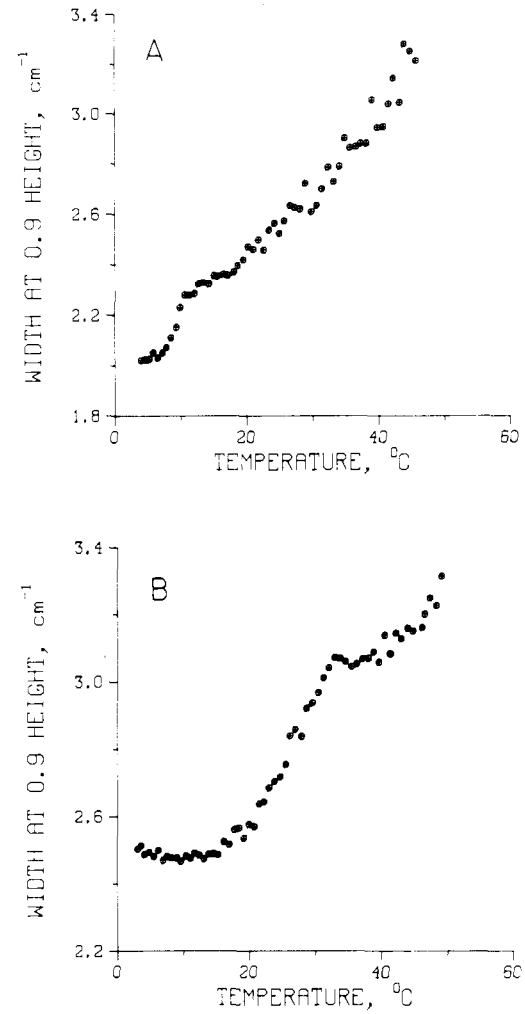

FIGURE 4: Plots of the full width at nine-tenths peak height vs. temperature for the CH_2 scissoring mode at 1468 cm^{-1} in (A) pure PS multilayers and (B) a PS–Ca^{2+} complex.

to distinguish acyl chain packing characteristics in the gel phase (Cameron et al., 1981; Cameron & Mantsch, 1982). We observe no splitting of this band in the spectra of the PS–Ca^{2+} complex. The band frequency is located at 1468 cm^{-1}; this indicates that the acyl chains are packed in a hexagonal subcell. The minor nature of the observed transition is likely due to the heterogeneity of the acyl chains in bovine PS. The distribution of acyl chain structural isomers due to the heterogeneity of the chains is also the most likely cause of the greater width of the contour in the spectra of the complex. However, bandwidth increases have been observed for the related methylene rocking band in long-chain alkanes while in the solid phase (Casal et al., 1982). Figure 2 clearly indicates the acyl chains in the PS complex to be highly ordered structures. The observations of a transition occurring in the crystalline chains of the PS–Ca^{2+} complex suggest that the structural rearrangement may be analogous to the solid–solid phase transitions observed in various hydrocarbon chain systems. An indication of the reproducibility of this transition comes from the fact that it was observed in three independent samples of PS–Ca^{2+} complexes. Such solid–solid phase transitions in long-chain aklanes and fatty acids have been observed by using infrared spectroscopy (Casal et al., 1982; Fischmeister, 1975; Maroncelli et al., 1982; Zerbi et al., 1981).

Interfacial Region: Carbonyl Stretching Vibrations. Figure 5A presents the infrared spectrum of pure PS in the carbonyl stretching region 1680–1780 cm^{-1}. The two principle features at 1742 and 1728 cm^{-1} are assigned to the C=O stretching

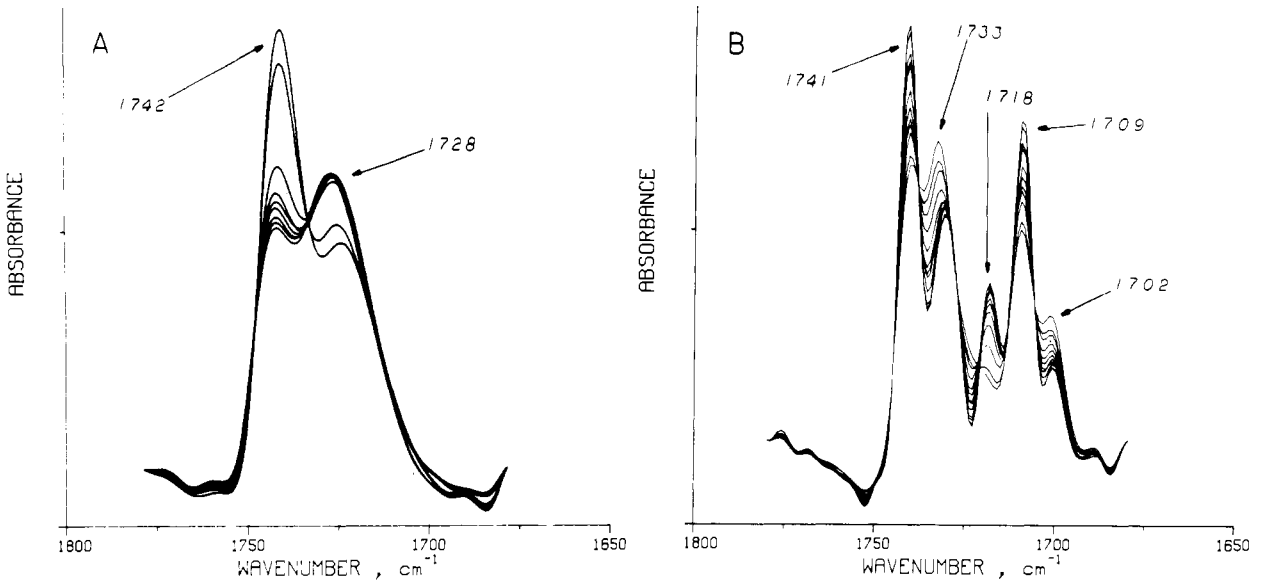

FIGURE 5: Temperature dependence of the infrared spectra of the C=O stretching region for (A) pure PS multilayers and (B) a PS-Ca^{2+} complex. The spectra are plotted in temperature intervals of ~4 °C over the range 5–50 °C. In (A), the band at 1742 cm^{-1} decreases in peak height with increasing temperature while the height of the band at 1728 cm^{-1} increases. For (B), the bands at 1733 and 1702 cm^{-1} increase in peak height with increasing temperature; all others decrease. The assignment of these bands to particular vibrational modes is given in the text. For the purpose of display, linear base lines extending from 1780 to 1680 cm^{-1} have been subtracted. Spectra have been Fourier self-deconvoluted with a 10-cm^{-1} half-width Lorentzian line and smoothed to K = 1.8 with a Bessel function (Kauppinen et al., 1981a,b).

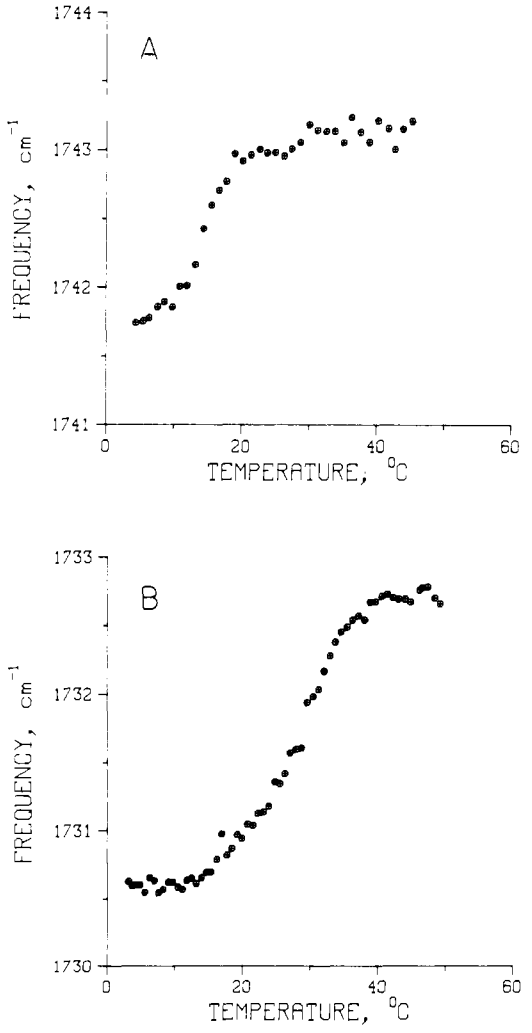

FIGURE 6: Plots of frequency vs. temperature for (A) the band at 1742 cm^{-1} in the spectrum of pure PS and (B) the band at 1733 cm^{-1} in the spectrum of the PS-Ca^{2+} complex.

modes of the carbonyl groups corresponding to the conformationally inequivalent *sn*-1 and *sn*-2 acyl chains, respectively (Bush et al., 1980; Mushayakarara & Levin, 1982; Mushayakarara et al., 1982).

Figure 6A shows the temperature dependence of the frequency parameter for the 1742-cm^{-1} band associated with the *sn*-1 chain. The phase transition for pure PS is reflected in the frequency shift of this peak between 8 and 16 °C.

The effect of Ca^{2+} on the carbonyl groups of PS is shown in Figure 5B. At least five bands are apparent in the region 1680–1780 cm^{-1}. The appearance of splitting in the infrared spectra of carbonyl bands has been observed previously in a low-temperature study of DPPC (Bush et al., 1980). The current interpretation suggests these bands arise from site-symmetry splitting. That is, when a molecular unit has its conformational freedom reduced by entering the crystalline state, its effective symmetry is also lowered because of intermolecular interactions. This reduction in symmetry may result in the splitting of degenerate vibrations and the introduction of new bands (Decius & Hexter, 1977). In the case of lipids, these new features have been attributed to individual rotational isomers of the acyl chains (Bush et al., 1980).

As noted, the frequencies at which the carbonyl bands appear are used to distinguish the conformation about the individual ester groups. Mushayakarara & Levin (1982) have suggested that the frequency range 1727–1744 cm^{-1} corresponds to the nearly all-trans carbonyl of the *sn*-1 chain. Due to the gauche bonds which allow the *sn*-2 chain to pack parallel to the *sn*-1 chain, the frequency of the *sn*-2 carbonyl band is lowered to the range 1716–1727 cm^{-1}.

Figure 5B shows that in addition to the frequencies mentioned, the spectrum of the PS-Ca^{2+} complex contains bands at 1709 and 1702 cm^{-1}. These frequencies fall outside the range normally observed for the carbonyl bands of PC's and PE's (Umemura et al., 1980; Mantsch et al., 1981). However, on the basis of Raman spectra of model compounds, frequencies in this range have been assigned to hydrogen bonding of the acyl chain carbonyl group which lowers the vibrational frequency (Mushayakarara & Levin, 1982). In addition, the infrared spectra of long-chain fatty acids in solution, a system known to be hydrogen bound at the carbonyl group, contain carbonyl absorptions at 1708 cm^{-1} (Sinclair et al., 1952;

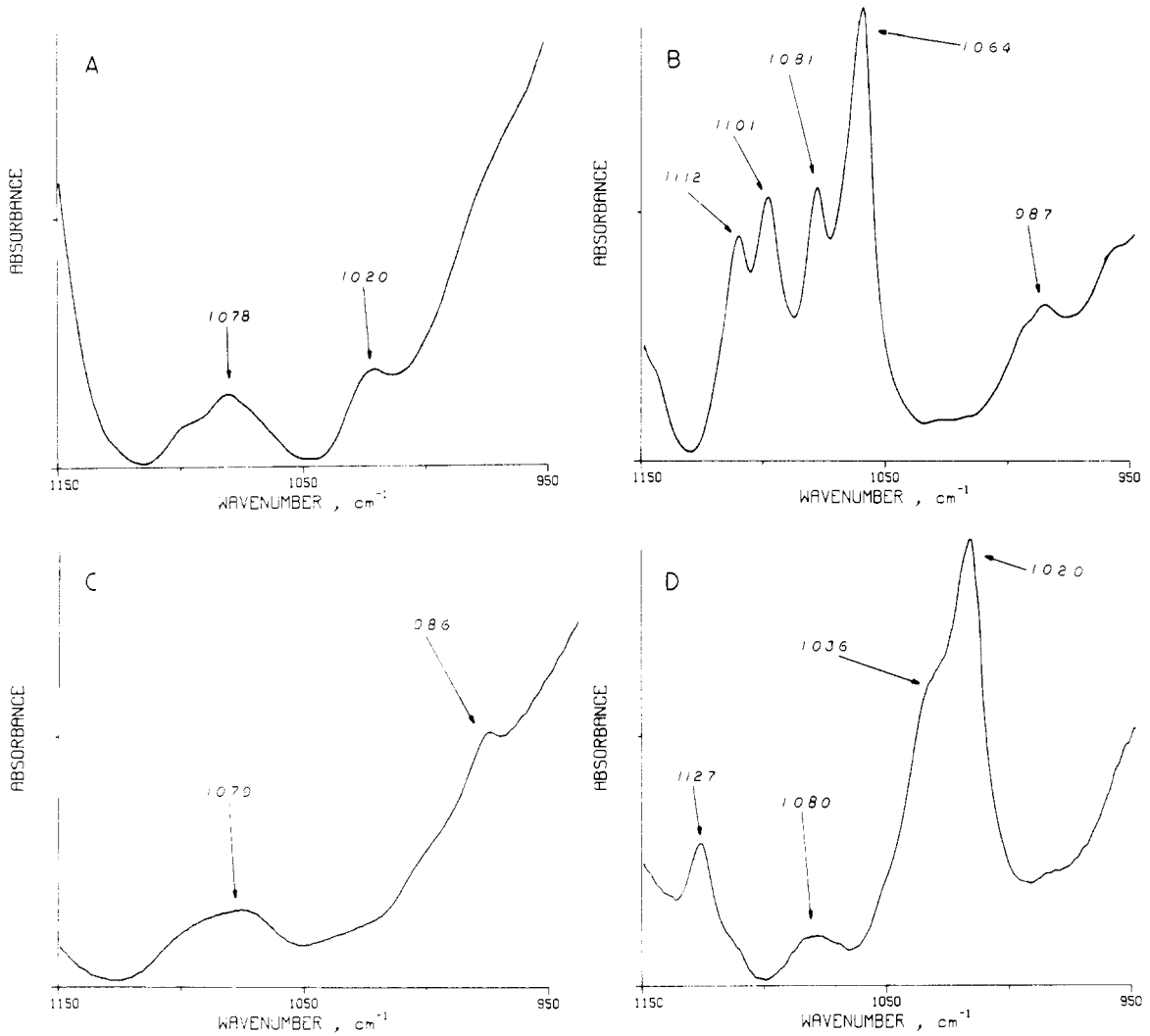

FIGURE 7: Infrared spectra of the symmetric PO_2^- stretching region between 1150 and 950 cm^{-1} in (A) pure PS multilayers, (B) a PS–Ca^{2+} complex, (C) 0.1 M K_2HPO_4 in 0.1 M NaCl–2 mM Tes–2 mM His–0.1 mM EDTA, and (D) 0.1 M $CaCl_2$ plus 0.1 M K_2HPO_4 [same buffer as in (C)].

Bellamy, 1975). Therefore, we tentatively assign the bands at 1709 and 1702 cm^{-1} to hydrogen-bound carbonyl groups in the PS–Ca^{2+} complex.

As described in the previous section, the bandwidth of the CH_2 scissoring mode provides evidence for a solid–solid phase transition occurring at 30 °C in the PS–Ca^{2+} complex. Figure 6B shows that the band at 1733 cm^{-1} in the spectrum of the PS–Ca^{2+} complex also reflects this transition.

Head Group Region: Phosphate Group Vibrations. The asymmetric and symmetric PO_2^- double-bond stretching bands occur at 1220 and 1080 cm^{-1}, respectively (Shimanouchi et al., 1964). Although these are the strongest infrared absorptions in the fingerprint region, studies of PC's and PE's have shown them to be almost invariant with temperature (Fringeli & Gunthard, 1981).

Figure 7A shows the region 950–1150 cm^{-1} of the spectrum of pure PS multilayers containing the PO_2^- symmetric stretching band at 1078 cm^{-1} superimposed upon a broad D_2O background. As with the PC's and PE's, no significant change in this band occurred with increasing temperature (data not shown). Figure 7B shows the same region in the spectrum of a PS–Ca^{2+} complex. Significant changes have occurred in the spectrum. The broad band centered at 1078 cm^{-1} in the spectrum of pure PS has split into four bands located at 1112, 1101, 1081, and 1064 cm^{-1} in that of the PS–Ca^{2+} complex.

These spectral changes may be interpreted by comparison with a simple model system. Figure 7C shows the 950–1150-cm^{-1} region of the spectrum of 0.1 M K_2HPO_4 in D_2O containing the symmetric PO_2^- stretching band. The PO_4^{3-} ion belongs to the high symmetry point group T_d (Cotton & Wilkinson, 1972; Lincoln & Stranks, 1968). Of the fundamental vibrational modes, only ν_3 and ν_4 are infrared active. When a metal cation coordinates to the free negatively charged oxygens, the symmetry is lowered by complex formation to C_{3v} or C_{2v}, and the degenerate vibrations are split (Lincoln & Stranks, 1968). This principle has been used extensively to determine the mode of coordination of acid anions such as NO_3^-, SO_4^{2-}, and CO_3^{2-} in inorganic coordination compounds (Nakamoto, 1978).

Figure 7D shows the effect on the PO_2^- stretching region of adding 0.1 M $CaCl_2$ to 0.1 M K_2HPO_4. By analogy with the spectrum of the PS–Ca^{2+} complex in Figure 7B, the lowering of the symmetry of the HPO_4^{2-} ion through coordination with Ca^{2+} has resulted in the splitting of the degenerate modes and the introduction of previously infrared-inactive vibrations. As in the case of the PS–Ca^{2+} complex, four bands are now apparent in the spectrum. In contrast with HPO_4^{2-}, the spectrum of 0.1 M $CaCl_2$ plus 0.1 M KH_2PO_4 is indistinguishable from that of 0.1 M KH_2PO_4 alone (data not shown). Therefore, the fact that only the dibasic anion HPO_4^{2-}, and not the monobasic anion $H_2PO_4^-$, reproduces the vibrational splitting in the presence of Ca^{2+} suggests that the Ca^{2+} ion is bound to the PS phosphate ester as a bidentate ligand.

The asymmetric PO_2^- stretching band between 1220 and

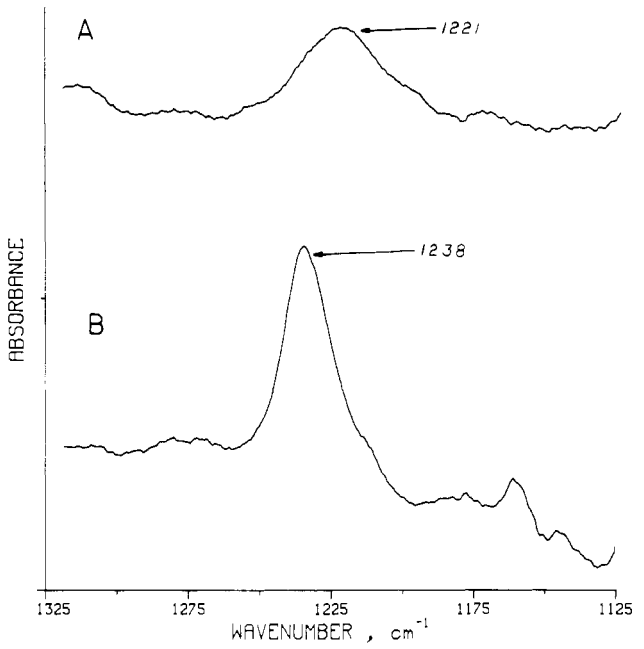

FIGURE 8: Infrared spectra of the asymmetric PO_2^- stretching region between 1325 and 1125 cm^{-1} in (A) pure PS multilayers and (B) a PS-Ca^{2+} complex.

1240 cm^{-1} provides further information about the environment of the phosphate ester. Figure 8A shows the band at 1221 cm^{-1} in the spectrum of the pure PS multibilayers while Figure 8B shows it at 1238 cm^{-1} in the spectrum of the PS-Ca^{2+} complex. A frequency of 1221 cm^{-1} is characteristic of a fully hydrated phosphate group, while a band at 1238 cm^{-1} is observed when the group is dehydrated (Fringeli & Gunthard, 1981). Together with the decreased bandwidth and increased intensity, the frequency shift indicates that the addition of Ca^{2+} results in dehydration of the phosphate group.

In contrast with the symmetric PO_2^- stretching band of pure PS, which shows no temperature-dependent changes, the analogous band at 1112 cm^{-1} in the spectrum of the PS-Ca^{2+} complex does undergo a nonlinear, temperature-induced transition centered at approximately 36 °C (data not shown). The observation of a head-group rearrangement in the PS-Ca^{2+} complex suggests that all regions of the PS molecule (acyl chain, interfacial, and head group) reflect a solid-solid phase transition between 30 and 40 °C.

Carboxylate Stretching Mode. The asymmetric stretching vibration due to the free COO^- group in the hydrophilic region of PS occurs at 1623 cm^{-1} and is shown in Figure 9A. Monitoring the frequency and bandwidth shows that the carboxylate group also senses the phase transition of pure PS (Figure 10A,B).

The effect of Ca^{2+} ions on the asymmetric carboxylate band in the spectrum of PS is shown in Figure 9B. The main effect is a substantial decrease in the width of the band, the frequency remaining constant. The temperature dependencies of these parameters are plotted in Figure 10A,B.

Recent proposals of the mode of action of Ca^{2+}-induced fusion of PS vesicles have postulated the displacement of interbilayer water by the Ca^{2+} in (Hauser et al., 1977). To test whether the observed spectral changes in the carboxyl region of the head group in the PS-Ca^{2+} complex are due to dehydration, an experiment was performed in which PS from a $CHCl_3$ solution was deposited on a CaF_2 plate and placed in the sample chamber of an FT-IR spectrometer under greatly reduced pressure. After several hours at 0.5 T, the frequency of the carboxylate band in the spectrum of the minimally hydrated PS was measured at 1640 cm^{-1} (data not shown). Since the frequency of the carboxylate band in the PS-Ca^{2+} complex is constant at 1622 cm^{-1}, this indicates a hydrated environment surrounding the COO^- group.

The effect of Ca^{2+} on the carboxylate group of PS, then, seems to be indirect. The temperature-invariant frequency and reduced bandwidth of the COO^- mode indicate that the carboxylate group exists in a hydrated, rigid conformation in the PS-Ca^{2+} complex.

Discussion

Previous reports of the interaction of Ca^{2+} with PS vesicles have shown that the effect of Ca^{2+} is to cause vesicle aggregation and fusion followed by collapse of the bilayers to form the so-called "cochleate lipid cylinders" (Papahadjopoulos et al., 1975; Portis et al., 1979). The mechanism by which Ca^{2+} mediates this fusion process has been postulated to involve the formation of a specific anhydrous complex between Ca^{2+} and the head groups of two opposing bilayers (Portis et al., 1979). Alternatively, an increase in surface tension caused by the

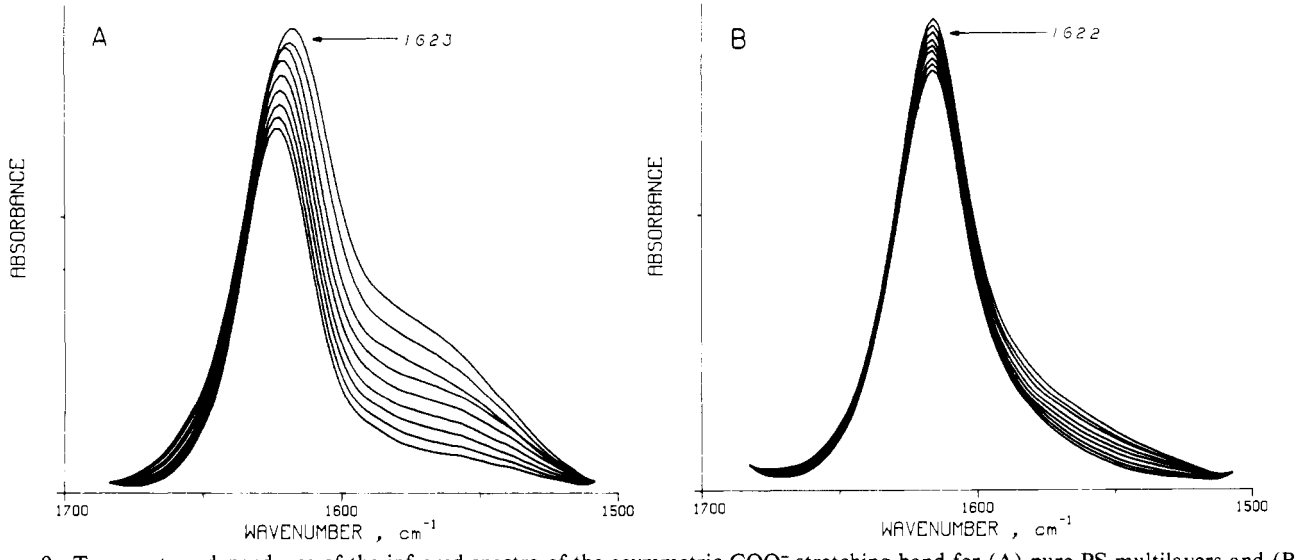

FIGURE 9: Temperature dependence of the infrared spectra of the asymmetric COO^- stretching band for (A) pure PS multilayers and (B) a PS-Ca^{2+} complex. Spectra decrease in peak height with increasing temperature and are plotted in intervals of ~4 °C over the range 5-50 °C. For the purpose of display, a linear base line extending from 1685 to 1510 cm^{-1} has been subtracted.

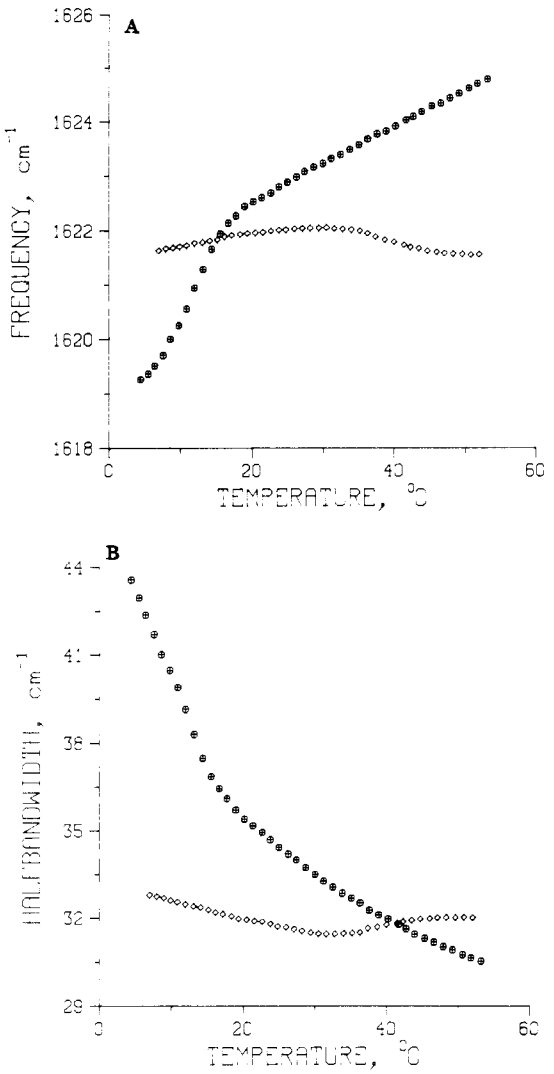

FIGURE 10: (A) Plots of frequency vs. temperature for the asymmetric COO^- stretching band at 1622 cm^{-1} in pure PS multilayers (⊕) and a PS–Ca^{2+} complex (◊). (B) Plots of the full width at half-maximum peak height vs. temperature for the asymmetric COO^- stretching band at 1622 cm^{-1} in pure PS multilayers (⊕) and a PS–Ca^{2+} complex (◊).

increased hydrophobic nature of the membrane surface due to ion binding may induce fusion (Ohki, 1982).

The observations made in the current infrared study regarding the structure of the PS–Ca^{2+} complex may be related to the various proposals that have been advanced to explain the Ca^{2+}-induced fusion of PS vesicles. Three separate aspects of the nature of the PS–Ca^{2+} complex may be examined by the infrared data: (a) the structure of the acyl chains; (b) the mode of binding of Ca^{2+} to the head group; and (c) the extent of hydration of the head group.

The temperature dependence of the acyl chain modes clearly demonstrates that the addition of Ca^{2+} induces an isothermal phase transition in PS membranes from the fluid to the crystalline state (Figure 2), consistent with previous X-ray and microcalorimetry results (Shipeley, 1973; Portis et al., 1979). Although in a highly ordered conformation, the packing arrangement of the chains in the Ca^{2+} complex allows considerable freedom of movement (Figure 3). Previous DSC results have reported the absence of any phase transition at temperatures below 100 °C in PS–Ca^{2+} complexes (Jacobson & Papahadjopoulos, 1975). However, monitoring the CH_2 scissoring region reveals the existence of an acyl chain rearrangement at 30 °C in the Ca^{2+} complex (Figure 4). Since the acyl chains exist in the crystalline state and the bandwidth of the CH_2 scissoring mode senses differences in hydrocarbon chain packing (Stein & Sutherland, 1953, 1954), it is reasonable to propose that this transition is analogous to a solid–solid phase transition of the type observed in solid *n*-alkanes (Maroncelli et al., 1982). This transition in the Ca^{2+} complex is not limited to the acyl chains; carbonyl and phosphate vibrations also reflect a transition occurring at 30–40 °C in the PS–Ca^{2+} complex. It must be emphasized that the observed transition is very minor in character, possibly due to the heterogeneity of the acyl chains and likely differences in packing among the various acyl chain structural isomers. Studies currently in progress of saturated, synthetic PS may clarify the nature of this transition.

The mode of binding of Ca^{2+} to the PS head group has been implicated in the mechanism of fusion (Portis et al, 1979). The current infrared results (Figure 7) demonstrate that the phosphate ester is the site of Ca^{2+} binding, as previously suggested (Kurland et al., 1979; Hauser et al., 1977). In addition, comparison of the infrared band splitting observed in the spectrum of PS with that of a Ca^{2+}–HPO_4^{2-} complex demonstrates that Ca^{2+} coordinates as a bidentate ligand to the phosphate ester (Figure 7B,D). However, it is not possible to discern from the infrared spectra whether the coordination is with a phosphate within the same bilayer or bridging between opposing bilayers. No evidence is seen for the specific chelation of Ca^{2+} to the carboxylate group, but, rather, evidence for the existence of a rigid conformation of the carboxylate in the PS–Ca^{2+} complex is observed (Figure 10A,B). Previous ^{31}P NMR results have shown that the line width of the ^{31}P signal in PS membranes is considerably broadened in the PS–Ca^{2+} complex (Hauser et al., 1977) and has a greatly increased relaxation rate (Kurland et al., 1979). The data indicate that the polar group in the Ca^{2+} complex exists in a rigid, immobilized conformation. These results could explain the immobilization of the carboxylate group without a specific Ca^{2+} coordination.

In addition to chelating with the phosphate ester, Ca^{2+} causes the dehydration of this portion of the head group (Figure 8). This is consistent with previous results that showed the phosphate group to be the site of the main hydration shell of phospholipids (Finer & Darke, 1974). However, the infrared spectrum of PS in a dehydrated film shows that the carboxylate group still remains hydrated in the PS–Ca^{2+} complex. This suggests that Ca^{2+} does not totally dehydrate the interbilayer space but rather raises the possibility of trapped interstitial water in the PS–Ca^{2+} complex. The possibility of some trapped water also is suggested by the spectra of the carbonyl region of the PS–Ca^{2+}+ complex (Figure 5). The bands at 1709 and 1702 cm^{-1} occur at frequencies characteristic of a hydrogen-bonded carbonyl (Mushayakarara & Levin, 1982). Since the splitting of the carbonyl mode indicates the presence of chain rotational isomers (Bush et al., 1980), hydrogen bonding could occur intramolecularly between the carbonyls of various chain isomers, or intermolecularly with trapped interstitital water. The use of isotopically labeled synthetic analogues of bovine PS may lead to a resolution of this question. Such experiments are currently in progress.

Registry No. Ca, 7440-70-2; K_2HPO_4, 7758-11-4.

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