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Calcium Ion Interactions with Insoluble Phospholipid Monolayer Films at the A/W Interface. External Reflection-Absorption IR Studies

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ABSTRACT External reflection Fourier transform infrared (FT-IR) experiments are reported for insoluble monomolecular films of an equimolar mixture of 1,2-dipalmitoylphosphatidylcholine (DPPC) and 1,2-dipalmitoylphosphatidylserine (DPPS) at the A/W interface as a function of surface pressure and Ca^{2+} ion presence. The separate components showed a surface pressure-induced conformational ordering of the acyl chains. The conformational ordering occurred more cooperatively for the DPPS.

Acyl chain perdeuteration of the DPPC permitted the observation of the response of the individual components in the binary mixture to changes in surface tension and to the presence of Ca^{2+}. Plots of surface pressure versus CH_2 or CD_2 stretching frequencies were analyzed with a two-state model. At each surface pressure within the two-state region, the fraction of disordered form was the same for each lipid component, suggesting that they are well mixed on the surface. Calcium ion (5 mM in the subphase) produces almost no effect on the pressure-induced acyl chain ordering of the DPPC in a single component film, whereas the same levels of Ca^{2+} induce acyl chain ordering at all surface pressures in both components of the binary mixture. Thus, unlike the bulk phase mixture of DPPC/DPPS, the binary lipids in this mixed monolayer film appear to retain their miscibility in the presence of Ca^{2+}. Finally, Ca^{2+}-induced dehydration of the phosphate group was observed through characteristic frequency shifts in the asymmetric PO_2^- stretching mode.

INTRODUCTION

Insoluble monomolecular films of phospholipids at the air/water (A/W) interface are widely used as model systems for the study of lipid structure and lipid-protein interaction in biological membranes (Phillips and Chapman, 1968; Möhwald, 1990; McConnell, 1991; Pérez-Gil et al., 1992; Hui and Yu, 1993). They also engender substantial interest in their own right as prototypes for understanding domain formation. Several groups, in particular those of McConnell (von Tscharner and McConnell, 1981; McConnell et al., 1984) and Möhwald (Lösche et al. 1985; Kjaer et al., 1987; Helm et al., 1987; Möhwald, 1988) and their respective co-workers, have developed the technologies of epifluorescence microscopy and synchrotron x-ray diffraction for investigating a variety of film properties including lateral pressure-induced phase transitions, domain formation, and orientational and positional order. In particular, epifluorescence micrographs have directly demonstrated the simultaneous coexistence of distinct domains of ordered and disordered phospholipids during the isothermal, surface pressure-induced, liquid-expanded to liquid-condensed monolayer interconversion.

To extend the elegant experimental approaches noted above to obtain direct molecular structure information, Dluhy and co-workers have developed the technique of external reflection Fourier transform infrared (FT-IR)[1] spectroscopy for the in situ examination of phospholipid films at the A/W interface (Dluhy and Cornell, 1985; Dluhy et al., 1988a, 1988b). They have demonstrated the feasibility of detecting the conformation-sensitive acyl chain CH_2 symmetric stretching vibrations that monitor the liquid crystal to gel state interconversion that takes place during compression of films of disaturated acyl chain phosphatidylcholines (PCs) (Mitchell and Dluhy, 1988). They have also demonstrated the feasibility of detecting the vibrational modes of the phosphate moiety, thereby facilitating studies of ion binding to the polar regions of the PCs (Hunt et al., 1989). In a collaborative study, this laboratory and Dluhy's have reported the occurrence of a surface pressure-induced phase transition in films of pulmonary surfactant (Dluhy et al., 1989). To extend the applicability of this technique into the domain of protein structure, our laboratory has described the miniaturization of the surface balance-FT-IR spectrometer combination that permits detection of the conformation-sensitive Amide I vibration of peptide or protein monolayer films on a D_2O subphase (Flach et al., 1993).

In the current work, external reflection FT-IR studies of insoluble monolayer films are extended in three ways. First, the applicability of the method to the study of the effect of Ca^{2+} ion on the individual components in mixed phospholipid films is demonstrated. This is achieved through perdeuteration of the acyl-chain of one of the components in the lipid mixture. The approach permits the separate examination of CD_2 or CH_2 stretching modes and hence conformational order of the individual film components. Second, we demonstrate the applicability of a two-state model which facilitates comparison of FT-IR conformational order information with the domain information directly observable from epifluorescence microscopy. Finally, we demonstrate the Ca^{2+}-induced dehydration of the phosphate group at the A/W interface in a mixed phospholipid monolayer film.

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[1] *Abbreviations used:* DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPS, 1,2-dipalmitoylphosphatidylethanolamine; FT-IR, Fourier transform infrared.

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EXPERIMENTAL PROCEDURES

Materials

1,2-Dipalmitoylphosphatidylcholine (DPPC), 1,2-dipalmitoylphosphatidylserine (DPPS), and acyl-chain perdeuterated DPPC (DPPC-d_{62}) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). EGTA (97% pure) was 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.

Surface films

Phospholipid samples were prepared by dissolving appropriate weights of the powdered lipids in a $CHCl_3$:MeOH (3:2, v/v) solution while stirring and heating gently, as needed. The aqueous subphase was a Tris buffer (10 mM Tris, 100 mM NaCl, pH 7.4) containing either 0.1 mM EGTA or 5 mM $CaCl_2$ prepared with double-distilled H_2O. The surface balance, described elsewhere (Boyle and Mautone, 1982), consisted of a Teflon trough, a 150-mesh stainless steel screen for changing surface area, and a roughened platinum sheet which served as a Wilhelmy plate. All were cleaned prior to use by soaking for 15 min in a chromic acid solution (Chromerge, Fisher Scientific, Fairlawn, NJ). In addition, the platinum plate was roughened, rinsed, and flamed. The cleanliness of the surface balance and subphase were verified before proceeding by raising and lowering the screen, while monitoring surface tension. When a constant surface tension value of 72.7 ± 1 dyne/cm was achieved, monolayers were spread. Monolayers were formed by spreading aliquots of the phospholipid solution (typically 15–25 μl) on the aqueous surface with a gas-tight syringe containing a Teflon-coated plunger while increasing the surface area to its maximum, ~68.00 cm^2. 10 min were allowed for solvent evaporation before film compression was initiated. Pressure-area isotherms were collected as monolayers were compressed at a rate of ~2.44 cm^2/min to the trough's minimum surface area of 19.21 cm^2. Films were expanded and then step-wise compressed to preset surface tension values as external reflection FT-IR spectra were acquired.

Reflection-absorption FT-IR spectroscopy

Details of the surface balance/IR spectrometer combination used in this study have been described elsewhere (Flach et al., 1993). The spectrometer used was a Bio-Rad (Digilab) FTS 40A, equipped with an external narrow band mercury-cadmium-telluride detector. External reflection IR spectra were produced by the coaddition of 512 interferograms collected at an angle of incidence centered at 30° (beam spread ±4°), taken at 4 cm^{-1} resolution, apodized with a triangular function, and Fourier-transformed with one level of zero-filling to produce spectral data encoded at 2-cm^{-1} intervals. When necessary, residual water vapor bands were removed, using an appropriate reference spectrum and manufacturer-supplied software. Baselines were flattened and spectra smoothed using a breakpoint of 0.8 for the determination of asymmetric PO_2^- double bond stretching frequencies by transferring data to a microcomputer and using software supplied by D. Moffatt of the National Research Council of Canada (Ottawa).

Theoretical considerations

IR modes detected via reflectance-absorbance spectroscopy may have slightly distorted band shapes and frequency maxima shifted to lower wavenumbers compared to pure absorbance spectroscopy. An absorbance spectrum depends on the extinction coefficient, k, of the absorber. If the absorber is spread as a film on a substrate, the reflectance-absorbance of the system depends on the optical constants, that is, the extinction coefficient, k, and the refractive index, n, of the film and the substrate. Since the refractive index of the film exhibits anomalous dispersion around an absorption maximum, a skewed reflectance-absorbance results.

A simulated reflectance-absorbance band (McIntyre, 1973) for the CH_2 symmetric stretching mode is shown in Fig. 1. The actual optical constants of water (substrate) in the region shown were used (Downing and Williams, 1975). The extinction coefficient of the film was taken to be Lorentzian in shape, while the refractive index has the derivative-like shape dictated by the Kramers-Kronig relation. The shift in the center of gravity of the band on going from transmission to reflection-absorption is 0.7 cm^{-1}. In addition, the lineshape is significantly asymmetric. Although the magnitude of the shift in frequency is small, the CH_2 stretching mode changes only by a few cm^{-1} during a lipid phase transition. Thus the observed shift is a significant fraction of the range available to the parameter. In addition, the band asymmetry led us to conclude that single symmetric Gaussian or Lorentzian functions would not produce good fits to the contour. Thus a different approach involving the actual experimental lineshape was used in the two-state model (see Results).

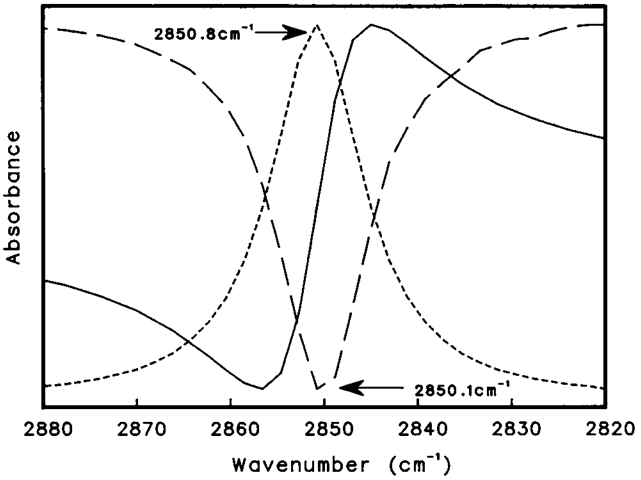

FIGURE 1 Spectral simulations. The optical constants for the film, i.e., n_2, the refractive index (——) and k_2 the extinction coefficient (- - - -) are plotted, along with the simulated reflectance-absorbance band (– – –) for the CH_2 symmetric stretching mode of a monolayer film at the air-water interface as a function of frequency (cm^{-1}). Assumed values: ν_{max} = 2850.8 cm^{-1}, full width at half-height = 12.0 cm^{-1}, n_2 = 1.5 and k_2 = 0.255. The optical constants for water were taken from Downing and Williams (1975). The peak maximum of the simulated reflectance-absorbance band is shifted to 2850.1 cm^{-1}.

RESULTS

Single component monolayers of DPPS and DPPC-d_{62}

As a basis for understanding surface pressure- and subphase Ca^{2+}-induced changes in mixed monolayer films composed of DPPS and DPPC-d_{62}, it was necessary to examine the individual components in some detail. Typical data for the CH_2 stretching vibrations of a monomolecular film of DPPS on Ca^{2+}-free buffer are shown in Fig. 2. Spectral data at various surface pressures are overlaid and plotted as reflectance-absorbance $A = -\log(R/R_o)$, where R_o is the reflectance from the air-buffer interface and R is the reflectance when the aqueous surface is covered with the lipid monolayer. Negative peaks are observed, as anticipated from the Fresnel equations with appropriate optical constants for data plotted in this manner (McIntyre, 1973; Dluhy, 1986). Changes in the frequencies of both the symmetric (2850 cm^{-1}) and asymmetric (2920 cm^{-1}) CH_2 stretching vibrations are evident as the surface pressure is altered. The fre-

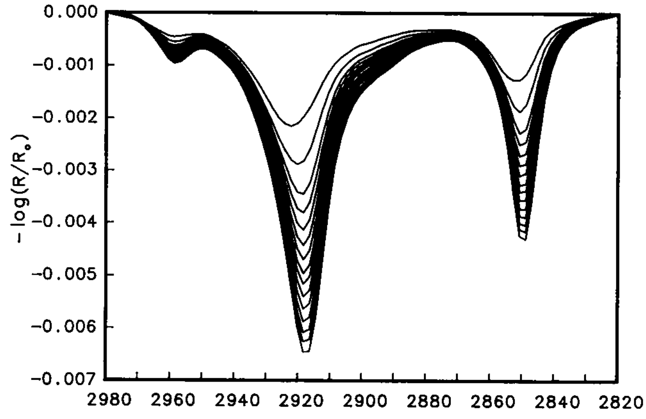

FIGURE 2 Overlaid external reflectance FT-IR spectra of the CH_2 stretching region obtained during the intermittent compression of a DPPS monolayer on Ca^{2+}-free buffer. Data are plotted as $-\log(R/R_o)$, where R is the single-beam reflectance spectrum of the lipid film at the A/W interface and R_o is the single-beam reflectance spectrum of the aqueous subphase. The spectrum containing the smallest negative peaks was obtained at a constant surface pressure of 3 dyne/cm and the spectrum with the largest at 37 dyne/cm. Spectra were baseline-corrected.

quency change in the 2920 cm^{-1} mode is plotted as a function of surface pressure (bottom abscissa) and approximate molecular area (top abscissa) in Fig. 3. These data, collected from three independent experiments, each covering a slightly different range of surface areas, are typical of the reproducibility of the current apparatus. The frequency decreases from 2924.7 to 2917.2 cm^{-1} as the molecular area decreases from 99 to 46 Å^2/molecule, and surface pressure increases from 1 to 38 dyne/cm. The CH_2 (CD_2) stretching frequencies in phospholipids have been widely used to monitor conformational ordering of the acyl chains in lipid bilayers. Snyder et al. (1978) have shown that a frequency decrease results from conformational ordering in the hydrocarbon chains, although quantitative correlations between the magnitude of the shifts and the extent of ordering have remained elusive. The observed decrease in frequency as surface pressure is increased thus reflects an increase in acyl chain conformational ordering in the DPPS monolayer. The ordering occurs over a narrow range of surface area. That is, 85% of the measured frequency shift occurs during compression of the film from 100 to 70 Å^2/molecule (surface pressure change from 1 to 16 dynes/cm).

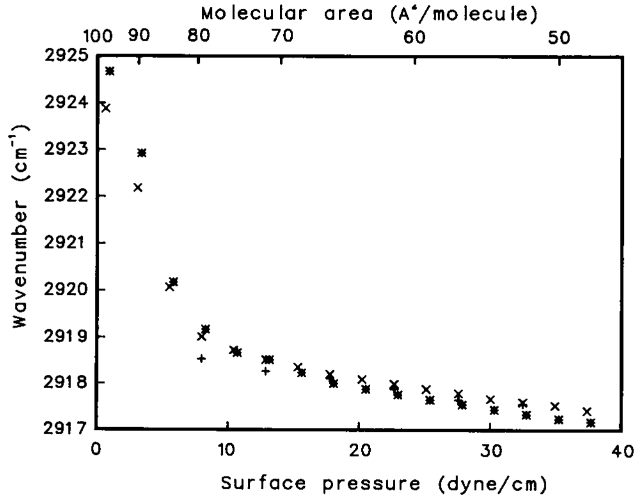

FIGURE 3 Frequency (cm^{-1}) of the asymmetric CH_2 stretch as a function of surface pressure in dyne/cm (*bottom abscissa*) and of approximate molecular area in Å^2/molecule (*top abscissa*) for a DPPS monolayer on a Ca^{2+}-free buffer. The data from three independent intermittent compression curves are plotted.

Fig. 4 displays the frequency variation of the CD_2 asymmetric stretching vibration with surface pressure in DPPC-d_{62} monolayers on an aqueous buffer in the absence and presence of calcium ions. The observed frequency decrease with increasing surface pressure in PC monolayers is independent of the presence of Ca^{2+}, and reflects conformational ordering of the acyl chains. Hunt et al. (1989) report similar results for both pressure-area and frequency-area curves of DPPC-h_{62} monolayers on subphases of pure H_2O and H_2O containing 50 mM Ca^{2+}. The ordering process in the Ca^{2+}-free DPPC-d_{62} monolayer is much less cooperative, that is, it occurs over a broader surface pressure range than that observed for the DPPS monolayer. A surface pressure change of 30 dyne/cm is required in the Ca^{2+}-free DPPC to induce a percentage of frequency shift similar (85% of the total) to that observed in the DPPS system with a pressure change of 16 dyne/cm. In contrast, pressure-area isotherms and external reflection FT-IR results for DPPS indicate that, in the presence of 5 mM Ca^{2+}, film spreading is incomplete and suggests PS-aggregation on the surface (results not shown).

Effects of Ca^{2+} on mixed monolayers of DPPS/DPPC-d_{62}

Fig. 5 *a* shows the surface pressure-% surface area curve for the intermittent compression of the mixed monolayer, Ca^{2+}-free system. At each point shown in the figure, a spectrum

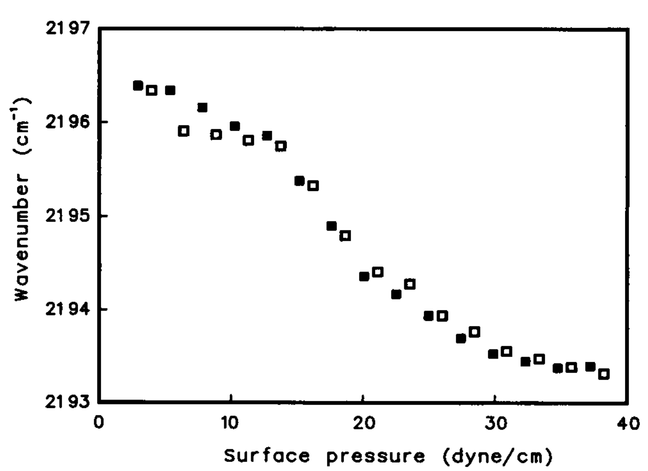

FIGURE 4 Frequency (cm^{-1}) of the asymmetric CD_2 stretch as a function of surface pressure (dyne/cm) for a DPPC-d_{62} monolayer on Ca^{2+}-free (□) and 5 mM Ca^{2+}-containing (■) buffer.

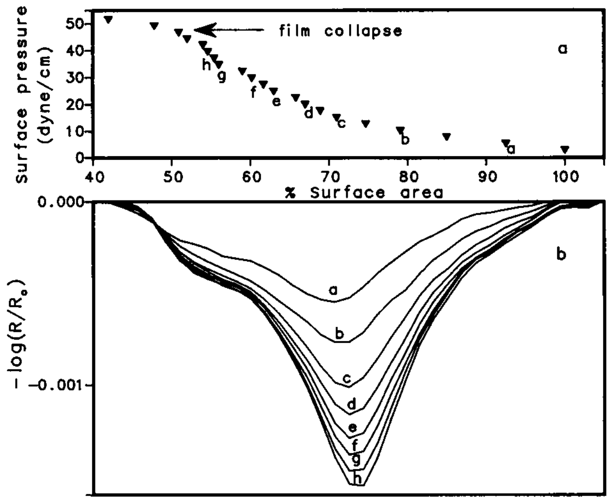

FIGURE 5 (*a*) The intermittent compression curve for an equimolar monolayer of DPPS/DPPC-d_{62} on Ca^{2+}-free buffer, plotted as surface pressure (dyne/cm) as a function of % surface area. (*b*) Overlaid external reflectance FT-IR spectra of the asymmetric CD_2 stretching region (DPPC-d_{62} component) of the same monolayer. Spectra labeled *a–h* are acquired at corresponding data points shown in the compression curve (*a*).

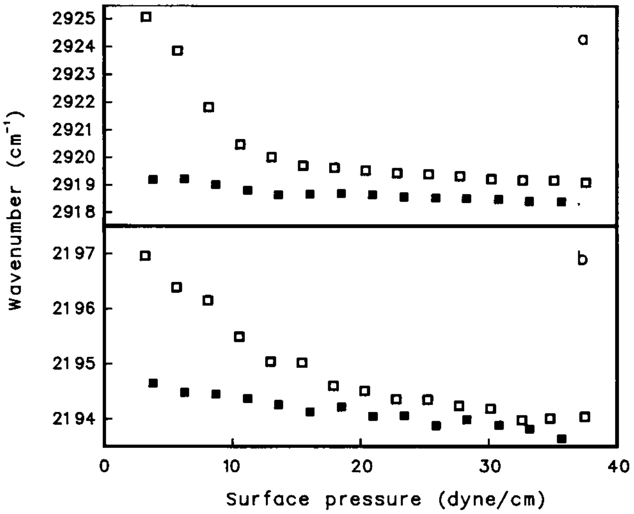

FIGURE 6 Frequency (cm^{-1}) as a function of surface pressure (dyne/cm) for: (*a*) the CH_2 asymmetric stretch (DPPS component) and (*b*) the CD_2 asymmetric stretch (DPPC-d_{62} component) of an equimolar DPPS/DPPC-d_{62} monolayer on Ca^{2+}-free (□) and on 5 mM Ca^{2+}-containing (■) buffer.

was acquired. Data labeled *a–h* refer to the spectra of the CD_2 asymmetric stretching region (DPPC-d_{62} component) in the same monolayer as shown in Fig. 5 *b*. As surface pressure increases, the peak intensity increases from 0.5 to 1.6 milliabsorbance units (mA) and the CD_2 stretching frequency decreases from 2196.4 to 2193.9 cm^{-1}. The CH_2 asymmetric stretch of the DPPS component behaves in a similar fashion (data not shown). The intensity increase is primarily due to an increase in the number of molecules per unit area on the surface. Changes in molecular orientation (tilt angle) with surface pressure increases and the Fresnel reflection coefficients as a function of film thickness may also effect intensity (Downing and Williams, 1975; Dluhy et al., 1986). The noise level in the spectrum displayed for the lowest pressure (peak intensity 0.5 mA) demonstrates the signal-to-noise ratio (about 50 at this signal level) realized with current instrument design.

The surface pressure dependence of the CH_2 and CD_2 asymmetric stretching modes in binary lipid monolayers in the presence and absence of subphase Ca^{2+} are displayed in Fig. 6, *a* and *b*, respectively. In the absence of Ca^{2+}, both the CH_2 and CD_2 stretching frequencies are strongly dependent on surface pressure, whereas in the presence of Ca^{2+}, the two film components display slightly different dependencies. The CH_2 frequency shifts (PS component) for the Ca^{2+}-free binary system (Fig. 6 *a*) from 2925.1 to 2919.1 cm^{-1} as surface pressure increases from 3 to 37 dyne/cm. In the presence of Ca^{2+}, the difference in frequency (2919.2–2918.4 cm^{-1}) is less than 1 cm^{-1} (13% of the available range) over the same pressure interval which indicates an ordered phase throughout. For the DPPC-d_{62} component (Fig. 6 *b*), the frequency shift from 2194.7 to 2193.6 cm^{-1} in the presence of Ca^{2+} is 37% of the total shift for the Ca^{2+}-free system (2197.0–2194.0 cm^{-1}), suggesting that the DPPS component is more ordered than the DPPC-d_{62} in the presence of Ca^{2+} at low surface pressures.

It is evident from Fig. 6 *a* that the CH_2 stretching frequency is higher for the Ca^{2+}-free system than for the Ca^{2+}-containing system at all surface pressures studied, again suggesting that Ca^{2+} effectively orders the DPPS component, even at low surface pressures. The PC component alone does not become ordered at low pressures in the presence of Ca^{2+} (Fig. 4), but requires the combined presence of PS and Ca^{2+} to induce acyl chain order. Quantitative evaluation of these data according to a two-state model is described below.

A two-state model for IR data interpretation

A two-state model for phospholipid acyl chain conformational order in bilayers (bulk phases) has been described and applied to the construction of phase diagrams from FT-IR melting curves for binary lipid systems (Dluhy et al., 1983a, 1985). For this purpose, spectral data collected at temperatures well below or above the thermotropic transition were used to define the frequency and bandwidth of the CH_2 or CD_2 stretching bands for the two physical states (i.e., gel and liquid crystal) at particular temperatures within the two-phase region. Two Lorentzian bands were generated and combined in varying proportions to create a series of simulated spectra that vary in the fraction of disordered component and then are used to optimally match the observed data. An important result of these simulations was that the observed frequencies and bandwidths are not linear functions of acyl chain order. In monolayer systems, epifluorescent micrographs at various surface pressures (McConnell et al., 1984; Peters and Beck, 1983) reveal domain formation corresponding to separate spatial regions containing ordered and disordered phospholipids. These observations indicate that a similar two-state approach may be appropriate for describing the response of the CH_2 or CD_2 stretching frequencies to surface pressure changes in the monolayer.

In considering possible applications of the two-state model to the current experimental data, it was recognized that lineshape distortions arise in the absorption-reflection experiment which preclude the use of symmetric functions (Gaussians, Lorentzians) to simulate band contours (see Theoretical Considerations). Therefore, the complete *experimental* bandshapes observed at the extremes of the surface pressure range are used to represent the two physical states. These are scaled by a relative extinction coefficient, added in varying proportions, and matched with observed bands at intermediate pressures with a least squares procedure yielding the fraction of each state (ordered and disordered) present in the original spectrum at that pressure.

Fig. 7 *a* displays a typical "best fit" result between an observed CD_2 asymmetric stretching band at surface pressure of 38 dyne/cm and a simulated band with an (optimized) fractional disorder of 0.14, whereas Fig. 7 *b* displays overlaid spectra where the fractional disorder is 0.24. The excellent agreement between calculated (optimized) and observed spectra (Fig. 7 *a*) is typical of the success of this data reduction protocol and demonstrates the applicability of the two-state model. The data in Fig. 7 *b* demonstrate the precision of the model.

Fig. 8 displays the fractional disorder for the individual lipid components in single and binary Ca^{2+}-free systems as a function of surface pressure. The fractional disorder for the single component systems demonstrate a higher degree of order in the PS versus the PC system at a comparable surface pressure. For the binary system, the fractional disorders are very similar for each component, indicating that the system is well mixed.

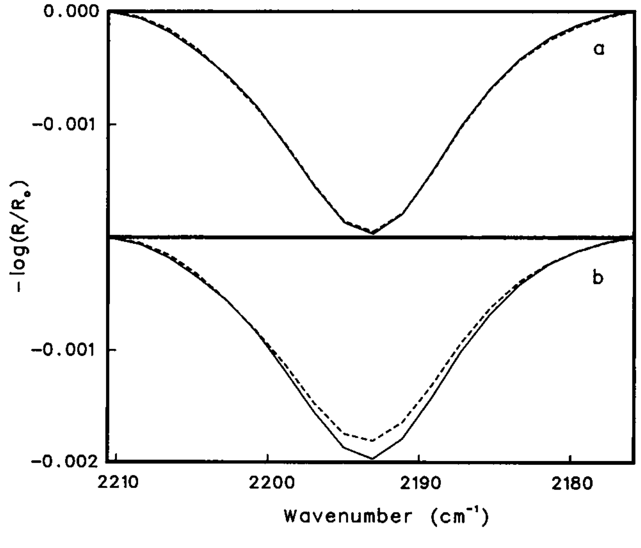

FIGURE 7 Overlaid external reflectance FT-IR spectrum (*solid line*) of the CD_2 asymmetric stretching band at surface pressure of 38 dyne/cm and a two-state model simulated band (*dashed line*). The fractional disorder of the simulated band shown in *a* is 0.14 representing the "best fit", and in *b* is 0.24.

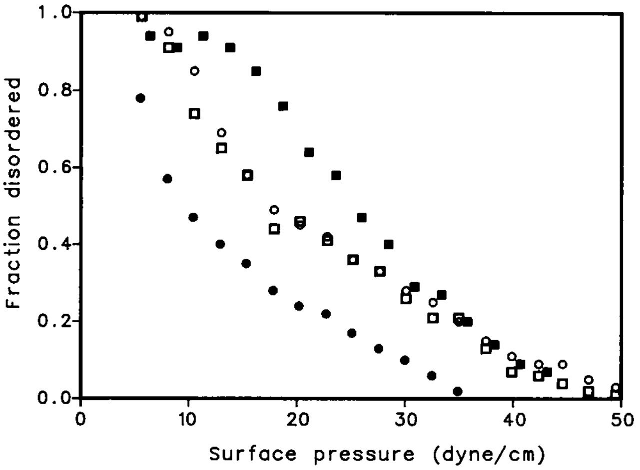

FIGURE 8 Fractional disorder for individual lipid components in single and equimolar DPPS/DPPC-d_{62} Ca^{2+}-free monolayer systems as a function of surface pressure (dyne/cm): pure DPPS (●), pure DPPC-d_{62} (■), DPPS component of mixture (○), and DPPC-d_{62} component of mixture (□).

Effects of Ca^{2+} and surface pressure on the PO_2^- moiety

FT-IR external reflection spectra of the asymmetric and symmetric PO_2^- double bond stretching region (1300–1070 cm^{-1}) for monolayers at a surface pressure of 38 dyne/cm are presented in Fig. 9. The spectra provide information about the hydration state of the phosphate group (Fringeli and Günthard, 1981; Dluhy et al., 1983b). The PO_2^- signal-to-noise ratio in this spectral region is reduced and the PO_2^- asymmetric stretching band has been smoothed prior to obtaining the frequencies reported in Table 1, however, the spectra displayed in Fig. 9 are as collected. The PO_2^- asymmetric stretching frequency (1200–1260 cm^{-1}) increases in the presence of Ca^{2+} for pure DPPC-d_{62} monolayers from ~1224 to 1229 cm^{-1} (Fig. 9 *a*) and mixed DPPS:DPPC-d_{62} monolayers from ~1228 to 1236 cm^{-1} (Fig. 9 *b*). Similar increases are observed in the same lipid systems at lower surface pressures (see Table 1). The larger shift observed in the binary lipid system indicates greater interaction of Ca^{2+} with the PS component of the mixtures. This result presumably reflects the effect of the negative charge in the PS headgroup as compared to the zwitterionic PC headgroup and has been observed in bulk phase systems of similar lipid composition (Flach and Mendelsohn, 1993). The frequency increase is consistent with dehydration of the phosphate. The PO_2^- symmetric stretching band (~1090 cm^{-1}) is observed to broaden in the binary lipid system in the presence of Ca^{2+}, while no change is observed for this mode in the DPPC-d_{62} lipid monolayer. The broadening may correspond to the site symmetry splitting observed for this band in bulk phase PS systems containing Ca^{2+} (Dluhy et al., 1983b).

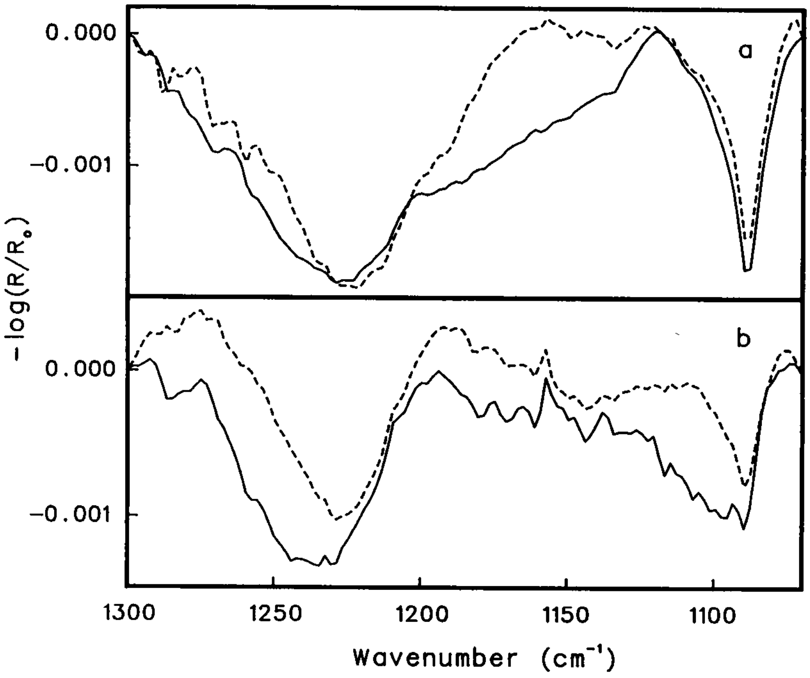

FIGURE 9 External reflectance FT-IR spectra of the asymmetric and symmetric PO_2^- stretching region for pure DPPC-d_{62} (*a*) and an equimolar DPPS/DPPC-d_{62} (*b*) monolayer on Ca^{2+}-free (*dashed line*) and 5 mM Ca^{2+}-containing (*solid line*) buffer. Residual water vapor bands have been subtracted and spectra are baseline-corrected.

Table 1 PO_2^- asymmetric stretching frequencies

Lipids	Surface pressure	PO_2^- asymmetric stretch Ca^{2+}-free	PO_2^- asymmetric stretch 5 mM Ca^{2+}
	dyne/cm	cm^{-1}	
DPPC-d_{62}	7	1213	1222
	38	1224	1229
Equimolar DPPS/DPPC-d_{62}	7	1228	1230
	38	1228	1236

The asymmetric PO_2^- stretching frequency in Ca^{2+}-free DPPC-d_{62} monolayers increases from ~1213 to 1224 cm^{-1} over a surface pressure range of 7 to 38 dyne/cm, while the same band in the Ca^{2+}-free binary lipid mixture observed at ~1228 cm^{-1} is independent of pressure. In the presence of Ca^{2+}, the PO_2^- asymmetric stretching frequency is observed to increase when surface pressure is increased for both the single and binary lipid systems. Similarly, the symmetric stretching frequency for the single lipid system is observed to be compression-sensitive and increases as surface pressure is increased, independent of the presence of Ca^{2+}. Hunt et al. (1989) have reported similar compression-sensitive behavior for the PO_2^- symmetric stretch in DPPC monolayers, regardless of the presence of Ca^{2+} in the subphase.

DISCUSSION

The existence of domains during the compression of monolayer films is now well established. Recent studies have reported alterations in domain size distribution induced by insertion of hydrophobic peptides (e.g., SP-C from lung surfactant (Pérez-Gil et al., 1992)). Molecular structure changes upon compression have usually been monitored in two ways. The first approach involves the generation of pressure/area isotherms, a technique poorly suited for acquisition of molecular structure information. The second utilizes the techniques of attenuated total reflectance (ATR) IR spectroscopy, which requires the transfer of films from the A/W interface to a solid support. The transfer process may alter the properties of the film from its untransferred state (McConnell et al., 1984; Helm et al., 1987). More recently, Möhwald and co-workers have reported synchrotron x-ray diffraction data from DMPA monolayers at the A/W interface and demonstrated the decoupling of translational and orientational order during the pressure-induced gel to liquid crystal interconversion (Kjaer et al., 1987).

The external reflection IR data presented here illustrate the potential of this technique, currently in its infancy, for acquiring molecular structure information from monomolecular films. The one component systems exhibit a liquid crystal gel transition in the acyl chains upon film compression. The spectra-structure correlations used to establish this result are well-determined from bulk phase measurements, where direct comparisons with primary thermodynamic methods such as differential scanning calorimetry are plentiful (Mendelsohn and Mantsch, 1986). Moreover, the two-state model as presented (Fig. 8) for quantitative analysis of the data yields the percentage of gel and liquid crystal phases present at each surface pressure within the two-phase region. The success of the model is an independent verification for the existence of domains at the surface. An advantage of the IR experiment, in addition to the direct availability of molecular structure information, is that the technique does not require the addition of a fluorophore as a probe for domain visualization. It has been occasionally observed that the probe molecule may itself perturb domain parameters (McConnell et al., 1984; Kjaer et al., 1987). A drawback of the current experiment is that the data were obtained from a macroscopic sample (beam area, about 1 cm^2). For future applications, we note that technology currently exists for execution of this experiment at the diffraction limit for the IR ($\sim 10\ \mu m$). As lipid domains of 10–20 μm are typical, the IR experiment offers the possibility of examining molecular structure within a single domain. Furthermore, the percentages of ordered and disordered phases will be directly comparable between IR and epifluorescence results. This comparison will be optimal using the same sample for both experiments, as monolayer properties are found to be very dependent on experimental parameters such as subphase composition, temperature, compression rate, and the presence of trace impurities.

The difference in the cooperativity of the phase transition between the two single lipid systems studied is evident in Figs. 3 and 4 and can possibly be explained by the differences between the two headgroups. The bulkiness of the choline moiety may hinder the packing of the molecules at the air/water interface, requiring a greater range of change in surface pressures to produce an ordering of the acyl chains. In addition, the presence of amino and carboxylate groups in the PS offers the potential for hydrogen bonding and other interactions which might reduce the area of the headgroup and are not available to the PC moiety. Limiting molecular areas obtained from surface pressure-molecular area isotherms are 44 and 40 $Å^2$/molecule for DPPC and DPPS, respectively (Phillips and Chapman, 1968; Mattai et al., 1989).

The extension to binary systems and investigation of the effects of Ca^{2+} on mixed monolayers further reveal the power of the IR technology. The Ca^{2+}-induced ordering in the PS component of the binary system is clearly evident from the position of and the lack of shift in the CH_2 asymmetric stretching mode compared with the calcium-free system under the same conditions of compression (Fig. 6 *a*). This is consistent with pressure-area curves of Losche et al. (1985) for negatively charged monolayers of DMPA, where, in the presence of 0.1 mM Ca^{2+}, a phase transition is not observed; that is, the transition is assumed to be complete at the pressures studied. At lower Ca^{2+} concentrations, phase transitions are found to occur at lower surface pressures than in Ca^{2+}-free systems. Control experiments with DPPC-d_{62} show no Ca^{2+}-induced ordering in PC alone (Fig. 4). Consequently, the ordering observed in the PC component of the binary PC/PS mixture (Fig. 6 *b*) immediately suggests that, under the conditions of this experiment, Ca^{2+} does not induce phase separation of the components. While we cannot completely rule out the simultaneous and separate coexistence of ordered PS and PC domains, this possibility is considered unlikely for the following reason. Had phase separation been induced by Ca^{2+}, we would have seen domains of PC in which the CD_2 stretching frequency responds in a fashion comparable to its behavior in the pure system in the presence of Ca^{2+} (Fig. 4). As shown in Fig. 4, Ca^{2+} has little effect on PC domain conformational order and a pressure-induced phase transition occurs. In contrast, the absence of a large pressure-induced response in the PC component in the mixed monolayer leads us to the current suggestion, namely, that the PC must be interacting with the PS component, presumably in mixed domains, for calcium to exercise an ordering effect on the PC. This result contrasts with bulk phase PC/PS vesicles containing Ca^{2+}, where lateral phase separation is suggested to occur (Flach and Mendelsohn, 1993; Silvius and Gagné, 1984). Phase behavior is expected to differ in the two cases as the Ca^{2+} bridging of apposed bilayers in PS/PC multibilayers (Wilschut, 1991) cannot occur in monolayers.

It is of interest to compare the monolayer results for the Ca^{2+}-free system with the bulk phase diagram deduced by Silvius and Gagné (1984). The current results are consistent with those of Silvius and Gagné for a saturated PC-PS mixture, in which there is no evidence for phase immiscibility. For the system containing 50 mole% PS, a temperature of 25°C (close to the boundary of the gel and two-phase regions) corresponds to a surface pressure of approximately 41 dyne/cm in the current work, whereas the two-phase and liquid crystalline boundary found at 30°C is comparable to a surface pressure of 8 dyne/cm for the monolayer. A temperature increase of 5°C thus translates into a surface pressure decrease of 33 dyne/cm. These numbers must be considered as a first approximation, as Silvius and Gagné used the dimyristoyl derivatives of PC and PS in their study. In contrast, the phase behavior of the Ca^{2+}-containing systems differs between the bulk and monolayer experiments. The phase diagram of Silvius and Gagné provides strong evidence of lateral phase separation for mixed bulk systems, a result supported by previous IR studies from this laboratory (Flach and Mendelsohn, 1993), whereas the monolayer systems show substantial miscibility.

Finally, the site of calcium ion interaction is readily evident from studies of the PO_2^- asymmetric stretching mode (Fig. 9, *a* and *b*). Ca^{2+} binding is observed to shift this frequency from 1221 to 1238 cm^{-1} in pure PS multilayers (Dluhy et al., 1983b). Similar shifts, observed prior to and during cochleate phase formation in bulk PS and PS/PC systems, are known to be the result of dehydration of the phosphate moiety (Flach and Mendelsohn, 1993). In the current case, single and binary lipid monolayers demonstrate an increase in the asymmetric PO_2^- stretching frequency resulting from the presence of Ca^{2+} and from an increase in surface pressure when Ca^{2+} is present. This differs from the response of bulk phase PC systems to the presence of Ca^{2+} where a change is not observed in the PO_2^- asymmetric stretching frequency. The difference in the compression sensitivity of the asymmetric PO_2^- stretching frequency in Ca^{2+}-free single versus binary lipid systems, where the frequency in the PC system responds to an increase in pressure while the binary system does not, indicates a stronger attraction between the hydration shell and the charged PS headgroup as compared to the zwitterionic PC headgroup. That is, an increase in surface pressure may be enough to squeeze water molecules from between PC headgroups, but Ca^{2+} is required for the replacement of H_2O in the PS headgroup. The compression insensitivity of the PO_2^- asymmetric stretch in the Ca^{2+}-free binary system is coincident with more disorder in the acyl chains of the PS component (see Fig. 6 *a*) at high surface pressures compared to the Ca^{2+}-containing system. That is, Ca^{2+} is more effective at ordering the acyl chains in the PS component than increasing pressure, similar to the ability of Ca^{2+} to dehydrate the PO_2^- group under conditions where high pressure is ineffective.

As the technology of this experiment improves, it will become feasible to evaluate other spectral regions for their structural information. We have reported an initial feasibility study for the detection, with the current apparatus, of the secondary structure-sensitive Amide *I'* vibration of insoluble protein monolayer films (Flach et al., 1993). The main impediment to their detection (the sharp H_2O rotation-vibration bands in the 1400–1900 cm^{-1} region) is partially overcome with a miniaturized system that permits the use of D_2O in the subphase. Further advances might be gained from polarization modulation experiments.

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