A Direct Test of the "Squeeze-Out" Hypothesis of Lung Surfactant Function. External Reflection FT-IR at the Air/Water Interface†

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ABSTRACT: The current theory of pulmonary surfactant function requires that very low surface tension be achieved and maintained in the alveolar surface film during compression (expiration). To effect this condition, it has been hypothesized that the unsaturated and/or fluid components of surfactant are selectively excluded or "squeezed out" from mixed monolayers containing both saturated and unsaturated phospholipids, leaving a surface film of essentially pure 1,2-dipalmitoylphosphatidylcholine (DPPC). External reflection Fourier transform infrared (FT-IR) spectroscopy has been employed to quantitatively test this hypothesis. Mixed monolayer films of acyl chain-perdeuterated 1,2-dipalmitoylphosphatidylcholine (DPPC-d_{62}) with 1,2-dioleoylphosphatidylglycerol (DOPG), 1-palmitoyl, 2-oleoylPG (POPG), 1,2-dipalmitoylPG (DPPG) were examined *in situ* at the air/water interface as a function of surface pressure. The relative intensities of CD_2 (CH_2) stretching vibrations of the deuterated (proteated) components permitted quantitative determination of the relative concentrations of each in the film. For 7:1 (mol:mol) mixtures of DPPC-d_{62}/DOPG, progressive, selective squeeze out of up to about 90% of the PG component is observed over a range of surface pressures from about 51 to 68 mN/m. The extent of maximal PG squeeze out was reduced to 61% for a 7:1 (mol:mol) mixture of DPPC-d_{62}/POPG. This phenomenon, which is at least partially reversible, appears to require relatively high rates of film compression. Squeeze out was reduced (<20%) for 7:1 (mol:mol) mixtures of DPPC-d_{62}/DPPG or for 7:3 mixtures of DPPC-d_{62}/POPG. Squeeze out requires that the lipid mixture achieve surface pressures greater than about 50–60 mN/m along with unsaturation (or at least conformational disorder) in the acyl chains of the non-DPPC component. Examination of the CH_2 stretching frequencies of the unsaturated phospholipids showed them to be conformationally disordered at the highest surface pressures achievable in single-component films but conformationally ordered in 7:1 binary mixtures with DPPC-d_{62} at high pressures. The DPPC-d_{62} component is conformationally ordered throughout. The relevance of these observations to the mechanism of pulmonary surfactant action is discussed.

Pulmonary surfactant is a mixture of lipids and proteins that functions, possibly as a monomolecular film, by lowering surface tension at the air/alveolar interface to near zero. The main phospholipid component of surfactant, 1,2-dipalmitoylphosphatidylcholine (DPPC),[1] is able to sustain high surface pressures in monolayer films at the air/water (A/W) interface under conditions of film compression. However, the spreading of gel phase DPPC at the A/W interface occurs far too slowly for it to be effective for the rates required *in vivo* (King & Clements, 1972; Notter et al., 1980; Goerke & Clements, 1986). Unsaturated phospholipids, present in surfactant in their liquid-crystalline phase, facilitate its spreading but diminish the ability of surfactant to withstand high surface pressures.

To reconcile these apparently contradictory, yet essential, attributes of surfactant (efficient spreading and the ability to sustain high surface pressures), it has been proposed (Watkins, 1968; Clements, 1977; Hildebran et al., 1979; Hawco et al., 1981a,b; Goerke & Clements, 1986) that unsaturated and/or disordered phospholipid constituents are preferentially "squeezed out" upon compression of monomolecular films. Any molecular description of the physical basis of surfactant function requires a critical evaluation of this hypothesis.

Several approaches have been brought to bear on this problem. Fluorescence polarization studies of bulk phase lipid transition temperatures, coupled with surface pressure measurements of monolayer films, have provided indirect evidence for preferential squeeze out (Egberts et al., 1989). Other evidence comes from the exclusion of radioactively labeled phospholipids from the surface (van Liempd et al., 1987). Recently, Nag and Keough (1993) suggested that the size distribution changes in condensed phospholipid domains during successive compression cycles of phospholipid mixtures, as viewed with epifluorescence microscopy, were consistent with squeeze out.

The feasibility of acquiring direct molecular structure information from phospholipid monolayers at the A/W interface under controlled conditions of surface pressure from external reflection FT-IR (IRRAS) spectroscopy has been demonstrated (Dluhy, 1986; Mitchell & Dluhy, 1988). Three advantages are gained from the use of this technology for

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[1] Abbreviations: ATR, attenuated total reflectance; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPC-d_{62}, acyl chain-perdeuterated DPPC; DPPG, 1,2-dipalmitoylphosphatidylglycerol; FT-IR, Fourier transform infrared; IRRAS, IR reflectance–absorbance; π, surface pressure.

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testing the squeeze-out hypothesis. First, the approach permits determination of the relative concentration of each component in mixed monolayer films. This is accomplished experimentally by requiring one monolayer component to have its acyl chains perdeuterated so that the CH_2 (CD_2) stretching vibrations from the proteated (deuterated) chains can provide isolated vibrations from each component for spectroscopic analysis. Second, the technique permits *in situ* evaluation of conformations adopted by the phospholipids at the A/W interface. Finally, uncertainties connected with either the transfer of films to solid substrates or the necessity to perform independent concentration measurements on the subphase and film are eliminated. In the current study, IRRAS experiments are performed to evaluate the squeeze-out hypothesis on mixed monolayer films of DPPC-d_{62} with DOPG, POPG, and DPPG under controlled conditions of surface tension. The compositions are chosen to mimic those of the surfactant phospholipid components.

MATERIALS AND METHODS

Materials. $CHCl_3$ (ACS grade), MeOH (ACS grade), and doubly-distilled H_2O were used throughout. NaCl was of the highest quality commercially available. DPPC, DPPC-d_{62}, POPG, and DOPG were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification.

Sample Preparation. Lipids and lipid mixtures were prepared at the desired mole ratio (usually 7:1 or 7:3 DPPC-d_{62}/PG) in $CHCl_3$/MeOH (2:1, v/v). DOPG and POPG were separately dissolved in $CHCl_3$.

Apparatus for IR Measurements at Controlled Surface Pressures. The apparatus for external reflection FT-IR at the A/W interface has been modified from that described previously (Flach et al., 1993a). The spectrometer used was a Digilab FTS 40A (Bio-Rad, Cambridge, MA) equipped with a HgCdTe detector. IR radiation was directed to the spectrometer exit port and focused with an angle of incidence of ~42° onto a Teflon trough into which two wells, a sample well and a reference well, had been milled. Connected to the sample well was instrumentation designed (Boyle & Mautone, 1982) for the control and measurement of surface pressure. The surface balance consisted of a Pt Wilhelmy plate connected to a Cahn balance, the output of which was fed to a servo-control unit (Klinetics Inc., Philadelphia, PA). The servo-control unit adjusts surface area by positioning a 150-mesh stainless steel screen in the subphase. The surface pressure was preset to the desired value; the height of the screen was under the control of the servo system to reach and maintain this setting. The trough and screen were cleaned prior to use by soaking in a chromic acid solution (Chromerge, Fisher Scientific, Fairlawn, NJ). The Pt plate was roughened, rinsed, and flamed prior to usage.

The aqueous surface was judged clean when a constant surface tension of 72 ± 1 mN/m was observed over the entire range of surface areas. Typically, 25–50 μL of the lipid sample was then applied to the surface of a 150 mM saline solution, while the surface area was being increased. This corresponded to 4 times the amount of sample required to achieve a surface area of 44 Å/molecule at minimal surface area of the trough. A solvent evaporation/equilibration period of 10–15 min was required. Initial pressures after equilibration were below the equilibrium pressures for the system; this precluded any initial occurrence of multilayers. The monolayer film was then compressed at a screen rate corresponding to an area change of 80 (slow) or 115 mm^2/s (fast). The minimum and maximum surface areas of the trough were 21.6 and 93.3 cm^2. The volume of the subphase was 40 cm^3, and all experiments were conducted at room temperature.

IR Data Acquisition. Typically, 64 interferograms were collected, coadded, apodized with a triangular function, and fast Fourier transformed with one level of zero-filling to produce spectral data encoded at ~2-cm^{-1} intervals, with 4-cm^{-1} spectral resolution. Data were collected in alternating fashion from the sample and reference wells. Although S/N ratios could in theory be improved by acquiring more scans, it was difficult to control π (especially at values > 60 mN/m) for the required times. Alignment of the sample and reference wells was achieved by rotation of the Teflon trough with a computer-controlled stepping motor. A time delay of 30 s was allowed for film reequilibration between trough rotation and commencement of data collection. Single beam data from sample and reference wells were made into ratios to produce an IRRAS spectrum. The geometry of the current experiment produces IRRAS peaks that are negative when plotted as absorbances ($-\log(R/R_0)$). This is anticipated (Dluhy, 1986) from the Fresnel equations for the three layer system (air/film/water) and the appropriate values for the real and imaginary parts of the refractive indices.

Data Manipulation. Baseline correction and water vapor subtraction were accomplished with software supplied by the instrument manufacturer. Spectra were neither smoothed nor deconvoluted prior to peak area measurements. Due to linewidth changes as π was altered, peak height measurements were less representative than peak area measurements for evaluation of relative amounts of each species at the interface. Frequency determinations and peak area calculations were accomplished with software written at the National Research Council of Canada.

RESULTS

Surface Film Stability. The squeeze-out hypothesis requires that exclusion of the unsaturated or conformationally disordered components take place at surface pressures greater than equilibrium (about 45 mN/m for DPPC). Thus, it was necessary to determine the maximum π values that could be achieved with various preparations relevant to the current investigation. The results, summarized in Table 1, are generally consistent with other studies that utilize standard surface film apparatus [e.g., Hawco et al. (1981a)]. The data demonstrate that films of fluid, unsaturated lipid or mixed films of 7:3 (mol:mol) of DPPC-d_{62} with DPPG or POPG cannot sustain high π levels. A monolayer film of pure POPG could not be compressed beyond 54 mN/m. In contrast, 7:1 mole ratio films of the DPPC-d_{62} with any of the three PG derivatives could be compressed to 68–69 mN/m at room temperature.

The current work requires the surface film to be stable at high surface pressures for a time sufficient for IR data acquisition. Films of DPPC-d_{62}/DOPG (7:1, mol:mol) that had been compressed slowly (compression rate of 80 mm^2/min) exhibit a rapid relaxation process (at initial surface pressures > 50 mN/m) which leads to a decrease in π. For this phospholipid mixture at this compression rate, π could not be controlled for the time required for IR data acquisition. In contrast, films of this mixture subject to rapid compression (115 mm^2/s) show a slower relaxation and permitted the acquisition of IR spectral data at constant π even at the highest π values (Tables 1 and 2). Films of DPPC-d_{62}/POPG (7:1, mol:mol) demonstrated sufficiently slow relaxation properties to permit IR data acquisition under conditions of both slow and rapid compression.

Table 1: Surface Characteristics of Representative Films

monolayer components	mole ratio	initial π (mN/m)[a]	final π (mN/m)[a]	π_2/π_1[b]	PG squeeze out (%)	compression rate (mm²/s)
DPPC-d_{62}/DPPG	7:3	41	54	51/45	4	28–545
DPPC-d_{62}/DPPG	7:1	38	69	68/43	9	28
DPPC-d_{62}/DOPG	7:1	41	68	68/51	89	115
DPPC-d_{62}/POPG	7:1	37	69	69/38	61	115
DPPC-d_{62}/POPG	7:1	38	68	68/45	38	80
DPPC-d_{62}/POPG	7:3	38	59	59/44	<5	115–545
POPG	na[c]	41	54	na	na	115–545
DOPG	na	39	54	na	na	115–545

[a] Initial and final surface pressures after film spreading and equilibration. [b] Pressure range (mN/m) from which the extent of squeeze out of the PG component was estimated, that is, π_1 (π_2) is the first (last) surface pressure at which IR data were collected. [c] Not appropriate.

Table 2: Asymmetric CH_2 and CD_2 Stretching Frequencies in Monolayer Films

monolayer	mole ratio	π_1 (mN/m)	ν_{CH_2} (cm^{-1})	ν_{CD_2} (cm^{-1})	π_2 (mN/m)	ν_{CH_2} (cm^{-1})	ν_{CD_2} (cm^{-1})
DPPC-d_{62}/DPPG	7:3	41	2918.6	2193.2	54	2918.6	2192.5
DPPC-d_{62}/DPPG	7:1	38	2919.9	2193.2	69	2920.6	2193.1
DPPC-d_{62}/DOPG	7:1	47	2916.0	2192.7	68	*b*	2194.0
DPPC-d_{62}/POPG	7:1	38	2922.4	2193.9	68	2917.7	2193.1
DPPC-d_{62}/POPG	7:3	44	2920.1	2194.8	59	2919.4	2194.8
DPPC-d_{62}	na[a]	4	n.a.	2196.3	46	na	2193.2
POPG	na	44	2923.1	na	54	2922.7	na
DOPG	na	39	2922.0	na	44	2922.3	na

[a] Not appropriate. [b] This frequency was subject to large experimental uncertainty due to the weakness of the band after squeeze out of the DOPG.

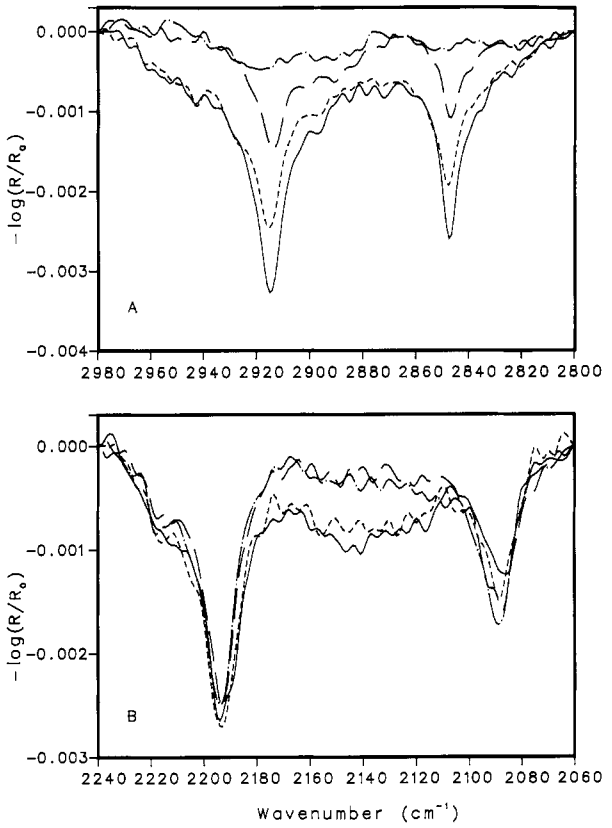

FIGURE 1: Overlaid external reflectance FT-IR spectra of DPPC-d_{62}/DOPG (7:1, mol:mol) monolayer upon stepwise compression at the following surface pressures (mN/m): 51 (—), 56 (– – –), 60 (— —), and 68 (— · —). (A) CH_2 asymmetric and symmetric stretching region of the DOPG component. (B) CD_2 asymmetric and symmetric stretching region of the DPPC-d_{62} component.

Typical FT-IR spectra for the C–H (2800–2980 cm^{-1}) and C–D (2060–2240 cm^{-1}) stretching regions of a 7:1 mixture of DPPC-d_{62}/DOPG are shown in Figure 1. The displayed data were acquired after rapid film compression in the physiologically relevant regions of π between 51 and 68 mN/m. The band intensities monitor, in part, the number of molecules of that species at the interface. As the pressure is raised from 51 to 68 mN/m, the integrated intensities of the CD_2 symmetric and asymmetric stretching vibrations (DPPC-d_{62} component) near 2090 and 2190 cm^{-1}, respectively, are altered by about ~2%. Since the IR experiment examines a constant surface area of the spread film, these data suggest that over this range of pressure, the area per molecule is altered by about the same value. In contrast, the CH_2 symmetric and asymmetric stretches (DOPG component) near 2850 and 2920 cm^{-1}, respectively, show dramatic responses to changes in surface pressure. As the pressure is increased from 51 to 68 mN/m, the intensity of each of these bands is reduced by about an order of magnitude. This result provides direct evidence that, as the film is compressed, the DOPG component is excluded from the surface. The relative intensities (measured as integrated peak areas) of the symmetric and asymmetric CD_2 and CH_2 stretches for the two phospholipids are each plotted as a function of π in Figure 2. The relative intensities of the CH_2 symmetric and asymmetric stretching bands and the DOPG bands are substantially reduced upon film compression.

Spectra of the CD_2 and CH_2 stretching regions for a 7:1 binary mixture of DPPC-d_{62}/POPG are shown in Figure 3. Effects similar to those seen for the DPPC-d_{62}/DOPG films are observed, that is, the POPG component is excluded from the surfaces at high pressures. As judged from the plot of relative intensities of the CH_2/CD_2 modes in Figure 4, the extent of squeeze out in this system is less than that in the DPPC-d_{62}/DOPG mixed film, that is, a greater fraction of the POPG remains at the A/W interface at high surface pressures than the DOPG component in the DPPC-d_{62}/DOPG mixed film. The effect of compression rate on squeeze out was evaluated for the DPPC-d_{62}/DOPG system, and the results are shown in Figure 5. At slow rates of compression, 38% of the PG is squeezed out at 68 mN/m, compared to about 61% at a faster compression rate. The squeeze-out process is also seen (Figure 4) to be substantially reversible, as expansion of the compressed film leads to the reappearance of the

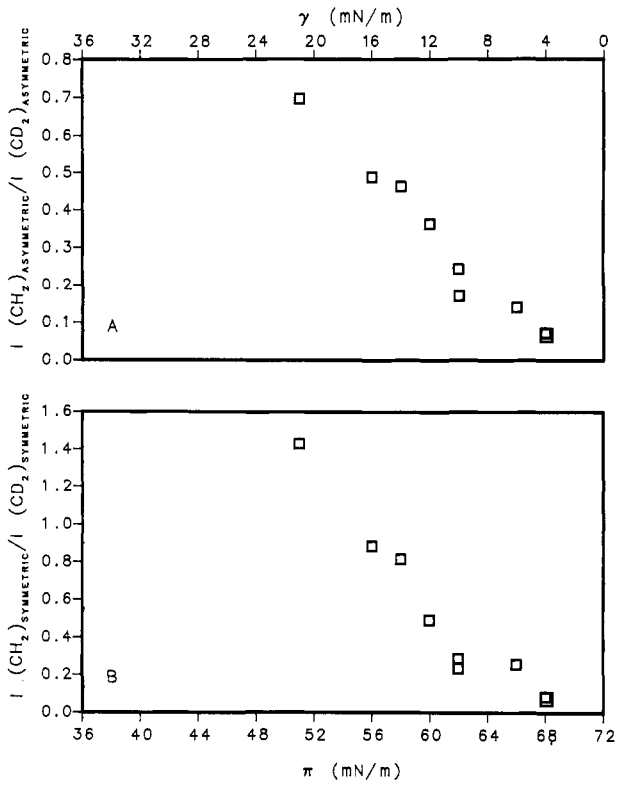

FIGURE 2: Ratio of the integrated band areas for (A) the asymmetric CH_2 stretching modes (DOPG component) and CD_2 stretching modes (DPPC-d_{62} component) as a function of surface pressure and for (B) the symmetric CH_2 stretching modes and CD_2 stretching modes as a function of surface pressure. Repeat experiments show a precision of about 15% of the measured ratio at each pressure.

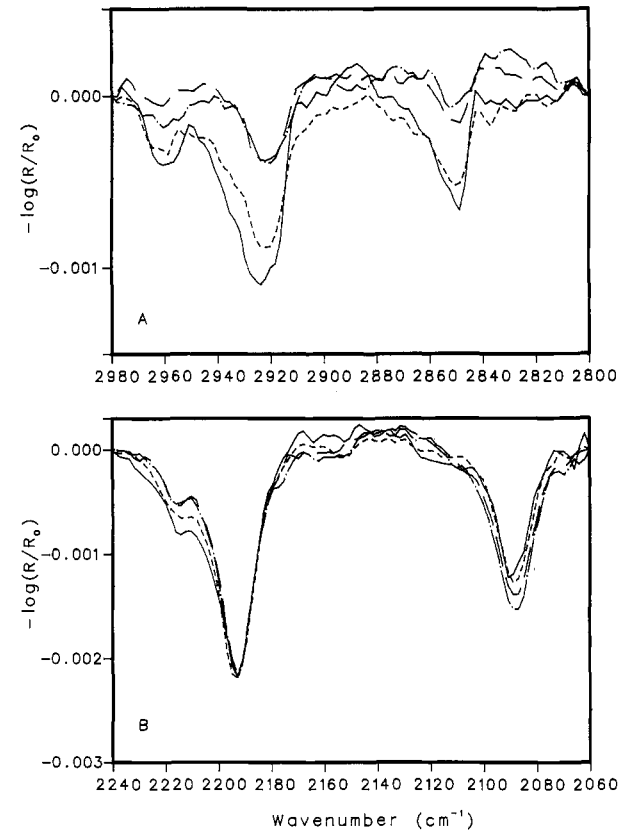

FIGURE 3: Overlaid external reflectance FT-IR spectra of DPPC-d_{62}/POPG (7:1, mol:mol) monolayer upon stepwise compression at the following surface pressures (mN/m): 49 (—), 61 (– – –), 67 (— —), and 69 (— · —). (A) CH_2 asymmetric and symmetric stretching region of the POPG component. (B) CD_2 asymmetric and symmetric stretching region of the DPPC-d_{62} component.

unsaturated component at the surface on a time scale as fast as our spectroscopic observations (about 2 min) permit. Subsequent recompression again reveals PG exclusion.

In contrast to the unsaturated PG systems, where squeeze out is immediately evident from the data, experiments with DPPC-d_{62}/DPPG show little or no exclusion of the PG component. Spectra of the CD_2 and CH_2 stretching regions for a 7:1 binary mixture of DPPC-d_{62}/DPPG are shown in Figure 6. In this instance, little systematic reduction in the intensity of the CH_2 stretching bands relative to the CD_2 stretching bands is observed upon compression. The intensity ratio plots are shown in Figure 7. At most, only a slight diminution is observed in the relative intensity of the bands from the proteated component as the surface pressure is increased.

In addition to IR band intensities directly demonstrating squeeze out at the A/W interface, qualitative information about the conformational state of the phospholipid acyl chains may be obtained from the precise frequencies of the CH_2 and CD_2 stretching modes. A shift to higher wavenumbers in either the symmetric or asymmetric stretching vibrations is known to arise from the introduction of gauche rotamers into the acyl chains (Snyder et al., 1978). Although the weakness of the bands and the somewhat variable nature of the background shape in the IRRAS experiment lead to some scatter in the frequency measurement (which uses a center-of-gravity algorithm and is thus sensitive to the entire shape of the band), some useful insights may be gained. Typical data are given in Table 2. DOPG alone in a monolayer film exhibits a frequency of 2922 cm^{-1} independent of surface pressure up to the collapse point of 44 mN/m. This frequency is characteristic of a disordered phase. In contrast, in 7:1 mixed monolayer films with DPPC-d_{62}, the frequency decreases to about 2916 cm^{-1}, characteristic of acyl chains possessing higher conformational order.

In monolayers of DPPC-d_{62}/POPG (7:1, mol:mol), the CH_2 asymmetric stretching frequency of the POPG component is altered from 2922.4 cm^{-1} at π = 38 mN/m to about 2917.7 cm^{-1} at surface pressures where maximum squeeze out is noted (68 mN/m). These values reveal conformational ordering in the residual POPG as π is increased. Finally, the DPPC-d_{62} component shows a CD_2 asymmetric stretching band at 2193.3 ± 0.5 cm^{-1} in the 7:1 binary mixtures with PG's. This value is essentially independent of surface pressure over the range π = 38–68 mN/m at the precision available here and is consistent with ordered (deuterated) acyl chains. DPPC-d_{62} alone undergoes a surface pressure-induced phase transition in which the conformational ordering is essentially complete at 15 mN/m (Flach et al., 1993b) and is accompanied by a decrease in asymmetric CD_2 stretching frequency from 2196.3 to 2193.2 cm^{-1}.

DISCUSSION

The current experimental design provides an unambiguous means to test for exclusion of particular components in monomolecular films at the A/W interface. The advantages of the IRRAS measurement are evident from Figures 1–6. The integrated intensities of the CD_2 and CH_2 stretching modes from the DPPC-d_{62} and PG components are a direct measure of their relative surface concentrations. As our experimental design permits rapid compression and maintenance of a constant π throughout the time required for data acquisition, a straightforward procedure for determination of squeeze out is achieved. The other advantage of the IR approach is that

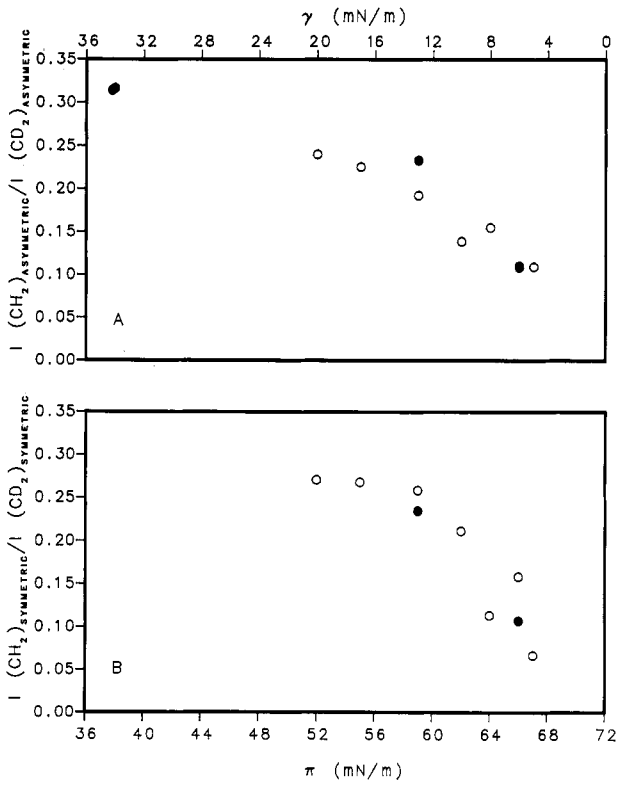

FIGURE 4: Ratio of the integrated band areas for (A) the asymmetric CH_2 stretching modes (POPG component) and CD_2 stretching modes (DPPC-d_{62} component) as a function of surface pressure and for (B) the symmetric CH_2 stretching modes and CD_2 stretching modes as a function of surface pressure. Reversibility of squeeze out for this monolayer is shown by comparing points from a first stepwise compression (○) and a second stepwise compression (●) after film reexpansion.

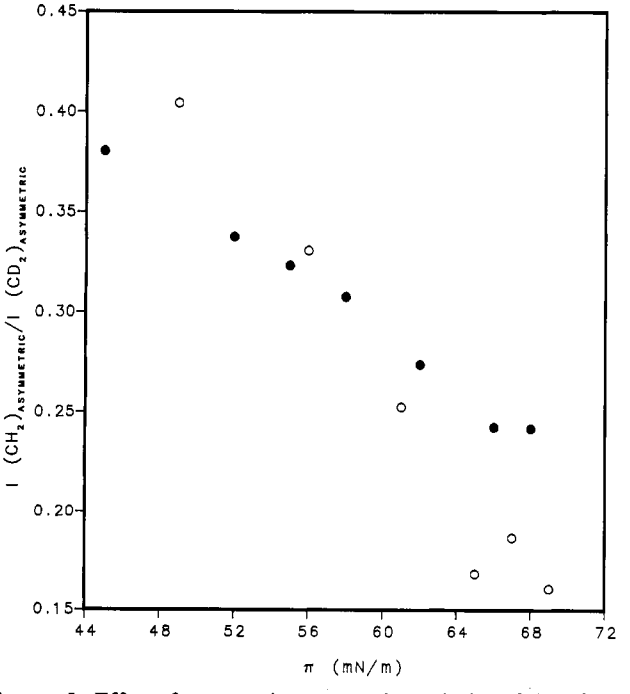

FIGURE 5: Effect of compression rate on the exclusion of the POPG component from a film of DPPC-d_{62}/POPG (7:1, mol:mol): compression rate of 80 mm²/s (●) and compression rate of 115 mm²/s (○).

the frequencies of the CH_2 (CD_2) stretching vibrations contain information about the conformational state of the PG (DPPC-d_{62}) component at the interface.

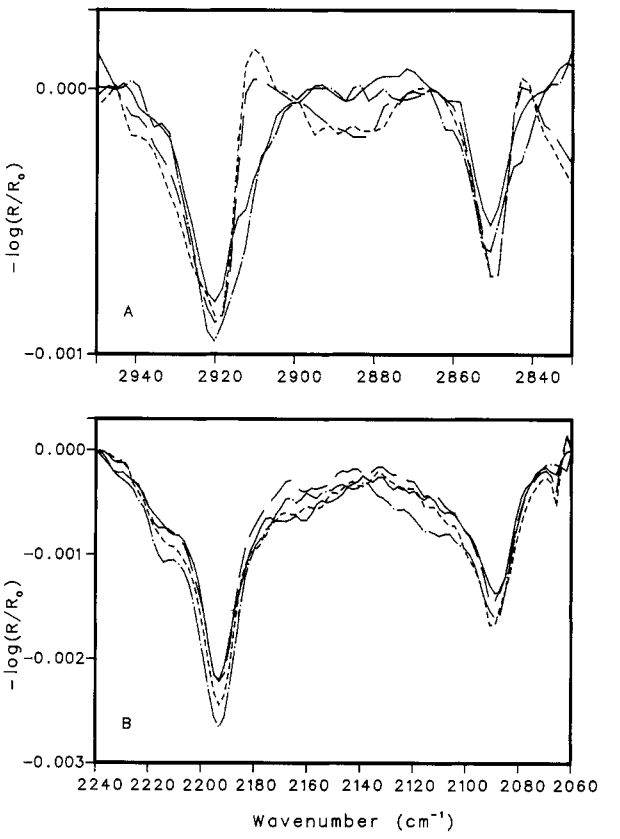

FIGURE 6: Overlaid external reflectance FT-IR spectra of DPPC-d_{62}/DPPG (7:1, mol:mol) monolayer upon stepwise compression at the following surface pressures (mN/m): 28 (—), 48 (– – –), 55 (— —), and 68 (— · —). (A) CH_2 asymmetric and symmetric stretching region of the POPG component. (B) CD_2 asymmetric and symmetric stretching region of the DPPC-d_{62} component.

Under conditions of rapid film compression, about 90% of the unsaturated (PG) component of DPPC-d_{62}/DOPG (7:1, mol:mol) film is excluded from the surface at a surface pressure of 68 mN/m. This quantitative analysis of squeeze out is subject to some uncertainty as changes in chain orientation upon compression might produce alteration in intensities. The effect is calculated to be small (C. R. Flach, J. W. Brauner, and R. Mendelsohn, unpublished results) with our experimental geometry. In theory, chain orientation in conformationally ordered chains may be precisely determined from examination of the CH_2 stretching bands with polarized radiation. In practice, the loss of IR intensity in the experiment would require additional collection time to achieve adequate signal-to-noise ratios. Due to surface film relaxation processes, it is not feasible to control surface pressure for the required duration.

Squeeze out is also noted for the POPG component in 7:1 films of DPPC-d_{62}/POPG, although in that instance, about 61% of the POPG is squeezed out at fast compression rates and 38% at low compression rates. In contrast to the data for unsaturated PG's, DPPG is not significantly squeezed out in binary mixtures with DPPC-d_{62}.

Two IR studies utilizing the techniques of ATR spectroscopy have been used to evaluate squeeze out. Rana et al. (1993) examined monolayers of DPPC-d_{62}/DPPG that had been transferred from the A/W interface to a Ge substrate and found no evidence for exclusion of the DPPG, in good accord with the current result. In an earlier study, Chung et al. (1990) had examined mixed monolayers of egg PG (mixed saturated and unsaturated chains) with DPPC-d_{62}. The authors concluded that squeeze out occurred, a result that

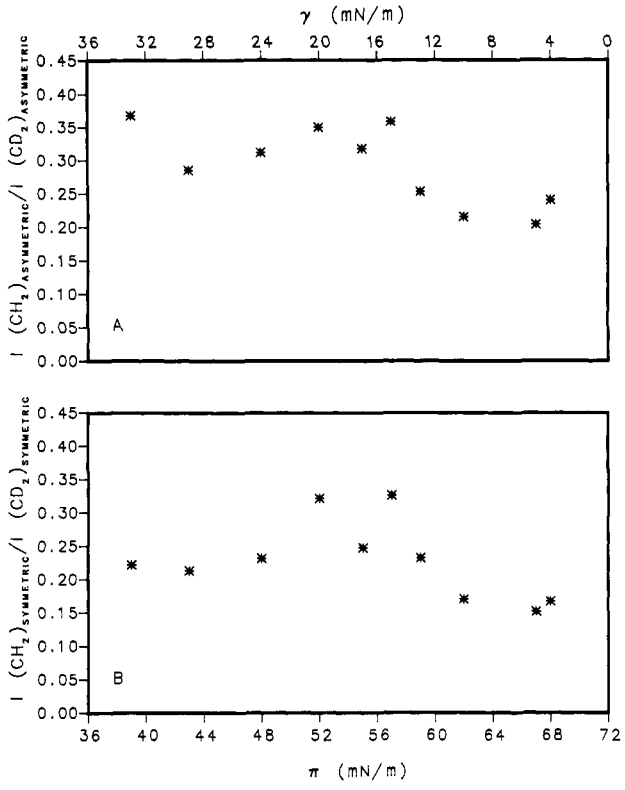

FIGURE 7: Ratio of the integrated band areas for (A) the asymmetric CH_2 stretching modes (DPPG component) and CD_2 stretching modes (DPPC-d_{62} component) as a function of surface pressure and for (B) the symmetric CH_2 stretching modes and CD_2 stretching modes as a function of surface pressure.

may also be in accord with the current work, assuming (reasonably) that bulk-phase egg PG is in a disordered state. The authors, however, report transfer ratios with unusually high values of up to 5.0, a result which suggests that they may not be transferring only monolayers onto the ATR substrates.

The external reflection approach utilized in the current work offers two advantages over the ATR experiments. First, films were examined *in situ* on the surface, without the possibility of experimental uncertainities arising from film transfer. Second, the frequencies observed are characteristic of the lipid conformation at the aqueous surface, rather than those arising from interaction of the lipid with the ATR substrate. The drawbacks of the approach are that the spectral signals are weak and that specialized accessories are required.

To achieve (in monolayers) the high pressures apparently needed for squeeze out, the lipid film must contain mostly conformationally ordered acyl chains, although the bulk phase of the non-DPPC component is required to be conformationally disordered. The phase behavior of binary mixtures of these phospholipids must therefore be considered to understand the composition dependence of the conformational order. Weidmann et al. (1993) reported bulk-phase DSC studies in mixtures of DPPC/DOPG. Although nonideal mixing was noted, nothing extraordinary (such as phase separation, which might imply the coexistence of ordered and disordered phases) was observed between 10 and 30 mol % of POPG that would account for the composition-dependent changes in the maximal π that can be achieved for the mixed monolayers in the current investigation. Thus, the driving force for squeeze out at high compression rates and low mole fraction of PG must depend on monolayer (rather than bulk phase) properties of these phospholipids. A major difference between monolayers and bulk phase morphology is the apearance of extensive domain structures in monomolecular phospholipid films. These structures, and their relationship to phase separation, must be considered in any theory of monolayer stability.

Other types of experiments have been used to infer squeeze out in monomolecular films. Hawco et al. (1981b) studied films of DPPC with four fluid PC's. They found, as was confirmed in the current investigation, that the minimum surface tension achieved depends on the rate of compression and on the relative proportions of fluid and rigid lipid in the monolayer prior to compression. Lower γ_{min} values were achieved at higher compression rates. While these results were considered suggestive of squeeze out of the fluid component, direct proof was not available. It is possible, for example, that some of the unsaturated component can be tolerated in monolayers at high pressures (as indeed has been noted for the DPPC-d_{62}/POPG system in the current work) while still allowing the film to achieve a low γ_{min}. In a different type of study, Egberts et al. (1989) compared the phase transition temperatures observed with surface tension measurements in monolayers to the fluorescence polarization measurements in vesicles of mixtures of DPPC with egg PC, egg PG, and soybean PI. The data are again consistent with squeeze out, but uncertainities in the location of the probe (diphenylhexatriene) molecule and possible ambiguities in comparing vesicles with monolayers render a quantitative interpretation uncertain.

The current IR studies directly indicate that squeeze out occurs at the A/W interface and requires, in addition to the DPPC, a lipid that is disordered at low pressures (in the current work, these contain unsaturated acyl chains). The extent of squeeze out is probably dependent on the rate of film compression. The third factor, which requires substantial further experimental investigation, suggests that the kinetic viscoelastic properties of the film are crucial to its function at the A/W interface. For example, Nag et al. (1991) have shown that even for a simple film consisting of one component (DPPC), higher rates of compression give smaller domain sizes. Clearly, the relationship of domain size to squeeze out must be quantitatively addressed. Other issues center around the possible role of the surfactant proteins which have been implicated in assisting the spreading of phospholipids or lung surfactant extracts at the A/W interface (Mautone et al., 1988; Dluhy et al., 1989; Chung et al., 1989; Cockshutt et al., 1990; Venkitaraman et al., 1990; Oosterlaken-Dijksterhuis et al., 1991; Pastrana et al., 1991). They therefore alter the viscoelastic properties of surface films and may have a role in squeeze out.

The major aim of this work is the quantitative testing of the squeeze-out hypothesis. The existence of the phenomenon is unambiguously demonstrated. The next stages of study require an understanding of the relationships between squeeze out, domain formation, kinetic film properties, and the mechanism of action of pulmonary surfactant. Our data, especially the observation of the reversible nature of the phenomenon, suggest that a relaxation process exists that requires the continuous application of a compressing force on the surface film to prevent the non-DPPC components from reentering the surface and reducing π. Thus, the extent to which squeeze out occurs is determined not only by the presence of acyl chain unsaturation but also by the rate of surface film compression. These aspects of surfactant action are inadequately handled by the current theory of surfactant function *in vivo*.

REFERENCES

Boyle, J., III, & Mautone, A. J. (1982) *Colloids Surf. 4*, 77–86.

Chung, J., Yu, S. H., Whitsett, J. A., Harding, P. G. R., & Possmayer, F. (1989) *Biochim. Biophys. Acta 1002*, 348–358.

Chung, J. B., Hannemann, R. E., & Frances, E. I. (1990) *Langmuir 6*, 1647–1655.

Clements, J. A. (1977) *Am. Rev. Respir. Dis. 115*, 67–71.

Cockshutt, A. M., Weitz, J., & Possmayer, F. (1990) *Biochemistry 29*, 8424–8429.

Dluhy, R. A. (1986) *J. Phys. Chem. 90*, 1373–1379.

Dluhy, R. A., Reilly, K. E., Hunt, R. D., Mitchell, M. L., Mautone, A. J., & Mendelsohn, R. (1989) *Biophys. J. 56*, 1173–1181.

Egberts, J., Sloot, H., & Mazure, A. (1989) *Biochim. Biophys. Acta 1002*, 109–113.

Flach, C. R., Brauner, J. W., & Mendelsohn, R. (1993a) *Appl. Spectrosc. 47*, 982–985.

Flach, C. R., Brauner, J. W., & Mendelsohn, R. (1993b) *Biophys. J. 65*, 1994–2001.

Goerke, J., & Clements, J. A. (1986) in *Handbook of Physiology* (Geiger, S. R., Ed.) Section 3, Vol. III, Part 1, pp 249–250, American Physiology Society, Bethesda, MD.

Hawco, M. W., Coolbear, K. P., Davis, P. J., & Keough, K. M. W. (1981a) *Biochim. Biophys. Acta 646*, 185–187.

Hawco, M. W., Davis, P. J., & Keough, K. M. W. (1981b) *J. Appl. Physiol. 51*, 509–515.

Hildebran, J. N., Goerke, J., & Clements, J. A. (1979) *J. Appl. Physiol. 47*, 604–611.

King, R. J., & Clements, J. A. (1972) *Am. J. Physiol. 233*, 715–726.

Mautone, A. J., Ritter, A., & Boyle, J., III. (1988) *Colloids Surf. 300*, 223–241.

Mitchell, M. L., & Dluhy, R. A. (1988) *J. Am. Chem. Soc. 110*, 712–718.

Nag, K., & Keough, K. M. W. (1993) *Biophys. J. 65*, 1019–1026.

Nag, K., Boland, C., Rich, N. H., & Keough, K. M. W. (1991) *Biochim. Biophys. Acta 1068*, 157–160.

Notter, R. H., Tabak, S. A., & Mavis, R. D. (1980) *J. Lipid Res. 21*, 10–22.

Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., van Golde, L. M. G., & Demel, R. A. (1991) *Biochemistry 30*, 8276–8287.

Pastrana, B., Mautone, A. J., & Mendelsohn, R. (1991) *Biochemistry 30*, 10058–10064.

Rana, F. R., Mautone, A. J., & Dluhy, R. A. (1993) *Biochemistry 32*, 3169–3177.

Snyder, R. G., Hsu, S. L., & Krimm, S. (1978) *Spectrochim. Acta 34A*, 395–406.

van Liempd, J. P. L. G., Boonman, A. A. H., Demel, R. A., Gieles, P. M. C., & Gorree, T. C. M. (1987) *Biochim. Biophys. Acta 897*, 495–501.

Venkitaraman, A. R., Hall, S. B., Whitsett, J. A., & Notter, R. H. (1990) *Chem. Phys. Lipids 56*, 185–194.

Wiedmann, T., Salmon, A., & Wong, V. (1993) *Biochim. Biophys. Acta 1167*, 114–120.

Watkins, J. C. (1968) *Biochim. Biophys. Acta 152*, 293–306.