Surface Chemistry of Binary Mixtures of Phospholipids in Monolayers. Infrared Studies of Surface Composition at Varying Surface Pressures in a Pulmonary Surfactant Model System†

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ABSTRACT: Phospholipid monomolecular films at the air/water interface were studied using Langmuir–Blodgett (L–B) surface chemistry, ^{31}P NMR spectroscopy, and infrared (IR) spectroscopy. These monolayers were composed of binary mixtures of acyl chain perdeuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (i.e., DPPC-d_{62}) with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (i.e., DPPG). This particular PC–PG binary mixture was chosen for study since this lipid system has been used as a model for pulmonary surfactant, especially in conjunction with the so-called "squeezing-out" hypothesis of pulmonary mechanics. This theory predicts that upon successive compression–expansion cycles, a surfactant surface film will reorganize to exclude all components except DPPC, thus resulting in a stable, low surface tension film. Several general results were obtained from these experiments. First, we have developed a combined spectroscopic assay using high-resolution ^{31}P NMR spectroscopy in combination with the C–H and C–D vibrational intensities obtained from the IR spectroscopy of binary mixtures in which one component is acyl chain perdeuterated. Using attenuated total reflectance IR spectroscopy of transferred L–B films, this combined spectroscopic approach allows us to quantitatively describe the fractional composition of each component in the binary monomolecular film. Second, when these methods are applied to transferred monolayer films of DPPC-d_{62} and DPPG (at an initial PC:PG mole ratio of 7:1), we find no evidence for a "squeezing-out" of the DPPG monolayer component at high surface pressure resulting in an enrichment of the DPPC component in the transferred monolayer film. On the contrary, the IR results indicate that at high surface pressures, both DPPC-d_{62} and DPPG are ordered and oriented. IR integrated intensities show that the fractional composition of each component in the monolayer does not change with increasing surface pressure, even up to 70 mN m^{-1} on multiple cycles. These results do not support the so-called "squeezing-out" hypothesis of surfactant physiology, at least for this particular PC–PG binary mixture.

Pulmonary surfactant is a heterogeneous lipid–protein complex which is secreted by type II cells of the pulmonary alveolus. The function of this surfactant is to reduce the surface tension in vivo at the air–alveolar interface to near zero [see, e.g., Scarpelli (1988)]. The lipid constitutents of pulmonary surfactant have been characterized chromatographically as belonging to as many as seven major phospholipid classes (King & Clements, 1972; Goerke & Clements, 1986; Bonnano et al., 1992). Lipid containing phosphocholine (PC)[1] can comprise up to 80–85% of surfactant phospholipids, with over half of the PC fraction belonging to the saturated species 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). The second most abundant lipid class is usually phosphoglycerol (PG), which accounts for approximately 5–10% of the total surfactant phospholipid. The remainder of the surfactant lipid consists of phosphoethanolamines, phosphoinositols, phosphoserines, lysophosphocholines, and sphingomyelin (Haagsman & van Golde, 1991).

The alveolar lining layer is composed of an aqueous hypophase containing all the phospholipid species as well as specific proteins associated with the pulmonary surfactant system, from which a continuous film can rapidly adsorb at the air–alveolar interface (King & Clements, 1972; Goerke & Clements, 1986). The in vitro spreading properties of what is currently believed to be the functional form of pulmonary surfactant have lead to the theory that the most physiologically relevant configuration for this surfactant is that of a monomolecular, lipid–protein film at the air–alveolar lining (King & Clements, 1972; Goerke & Clements, 1986). This contention has never been definitively proven, and other configurations may also be present (Scarpelli, 1988). Furthermore, this theory suggests, by extrapolation, that the most relevant model system for studying the surface-active properties of pulmonary surfactant constituents in vitro would be a two-dimensional monomolecular film at the A/W interface of a Langmuir film balance (Notter, 1984; Goerke & Clements, 1986). In this fashion, the in vivo surfactant surface properties operative in the alveolus may be modeled in vitro during the compression and expansion cycles of monomolecular films at the A/W interface.

At present, the role of the various phospholipid components in surfactant physiology is still incompletely understood. Although a monomolecular film of DPPC, by itself, can reduce the surface tension of water to near zero in vitro, the kinetics

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[1] Abbreviations: ATR, attenuated total reflectance; A/W, air/water; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; acyl chain perdeuterated DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-d_{62}; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; FT-IR, Fourier transform infrared; IR, infrared; L–B, Langmuir–Blodgett; LC, liquid-condensed; LE, liquid-expanded; π–A, pressure–area; π_t, phase transition onset pressure; PC, phosphocholine; PG, phosphoglycerol; SC, solid-condensed.

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of spreading for DPPC alone are much too slow to be effective in vivo (Colacicco et al., 1976; King & MacBeth, 1981; Yu & Possmayer, 1986). Therefore, other surface components affecting monomolecular film structure (e.g., PG, proteins, pH changes, and inorganic ions) have been implicated in the spreading mechanism (Scarpelli et al., 1965; Colacicco et al., 1976; Hagwood et al., 1985). The presence of these secondary surfactant components would seem to be physiologically required, since negatively charged phospholipids, like PG, as well as acyl chain unsaturation tend to "fluidize" the otherwise solid DPPC film (Lee, 1977; Evans et al., 1980; Jacob et al., 1980). This increased fluidity should increase the rate of phospholipid adsorption into the surface monolayer, which is necessary for the rapid rate of surface film formation needed in the alveolus during the respiratory cycle (Notter, 1984; Goerke & Clements, 1986). However, these same secondary surfactant components that promote spreading at high surface tensions also enhance the collapsibility of a formed and compressed monolayer, thereby rendering any low surface tension surface film unstable (Goerke, 1981; Notter, 1984; Goerke & Clements, 1986). Therefore, in order for the surfactant monolayer to rapidly adsorb at high surface tension and remain stable at low surface tension, it has been postulated that the non-DPPC components of the surfactant system, such as PG, must be "squeezed-out" of the surface film to ensure alveolar stability once the monolayer has spread and has been compressed (Hildebran et al., 1979; Goerke & Clements, 1986).

Monomolecular films have been studied at the A/W interface in order to examine the roles of the secondary surfactant components in vitro. These monolayer films have consisted of mixtures of DPPC in combination with other synthetic lipids which mimic the components of the pulmonary surfactant system (Clements, 1977; Hildebran et al., 1979; Goerke & Clements, 1986). In these cases, the surface pressure–molecular area (π–A) isotherms of these mixed lipid monomolecular films show that a very high surface pressure can be achieved (i.e., the surface tension can be lowered to near zero). It has been postulated that the mechanism of this action involves the reorganization of the surface monomolecular film upon compression by "squeezing out" all the non-DPPC components. This would result in the selective enrichment of the DPPC component in the monolayer, which could provide for the lowered surface tension (Hildebran et al., 1979; Notter, 1984).

Unfortunately, the experimental evidence for the surfactant "squeeze-out" hypothesis is almost entirely based on conventional monolayer surface film π–A curves, which can provide only an indirect means of understanding the surface film composition of mixed monolayers (Scarpelli, 1988). Therefore, in order to understand the molecular-level nature of the structural interactions occurring in mixed lipid monomolecular films, we have used in this present work a combination of surface chemistry, Langmuir–Blodgett monolayer transfer techniques, infrared spectroscopy, and ^{31}P NMR spectroscopy to characterize lipid monolayers composed of PC and PG with the aim of directly testing the "squeeze-out" hypothesis of surfactant physiology using defined binary mixtures of synthetic phospholipids.

Infrared spectroscopy has several advantages as a spectroscopic tool for research in lipid biophysics (Cameron & Dluhy, 1986). This technique can directly measure lipid acyl chain conformation and orientation in ordered systems [see, e.g., Mendelsohn and Mantsch (1986)]. In addition, IR spectroscopy can easily be applied to the study of binary lipid mixtures by the deuteration of the acyl chains of one component, which shifts its vibrational frequency to a region of the spectrum which is independent of the other lipid component (Dluhy et al., 1985). This allows researchers to independently monitor the conformation and orientation of each component in a binary mixture. IR spectroscopy has also proven itself to be an extremely surface-sensitive spectroscopic tool, so that it is now routinely possible to study ordered monolayers transferred onto solid substrates in the attenuated total reflection (ATR) configuration (Cornell et al., 1989; Dluhy & Cornell, 1991), or the in situ study of monomolecular films directly at the A/W interface (Mitchell & Dluhy, 1988; Dluhy et al., 1989; Dluhy & Cornell, 1991). These modern techniques of biophysical IR spectroscopy have recently been brought to bear on problems of pulmonary surfactant biophysics (Mautone et al., 1987; Dluhy et al., 1989; Reilly et al., 1989; Pastrana et al., 1991).

In the current work, binary mixtures of acyl chain perdeuterated DPPC (DPPC-d_{62}) with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were studied. Several general results are obtained from the data presented here. First, it is possible to use high-resolution ^{31}P NMR spectroscopy in combination with the C–H and C–D vibrational intensities obtained from the IR spectroscopy of binary mixtures to quantitatively describe the fractional composition of binary mixtures of phospholipids by IR spectroscopy. Second, when these methods are applied to transferred monolayer films of DPPC-d_{62} and DPPG (at an initial mole ratio of 7:1 PC to PG), we find no evidence for a "squeezing-out" of the DPPG monolayer component at high surface pressure resulting in an enrichment of the DPPC component in the transferred monolayer film. On the contrary, the IR results indicate that at high surface pressures, both DPPC-d_{62} and DPPG are highly ordered and oriented. The evidence presented here shows that the fractional composition of each component in the monolayer does not change with increasing surface pressure, even up to 70 mN m^{-1}. These results are in contrast to those obtained by Chung et al. (1990), who studied a similar system, albeit with unsaturated PG components, and obtained IR spectra which they interpreted as confirming an exclusion of the PG component from the surface. Our results indicate the complete lack of any such surface reorganization, and do not support the so-called "squeezing-out" hypothesis of surfactant physiology, at least for a PC–PG mixture containing saturated acyl chains.

EXPERIMENTAL PROCEDURES

Preparation of Synthetic Lipids. The phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), acyl chain perdeuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC-d_{62}), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were obtained from Avanti Polar Lipids (Alabaster, AL) at 99% state purity. These lipids were further purified before use by successive recrystallizations, as previously described (Mitchell & Dluhy, 1988). Each lipid was dissolved in a $CHCl_3$–MeOH (4:1) stock solution at a concentration of approximately 2 mg mL^{-1}. Organic solvents were freshly distilled prior to use. The exact stock solution concentrations of each lipid were verified by an inorganic phosphorus assay (Chen et al., 1956). Binary lipid mixtures at specific mole ratios were prepared by addition of the appropriate aliquots from the individual lipid stock solutions. The exact fractional composition of each phospholipid component in the binary mixtures was ascertained by inorganic phosphorus assay combined with ^{31}P NMR spectroscopy. The details of this procedure are provided below.

Surface Chemistry of Phospholipid Monolayer Films. Phospholipid monomolecular films were formed by spreading 20 μL of a 2 mg mL^{-1} $CHCl_3$–MeOH (4:1) lipid stock solution onto the air/water (A/W) surface of a PTFE (i.e., Teflon)-coated aluminum trough. The surface film was then allowed to equilibrate for 10 min before film compression was begun. Phospholipid pressure–area (π-A) isotherms were recorded on an automated, computer-controlled, dual-area Langmuir–Blodgett (L–B) film balance from Joyce-Loebl, Ltd. (Gateshead, England). This film balance design utilizes a constant-perimeter PTFE-coated fiberglass tape to control the size of two trough areas, each of which can be varied from approximately 440 cm^2 to approximately 90 cm^2 in surface area. During the acquisition of the lipid π–A isotherms reported here, the film balance surface area was compressed at the rate of 0.5 cm^2 min^{-1}. This rate of surface area change was used for both the compression and expansion phases of multicycle isotherms. The temperature of the film balance subphase was held constant during the isotherms at 22 ± 1 °C by flowing a thermostated H_2O solution through the hollow body of the aluminum trough. The trough subphase was 150 mM NaCl, pH 5.6. Subphase water was obtained from a Barnstead (Dubuque, IA) ROpure/Nanopure reverse osmosis/deionization system and had a nominal resistivity of 18 mΩ-cm. Monolayer surface pressures were recorded by differential weight measurements using a filter paper Wilhelmy plate (Whatman no. 1) suspended from a microbalance.

Substrates for Monolayer Film Deposition. Phospholipid monolayers were transferred onto monocrystalline germanium substrates for subsequent attenuated total reflectance (ATR) infrared analysis. Parallelogram Ge ATR crystals (50 × 10 × 2 mm) with a 45° face angle and a total surface area of 12.6 cm^2 were obtained from Wilmad Glass Co. (Buena, NJ). These crystals were routinely cleaned first by mechanical polishing with a 0.3-μm alumina micropolish (Buehler, Lake Bluff, IL) followed by extensive rinsing with deionized H_2O. Crystals were then immersed in $CHCl_3$, followed by bath sonication for 15 min. Final cleaning was performed in a plasma cleaner (Harrick Scientific Corp., Ossining, NY) under argon for 10 min immediately prior to monolayer transfer.

Langmuir–Blodgett Monolayer Film Deposition. Phospholipid monolayer transfer onto the Ge substrates was accomplished using the following protocol. A Ge ATR crystal was immersed in the subphase immediately after cleaning, after which the lipid monolayer in $CHCl_3$–MeOH (4:1) solution was spread onto the water surface. After the equilibration period, the monolayer was compressed to the desired final surface pressure (either 40 or 70 mN m^{-1}) at a rate of 0.5 cm^2 min^{-1}. The Ge crystals were then vertically raised through the compressed monolayer film at a rate of 20 mm min^{-1} while the surface pressure was held constant (estimated deviation from the set pressure: <±1 mN m^{-1}), and the monolayers were deposited in a single pass through the interface. This type of monolayer transfer corresponds to the classical Z-type deposition previously described (Kuhn et al., 1972) with one monolayer supported with its headgroup down on each crystal face. Deposition of a single monolayer was assured by monitoring the ratio of the L–B film balance surface area decrease at constant π to the surface area of deposition on the ATR crystal (i.e., the transfer ratio). Transfer ratios of unity signify a single monomolecular film was deposited on each crystal. Under the experimental conditions employed in this study, the transfer of the binary phospholipid monolayer films produced supported monolayers on Ge with transfer ratios of unity.

IR Spectroscopy of Monolayer Films. Infrared spectra of phospholipid monolayer films on Ge substrates were obtained using a Digilab FTS-40 Fourier transform infrared spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) equipped with a narrow-band, liquid N_2-cooled HgCdTe detector (Infrared Associates, Orlando, FL) at 4-cm^{-1} resolution with triangular apodization and one level of zero-filling. The Ge ATR crystals were mounted in a 4× ATR beam condenser (Spectra-Tech, Inc., Stamford, CT) placed within the sample compartment of the IR spectrometer. The incoming IR radiation was polarized using an Al wire-grid polarizer on KRS-5 (Model IGP225, Molectron Detector Inc., Portland, OR). ATR monolayer absorbance spectra were obtained by ratioing the single beam IR spectrum of a monolayer-covered crystal to that of a blank Ge crystal. Typically, 1024 scans were collected to improve the signal-to-noise ratio; the spectra presented here have not been smoothed.

The C–H asymmetric stretching vibration at 2920 cm^{-1} along with its band intensity was monitored as an empirical indicator of acyl chain conformation and configuration for the protiated lipids DPPC and DPPG. Analogously, the C–D asymmetric stretching vibration at 2190 cm^{-1} along with its band intensity was monitored as an empirical indicator of acyl chain conformation and configuration for the deuterated lipid DPPC-d_{62}. Vibrational frequencies were calculated using a center-of-gravity algorithm (Cameron et al., 1983) and are accurate to better than ±0.1 cm^{-1}. Detailed data reduction procedures for phospholipid infrared spectra have been previously described (Casal & Mantsch, 1984; Cameron & Dluhy, 1986). Integrated vibrational band intensities were calculated between wavenumber limits 2870 and 2825 cm^{-1} for the symmetric C–H stretching band, and between 2125 and 2045 cm^{-1} for the symmetric C–D stretching vibration.

^{31}P NMR Spectroscopy. Aliquots of standard solutions of DPPC-d_{62}–DPPG binary mixtures at various mole ratios or of the DPPC-d_{62}–DPPG monolayer spreading solutions were prepared for ^{31}P NMR spectroscopy by evaporating the organic solvent with a gentle stream of Ar and then dissolving the resulting phospholipid film in 0.6 mL of $CDCl_3$ and 1.2 mL of a ^{31}P NMR potassium line-narrowing reagent (Meneses & Glonek, 1988; described in further detail below). The samples for NMR spectroscopy that resulted from addition of $CDCl_3$ and the line-narrowing reagent to the dry lipid film were biphasic; the upper aqueous layer was removed by aspiration. The samples were adjusted in the NMR tube turbine so that only the $CDCl_3$ phase was in the transmitter/receiver coil.

This NMR line-narrowing reagent was prepared as originally outlined by Meneses and Glonek (1988) with modifications which have been previously described (Rana et al., 1991). Briefly, the line-narrowing reagent was prepared by titrating a 0.2 M suspension of the free acid form of EDTA with KOH until the pH was 6.00. The solution was freeze-dried, and the salt was dissolved in enough H_2O to yield a 0.2 M solution of EDTA. Four volumes of freshly distilled methanol were then added. Additional water was added if the line-narrowing reagent appeared cloudy due to any undissolved salt.

^{31}P NMR spectra were obtained on a Bruker AMX 400 NMR spectrometer operated at 161.977 MHz with a 5-mm probe at ambient temperature. The field frequency was locked to $CDCl_3$. Typically, 1000–2000 transients were collected using a sweep width of 1639.34 Hz (10.121 ppm), a flip angle of 45°, and an acquisition time of 1.25 s. The T_1 values of the DPPC-d_{62} and DPPG signals were measured as 1.61 and

1.59 s, respectively. An interpulse delay of 2 s was used which was found to be sufficient to allow the nuclei to completely relax while minimizing the delay time (Rana et al., 1993). Broad-band decoupling was employed using the WALTZ16 pulse sequence. The decoupler was gated on only during the acquisition in order to suppress NOE effects. Before computation of the Fourier transform, the FIDs were multiplied by an exponential window function with the line-broadening exponent set at a value of 1. The digital resolution was 0.80 Hz per data point, and typical line widths at half-height were about 1.6 Hz.

RESULTS

Surface Chemistry of Pure and Mixed Lipid Monolayers. The characteristic dynamic π–A behavior of pure DPPC, DPPC-d_{62}, and DPPG monolayers at the A/W interface when subjected to three successive compression–expansion cycles is shown in panels A, B, and C, respectively, of Figure 1. For these experiments, the monolayer was not allowed to relax between cycles. The subphase was 150 mM NaCl, pH 5.6, and the temperature was held at 22 ± 1 °C. The π–A isotherm measured for DPPC when compressed as a static film (the first compression) is in close agreement with previously published isotherms (Phillips, 1972). Four regions are discernible in the π–A isotherm of DPPC: (1) the LE phase (105–85 Å^2); (2) the LE/LC transition (85–60 Å^2); (3) the LC/SC phase (60–50 Å^2); and (4) a high-pressure phase above 50 Å^2. The hysteresis between the first and second compression–expansion cycles is typical for DPPC monolayers and is characterized by displacement of the isotherms to lower molecular areas. It has been suggested that this hysteresis occurs primarily as a result of the loss of DPPC molecules from the interface at high surface pressures (Notter et al., 1982). This is unlikely in the present case, since the second and third compression and expansion cycles are nearly identical. It seems more plausible that the π–A hysteresis for DPPC is due to differences between compressing the film under static conditions (the first compression) and dynamic conditions (the second and third compressions).

The π–A isotherms shown in Figure 1B indicate that at higher surface pressures the DPPC-d_{62} monolayer at the A/W interface is less stable than that of DPPC. While the "lift-off" point of the DPPC-d_{62} isotherm is very close to that of DPPC, the onset of the LE/LC transition (π_t) does not occur until 17 mN m^{-1} for DPPC-d_{62} compared to a π_t value of 10 mN m^{-1} for DPPC. The increased π_t value for DPPC-d_{62} is consistent with the thermotropic behavior of DPPC and DPPC-d_{62} in the bulk aqueous phase. The midpoint of the bulk gel-to-liquid-crystal phase transition temperature is shifted from 41.5 °C for pure DPPC to 38.5 °C for hydrated DPPC-d_{62} dispersions as a result of fatty acyl chain perdeuteration (Gaber et al., 1978; Klump et al., 1981), indicating slightly weaker interactions between the lipid acyl chains for the deuterated sample. A slight increase in the π_t value may be generally associated with acyl chain deuteration in Langmuir monolayers. For example, an increase in the π_t value from 5 to 12 mN m^{-1} at 22 ± 1 °C has been observed to occur for the perdeuterated pentadecanoic acid relative to the proteated parent compound (S. Widayati and R. A. Dluhy, unpublished results). DPPC-d_{62} exists in the LC/SC phase until 65 mN m^{-1}, at which point it appears as if the film collapses. The hysteresis which occurs in the DPPC-d_{62} isotherms for the second and third compression–expansion cycles is more dramatic than for DPPC, with the shifts to lower molecular areas being more pronounced. In addition, the collapse pressure decreases for each successive compression–expansion cycle. The decreased stability of the DPPC-d_{62} monolayer may be attributable to the reduced energy of the van der Waals interactions between adjacent CD_2 residues in the fatty acyl chains of DPPC-d_{62} compared to those occurring between the CH_2 groups of the DPPC hydrocarbon chains. Molecular modeling calculations for CH_3 vs CD_3 groups have shown a slight decrease in the calculated van der Waals energies upon deuterium substitution, a result which tends to support this interpretation (Allinger & Flanagan, 1983).

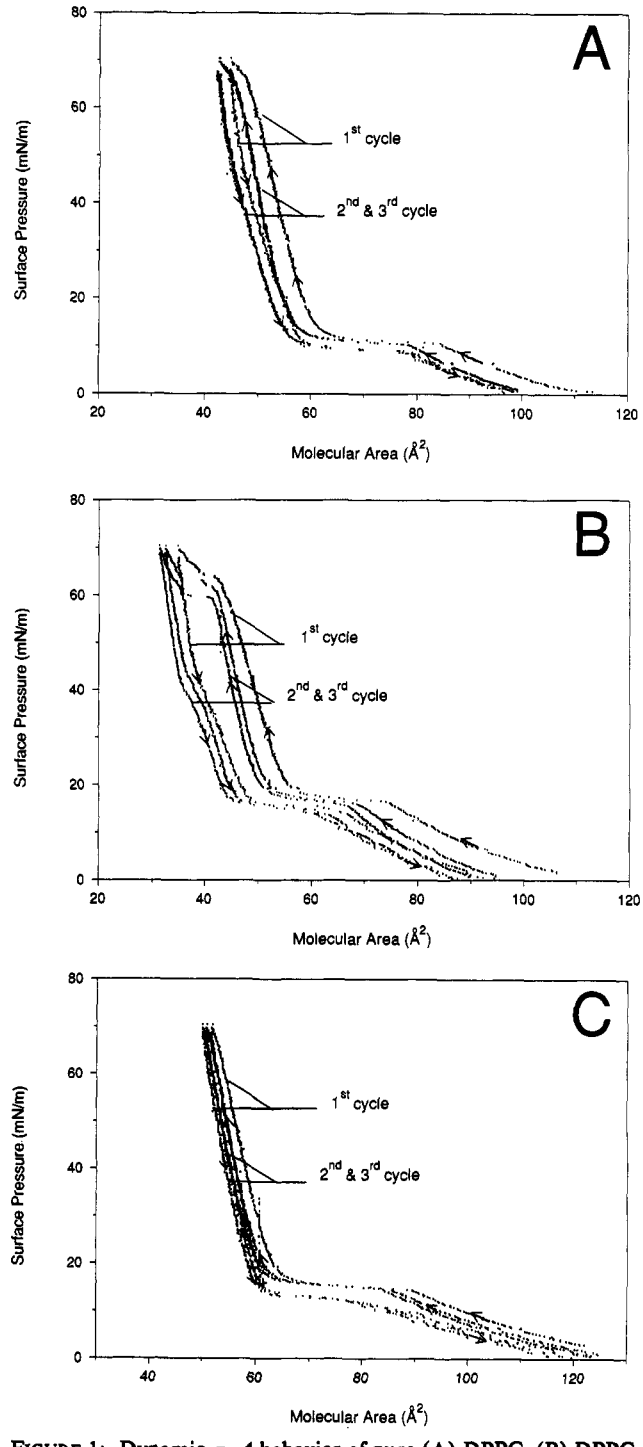

FIGURE 1: Dynamic π–A behavior of pure (A) DPPC, (B) DPPC-d_{62}, and (C) DPPG at the A/W interface for three successive compression–expansion cycles. The monolayer film was not permitted to relax between cycles. The subphase was 150 mM NaCl, pH 5.6. The subphase temperature was held at 22 ± 1 °C.

The monolayers of pure DPPG on a subphase of 150 mM NaCl, pH 5.6, appear to be very stable. Under these

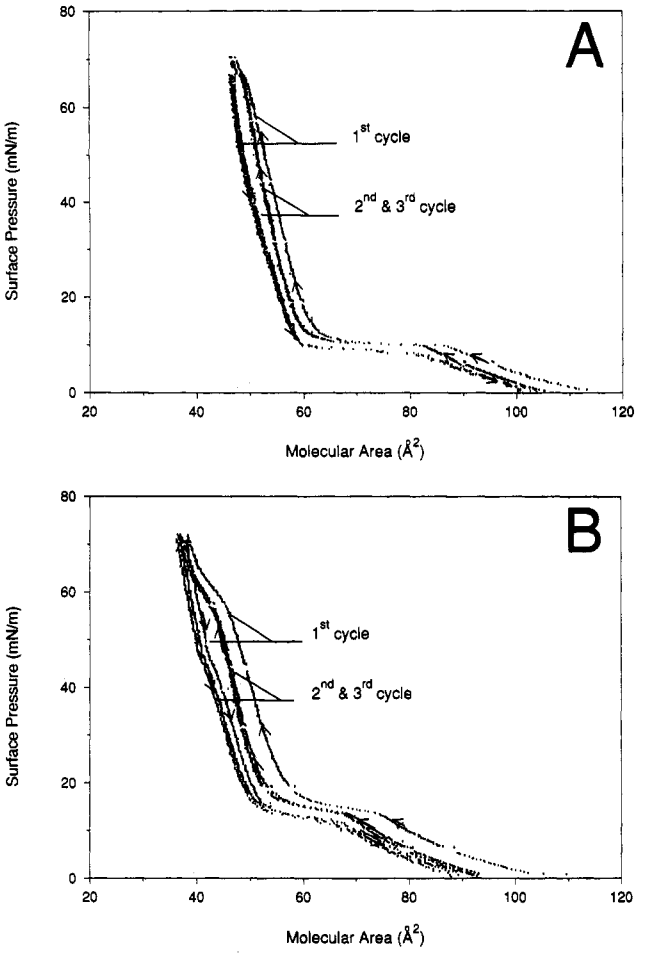

FIGURE 2: Dynamic π–A behavior of 7:1 mixed monolayers of (A) DPPC–DPPG and (B) DPPC-d_{62}–DPPG at the A/W interface for three successive compression–expansion cycles. The monolayer film was not permitted to relax between cycles. The subphase was 150 mM NaCl, pH 5.6. The subphase temperature was held at 22 ± 1 °C.

conditions, DPPG monolayers can be compressed to 72 mN m^{-1} without collapsing. The extent of the hysteresis experienced by the DPPG film as a result of multiple compression–expansion cycles is comparable to DPPC. Our results are consistent with those of Sacré and Tocanne (1977). These workers have shown that the π–A behavior of DPPG is sensitive to the pH and ionic strength of the subphase. At pH 6, only the LC/SC phase is observed for DPPG. The LE phase begins to appear as the salt concentration is increased in the subphase and is stabilized at near 100 mM salt. Under these conditions, the π_t is about 12 mN m^{-1}, which agrees closely with our value of 14 mN m^{-1} obtained at 150 mM NaCl.

Panels A and B of Figure 2 show the π–A behavior of 7:1 (mol/mol) DPPC–DPPG and DPPC-d_{62}–DPPG mixed monolayers, respectively. The 7:1 DPPC–DPPG monolayer film displays π–A behavior that is very similar to pure DPPC films (Figure 2A). The π_t of the DPPC–DPPG 7:1 monolayer is nearly identical to that of pure DPPC, and this mixed monolayer can be compressed up to 72 mN m^{-1} without any evidence of film collapse. However, the extent of the hysteresis observed in the repetitive cycling of the 7:1 DPPC–DPPG monolayer is much less than for pure DPPC films. This suggests that the presence of DPPG may serve to stabilize the DPPC monolayer at the A/W interface. The π–A behavior of a DPPC-d_{62}–DPPG (7:1 mol/mol) mixed monolayer at the A/W interface shows that this binary mixture also forms a stable monolayer film (Figure 2B). The π_t for the DPPC-d_{62}–DPPG 7:1 mixed monolayer is reduced to 14 mN m^{-1} from the 17 mN m^{-1} observed in the single-component DPPC-d_{62} monolayer film. Furthermore, the 7:1 DPPC-d_{62}–DPPC monolayer does not appear to collapse at high surface pressures although an inflection in the π–A isotherm of this mixed monolayer film takes place at 63 mN m^{-1}. As with the 7:1 DPPC–DPPG film, the hysteresis occurring in the 7:1 DPPC-d_{62}–DPPG monolayer for the second and third compression–expansion cycle is less pronounced than for the pure DPPC-d_{62} films.

Binary mixtures of phospholipids in which one component has perdeuterated fatty acyl chains have been used extensively to evaluate the contribution that each component makes to the bulk phase physical properties of the mixture (Dluhy et al., 1985). We wish to extend this approach to monolayer films, and use a 7:1 binary mixture of DPPC-d_{62}–DPPG to examine the structural and compositional changes occurring at the A/W interface as a result of dynamic film compression and expansion for a phospholipid mixture which mimics the PC–PG composition of mammalian pulmonary surfactant (King, 1984). While the π–A behavior of the 7:1 DPPC–DPPG and DPPC-d_{62}–DPPG mixed monolayers is generally the same, some notable differences do exist. In particular, the 7:1 DPPC-d_{62}–DPPG monolayer is more expanded and undergoes greater hysteresis than the 7:1 DPPC–DPPG film. Also, the structural rearrangement occuring in the 7:1 DPPC-d_{62}–DPPG monolayer film at high surface pressures is more pronounced and occurs at lower π values than for the 7:1 DPPC–DPPG mixture. Significantly, the 7:1 DPPC-d_{62}–DPPG monolayer does not collapse at 72 mN m^{-1} (vide infra). Therefore, even with the minor differences in surface chemistry properties between the proteated and deuterated binary phospholipid mixtures, the 7:1 DPPC-d_{62}–DPPG mixture can serve as a suitable surfactant model, providing relevant insight into the structural and compositional fate of each component in the mixture.

Effect of Dynamic π–A Behavior on the Composition of DPPC-d_{62}–DPPG 7:1 Mixed Monolayers. Changes occurring in the surface composition of the DPPC-d_{62}–DPPG 7:1 mixed monolayers as a result of film compression–expansion were monitored using ATR-IR spectroscopy. Results from this laboratory have demonstrated that ATR-IR spectroscopy of monomolecular Langmuir–Blodgett films can be used to measure the composition of binary mixtures of phospholipids at the A/W interface in which the fatty acyl chains of one of the components are perdeuterated (Rana et al., 1993). This approach involves transferring the monolayer film at the A/W interface to a Ge ATR crystal using L–B methods, and examining the IR spectra of the resulting monolayer film. Using an analysis method described below, the relative integrated intensity ratios of the C–H vs C–D stretching bands yield the mole fraction of each species present at the A/W interface. Although the asymmetric stretching C–H and C–D bands are more intense, the symmetric C–H and C–D stretching bands are better suited for this analysis, since these bands arise from isolated vibrational modes. The intensities of the asymmetric C–H and C–D stretching bands contain overlapping contributions from a broad Fermi resonance band due to the C–H or C–D bending modes (Bunow & Levin, 1977; Snyder et al., 1982).

Isotopic substitution not only causes wavenumber shifts in IR bands, but also influences the intensity of IR bands (Pinchas & Laulicht, 1971). Therefore, in order to use the analysis of C–H and C–D band intensities to determine the fractional composition of binary mixtures, the relative absorption

coefficients of the C–H vs C–D stretching bands must be taken into account. While the alteration in the intensity of vibrational bands upon deuterium substitution is simply described for diatomic systems in the gas phase, the theory becomes complex for polyatomic molecules. We have therefore empirically determined the effect of fatty acyl chain perdeuteration on the integrated intensity of the C–D symmetric stretching band of DPPC-d_{62}.

Our method of determining fractional composition from the IR C–D vs C–H relative integrated areas uses a series of DPPC-d_{62}–DPPG binary mixture standard solutions made at different mole ratios. The IR spectra of these binary multilayer films were obtained along with the ^{31}P NMR spectra of these standards in solution. The ratioed integrated intensities of the C–D vs C–H symmetric stretching bands (A_{CD}/A_{CH}) obtained from the ATR-IR spectra of the lipid multilayers in the DPPC-d_{62}–DPPG binary mixtures were then compared with the corresponding integrated intensity ratios of the PC–PG peaks in the high-resolution ^{31}P NMR spectra of these solutions. This IR–NMR comparison method essentially establishes a calibration curve for the IR C–D:C–H integrated intensity ratios in the DPPC-d_{62}–DPPG binary mixture vs mole ratio as established by the NMR integrated intensity ratio. A detailed description of the methodology employed in this analysis will be published elsewhere (Rana et al., 1993). Integrated band areas were calculated between wavenumber limits of 2870 and 2825 cm^{-1} for the symmetric C–H stretching band, and between 2125 and 2045 cm^{-1} for the symmetric C–D stretching vibration. Under these conditions, we found the ratio of the integrated intensities of the symmetric C–D vs C–H stretching bands (A_{CD}/A_{CH}) for a 50/50 mol % mixture of DPPC-d_{62}–DPPG in multilayer form was 1.05. In addition, plotting A_{CD}/A_{CH} vs mole fraction as determined by NMR for a series of DPPC-d_{62}–DPPG binary mixtures leads to a linear relationship with the slope of the best-fit line equal to 1.07 and a regression coefficient (r^2) equal to 0.993172 (Rana et al., 1993).

The exact composition of the 7:1 DPPC-d_{62}–DPPG spreading solutions was determined using high-resolution ^{31}P NMR spectroscopy prior to forming the monolayer films at the A/W interface. High-resolution ^{31}P NMR spectroscopy is uniquely well suited for quantifying the relative molar amounts of individual phospholipid classes on the basis of head-group structure in a mixture of phospholipids. In the presence of a line-narrowing reagent, base-line resolution is achieved over a very narrow chemical shift region for the ^{31}P NMR signals arising from most of the phospholipid classes (Meneses & Glonek, 1988). Figure 3 shows a representative high-resolution, proton-decoupled ^{31}P NMR spectrum of a typical 7:1 DPPC-d_{62}–DPPG spreading solution. The well-resolved signals at –0.84 and 0.57 ppm (ppm measured with respect to 85% phosphoric acid) arise from the phosphorus nuclei of DPPC-d_{62} and DPPG, respectively.

L–B monolayer film transfers onto Ge ATR crystals were performed on the 7:1 DPPC-d_{62}–DPPG mixed monolayers at 40, 60, and 70 mN m^{-1} during the first compression. The surface monolayer film was also transferred to the ATR crystals at 70 mN m^{-1} during the fourth compression after the film had experienced three compression–expansion cycles. For the multiple compression–expansion cycles, the monolayer film was compressed to 72 mN m^{-1}. The measured transfer ratios for the L–B monolayer transfers on the fourth compression cycle were unity. The fractional composition of the PC and PG components in the transferred monolayer was calculated using the A_{CD}/A_{CH} ratio obtained from the monolayer spectrum in combination with the ^{31}P NMR–IR intensity ratio correlations established from the bulk phase.

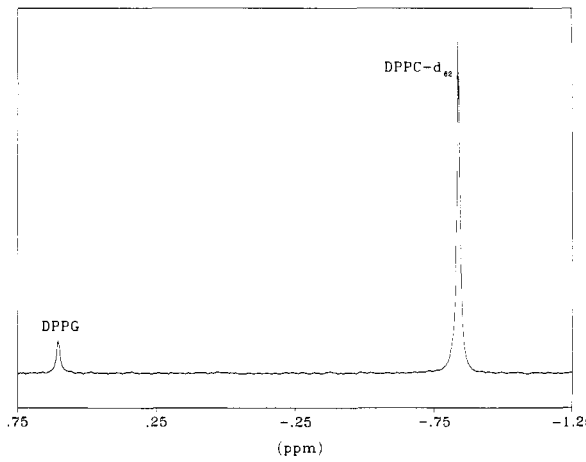

FIGURE 3: High-resolution ^{31}P NMR spectrum of a typical 7:1 (mol/mol) DPPC-d_{62}–DPPG spreading solution used to form the monomolecular films at the A/W interface.

Representative ATR-IR spectra between 3200 and 2000 cm^{-1} are shown in panels A, B, C, and D of Figure 4 for monomolecular films transferred at 40, 60, and 70 mN m^{-1} during the first compression and at 70 mN m^{-1} during the fourth compression, respectively. In Figure 4, the bands between 3000 and 2800 cm^{-1} arise from the C–H stretching vibrations of the DPPG hydrocarbon chains, whereas the bands occurring between 2250 and 2050 cm^{-1} are due to the C–D stretching modes of the perdeuterated fatty acyl chains of DPPC-d_{62}. The symmetric C–H and C–D methylene stretching bands are centered near 2850 and 2088 cm^{-1}, respectively. It is evident from the spectra that the relative levels of DPPC-d_{62} and DPPG in the monomolecular film are unaltered by compression to 70 mN m^{-1} and by multiple compression–expansion cycles. The results of these experiments are summarized in Table I which shows the mole fractions of DPPC-d_{62} and DPPG determined from the ATR-IR spectra of monomolecular L–B films with respect to the composition of the corresponding spreading solutions determined two ways: (i) from inorganic phosphorus assay of the individual stock solutions; (ii) from ATR-IR and high-resolution ^{31}P NMR spectroscopy of the original spreading solution. Clearly, our data indicate that the monolayer film at the A/W interface does not become enriched with the DPPC-d_{62} component with increasing surface pressure or as a consequence of multiple compressions.

Effect of Dynamic π–A Behavior on the Structure of DPPC-d_{62}–DPPG 7:1 Mixed Monolayers. The positions of the conformationally sensitive asymmetric and symmetric C–H and C–D stretching bands centered near 2920 and 2850 cm^{-1} and 2193 and 2088 cm^{-1}, respectively, have been used to empirically monitor the thermotropic phase transitions of phospholipid dispersions in the bulk aqueous phase (Cameron & Dluhy, 1986). The peak positions of the C–H stretching bands have also been used successfully to characterize the surface phase transitions occurring in monolayer films located at the A/W interface using in situ external reflectance IR spectroscopy (Mitchell & Dluhy, 1985; Hunt et al., 1989; Dluhy et al., 1989) or in L–B films transferred onto Ge ATR crystals (Dluhy & Cornell, 1991). Decreases in the peak position of the C–H and C–D stretching bands have been correlated with an increase in the relative number of trans conformers in the lipid fatty acyl chains, and therefore reflect an ordering of the lipid molecules (Cameron & Dluhy, 1986).

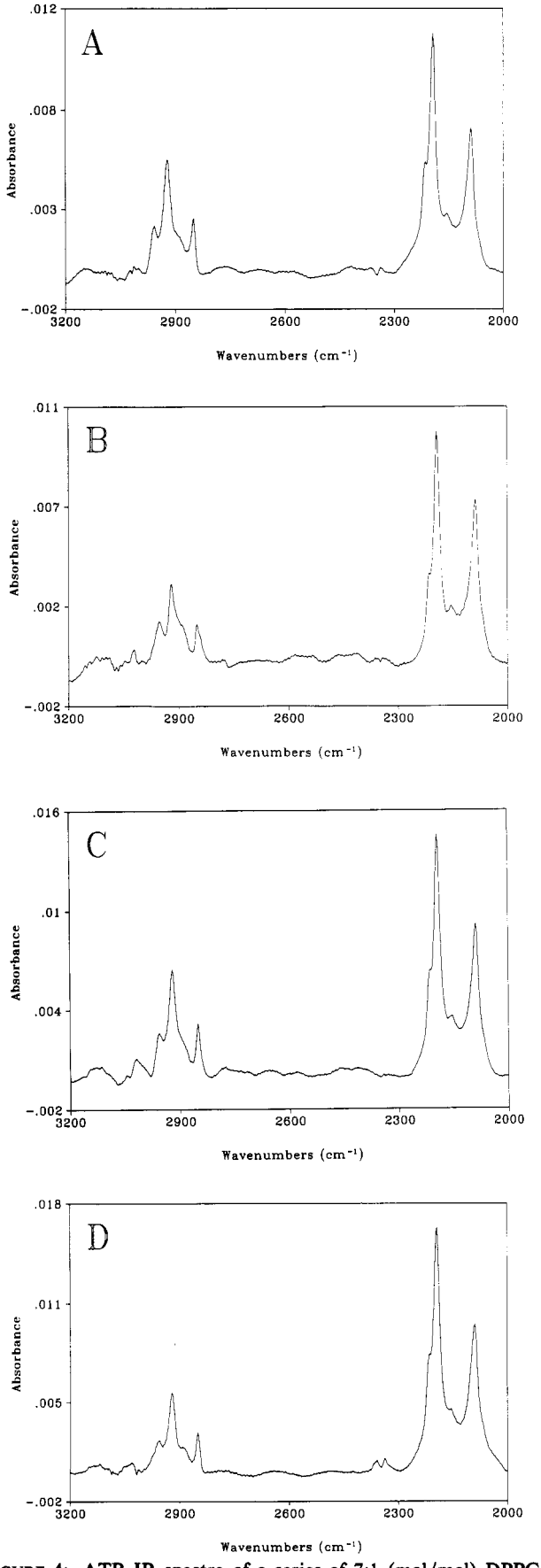

FIGURE 4: ATR-IR spectra of a series of 7:1 (mol/mol) DPPC-d_{62}–DPPG mixed monolayers transferred from the A/W interface to Ge ATR crystals. The surface pressures and compressions of transfer were the following: (A) 40 mN m^{-1}, first compression; (B) 60 mN m^{-1}, first compression; (C) 70 mN m^{-1}, first compression; and (D) 70 mN m^{-1}, fourth compression.

Table I: Effect of Film Compression on the Composition of DPPC-d_{62}–DPPG (7:1 mol/mol) Binary Monolayer Films

surface pressure (mN m^{-1})	no. of cycles	method	mol % DPPC-d_{62}	mol % DPPG
40	1	P assay	88	12
		^{31}P NMR	90	10
		IR(multilayers)	92 ± 1[a] (2)[b]	8 ± 1 (2)
		IR(LB monolayers)	86 ± 4 (5)	14 ± 4 (5)
60	1	P assay	88	12
		^{31}P NMR	90	10
		IR(multilayers)	90	10
		IR(LB monolayers)	93	8
70	1	P assay	88	12
		^{31}P NMR	90	10
		IR(multilayers)	90	10
		IR(LB monolayers)	87 ± 3 (4)	13 ± 3 (4)
70	4	P assay	88	12
		^{31}P NMR	90	10
		IR(multilayers)	91 ± 1 (3)	9 ± 1 (3)
		IR(LB monolayers)	90 ± 2 (4)	10 ± 2 (4)

[a] Uncertainties are expressed as standard deviation. [b] Numbers in parentheses are the number of independent samples used in the calculation of the standard deviation.

Table II: Effect of Film Compression on the Peak Position of the C–H and C–D Stretching Vibrations in Mixed Monolayer Films of DPPC-d_{62}–DPPG (7:1 mol/mol)

surface pressure (mN m^{-1})	no. of cycles	C–D ν_{asym} (cm^{-1})	C–H ν_{asym} (cm^{-1})
40	1	2193.53 ● 0.32[a] (5)[b]	2921.28 ± 0.44 (5)
60	1	2193.47 ● 0.048 (3)	2920.81 ± 1.34 (3)
70	1	2193.39 ± 0.10 (4)	2920.51 ± 0.35 (4)
70	4	2193.34 ± 0.34 (4)	2920.90 ± 0.34 (4)

[a] Uncertainties are expressed as standard deviation. [b] Numbers in parentheses are the number of independent samples used in the calculation of the standard deviation.

In order to characterize the structural changes taking place within the mixed monolayer film at high surface pressures, we have monitored the frequencies of the C–H and C–D stretching vibrations. Table II shows the peak positions of the asymmetric C–H and C–D stretching bands for 7:1 DPPC-d_{62}–DPPG mixed monolayers transferred onto Ge ATR crystals from the LC phase at surface pressures above and below 63 mN m^{-1} in the first compression–expansion cycle. The frequency of these bands was also determined at 70 mN m^{-1} for the fourth compression in order to monitor changes taking place in the film as a result of multiple compression–expansion cycles.

In the condensed monolayer phase, the conformation of the DPPC-d_{62} fatty acyl chains is essentially unchanged as the monolayer compressed from 40 to 72 mN m^{-1}. The observed wavenumbers of the C–D asymmetric stretching band are typical for perdeuterated phospholipids in the bulk gel phase (Dluhy et al., 1985) or in the condensed monolayer phase (F. R. Rana and R. A. Dluhy, unpublished results), indicating that the DPPC-d_{62} fatty acyl chains are in a nearly all-trans geometry. The observed wavenumbers of the C–H stretching vibrations for the DPPG component of the binary mixture also indicate an ordered structure for the PC protreated hydrocarbon chains. The wavenumber peak position of the asymmetric C–H stretching band decreases by about 0.8 cm^{-1} as the 7:1 DPPC-d_{62}–DPPG monolayer is compressed from 40 to 72 mN m^{-1}. This small shift in the wavenumber peak position may be related to a solid–solid domain transition among ordered hydrocarbon chains that has been described by syncrotron X-ray diffraction for monolayer films of DPPA (i.e., 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid) at the

A/W interface under conditions of high surface pressure (Kjaer et al., 1987). However, the significance of such a small, but reproducible, wavenumber shift in the C–H stretching bands of the DPPG component of the binary mixture is unclear at present.

The orientation of the DPPC-d_{62} and DPPG fatty acyl chains in the mixed monolayer was determined from the IR dichroism of the symmetric and asymmetric C–H and C–D stretching bands using procedures which have been previously described (Cornell et al., 1989). The orientational distribution for the lipid acyl chains is described by the average tilt angle, θ, formed between the lipid fatty acyl chain and the surface normal. Analysis of the dichroic ratios in the C–H region for the DPPG component of the transferred monolayer shows that the proteated fatty acyl chains of DPPG are oriented perpendicular to the plane of the Ge crystal. This orientation is observed irrespective of whether the monolayer transfer was made at surface pressures above or below the π–A inflection observed at 63 mN m^{-1}. This same result is obtained for the DPPC-d_{62} component in the monolayer. That is, the dichroic ratios in the C–D stretching bands indicate that the deuterated acyl chains of DPPC-d_{62} are oriented perpendicular to the ATR crystal surface. This orientation is also found at all surface pressures studied between 40 and 72 mN m^{-1}.

DISCUSSION

In recent years, biophysical approaches have been used to study the functional mechanism of pulmonary surfactant. This work is beginning to yield insight into the molecular-level interactions occurring in pulmonary surfactant. A central issue in surfactant physiology that has yet to be proven is the validity of the "squeezing-out" hypothesis. An important test of this hypothesis would be to unambiguously measure the compositional changes taking place at the A/W interface upon film compression for well-defined model systems. As described in this paper, we have recently developed a combined spectroscopic approach which can be used to directly test the squeeze-out hypothesis using defined phospholipid mixtures. By systematically applying this approach, it should be possible to identify which, if any, monolayer components are driven from the interface upon monolayer compression. In the current study, we have attempted to monitor the fate of a saturated PG component in a mixed PC–PG monomolecular film by using ATR-IR spectroscopy to observe the compositional and structural changes taking place at the A/W interface for a 7:1 (mol/mol) binary mixture of DPPC-d_{62}–DPPG when this film experiences multiple compression–expansion cycles.

The evidence for the enrichment of DPPC at the A/W interface during surface compression at the expense of other lipid components in phospholipid mixtures is indirect and based on the π–A behavior of mixed monolayers of DPPC with unsaturated PG's or PC's (Watkins, 1968; Hildebran et al., 1979; Bangham et al., 1979; Notter et al., 1980a,b; Hawco et al., 1981; Fontanges et al., 1985; Boonman et al., 1987). These π–A curves typically show a plateau at intermediate surface pressures which corresponds to the collapse pressure of the unsaturated lipid component of the mixture. Successive compression–expansion cycles of these binary mixtures produce a hysteresis in the π–A isotherms characterized by displacements to lower molecular areas. This behavior is interpreted to indicate that the fluid lipid has been driven out of the interface at high surface pressures as a result of film collapse. The present experiments, however, clearly show that this is not the case for the DPPC-d_{62}–DPPG binary mixture. The IR spectra of the transferred monolayer film show that the surface composition of the PC–PG mixed monolayer does not change as a result of either compression up to 70 mN m^{-1} or upon multiple compression–expansion cycles (Figure 4 and Table I).

Selective "squeeze-out" of phospholipid monolayer components from the A/W interface requires the formation of surface domains that are enriched in DPPC. The work of El Mashak et al. (1982) indicates that, at $\pi < 50$ mN m^{-1}, lateral phase segregation does not occur for mixed monolayers of PC and PG when both components have saturated fatty acyl chains. Monolayers formed from saturated PC's and PG's appear to be highly miscible regardless of the pH, the ionic strength, or the presence of divalent cations such as Ca^{2+}.

The IR data presented here unambiguously show that each component of this mixed monolayer exists in a highly ordered and oriented conformation irrespective of surface pressure above 40 mN m^{-1}. There is no evidence for the selective enrichment of the PC component of this monolayer at the expense of the PG component. For the case of saturated PC and PG monolayer components at physiological mole ratios, at least, the so-called "squeeze-out" model of surfactant physiology does not seem to apply. These results imply that saturated PG components of native pulmonary surfactant may remain at the air–alveolar interface when the surface tension of the film approaches zero. In some isolates, these saturated PG components have been identified as the major PG constituent (King, 1984). This is consistent with the π–A behavior of pure DPPG at physiological ionic strengths which form stable films that can be compressed to 72 mN m^{-1} (Figure 1C). Pastrana et al. (1991) have argued that the dynamic surface behavior of DPPC–DPPG 7:1 (w/w) mixed monolayers containing 5% surfactant protein C (SP-C) indicates that none of the components are lost from the surface. However, quantifying the levels of individual surface components was not possible in this study. From the hysteresis curves, it appears as if DPPG stabilizes both DPPC and DPPC-d_{62} monolayers when present at levels that approach 10 mol % (Figure 2A,B). Collectivley, these data suggest that the role of saturated PG's in native pulmonary surfactant may be to provide additional stability to the DPPC components at the alveolar–air interface. We are currently in the process of characterizing the dynamic compositional and structural behavior of unsaturated PG components in DPPC–PG mixed monolayers.

One study similar to this one has previously been reported by Chung et al. (1990). In this study, the compositional changes in a 1:1 mixed monolayer film of DPPC–egg yolk PG and DPPC-d_{62}–egg yolk PG were measured using ATR–IR spectroscopy of L–B films. A surface enrichment of DPPC and DPPC-d_{62} from 50 to 80 mol % was observed to occur as the films were compressed from 20 to 60 mN m^{-1}. In contrast to our work, the PG component used in this study was comprised of predominantly heterogeneous unsaturated fatty acids and was present at 50 mol % in the binary mixture. This fact, by itself, may completely account for the different results obtained in the two studies.

Several other aspects of the work of Chung et al. (1990), however, make direct comparison with our current study problematic. Two primary differences between this current IR study and the previous one involve the nature of the monolayer transfers and the method used to correlate the observed IR intensities with fractional composition. In the first case, Chung et al. reported transfer ratios of between 3 and 5, even at low surface pressures. This result indicates that these researchers were studying an uncharacterized

multilayer sample, rather than the ordered monolayers under consideration here. Second, their method of correlating measured IR peak heights with fractional composition involves an indirect determination of the number density of surface methylene groups, while the method described here relies on an exact calibration of the IR integrated areas with a mole fraction which is experimentally determined using high-resolution ^{31}P NMR spectroscopy. Both of these conditions are likely to contribute to the observed experimental differences between the two studies.

As demonstrated in this paper, the coupling of L–B techniques with ATR-IR spectroscopy is a powerful approach for monitoring the dynamic film composition of monolayers at the A/W interface. Any multicomponent quantitative analysis method where the absolute absorption constants for the components are unknown requires that proper calibrations are performed. In our case, this can be accomplished by using solution-phase, high-resolution ^{31}P NMR spectroscopy of the spreading solutions in conjunction with ATR-IR spectroscopy. We are currently applying this technique to systematically determine if other phospholipid or protein components are excluded from the A/W interface at high surface pressures.

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