Pulmonary Surfactant Proteins SP-B and SP-C in Spread Monolayers at the Air-Water Interface: I. Monolayers of Pulmonary Surfactant Protein SP-B and Phospholipids

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ABSTRACT The effects of pulmonary surfactant protein SP-B on the properties of monolayers of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG), and a mixture of DPPC:DPPG (7:3, mol:mol) were studied using spread films at the air-water interface. The addition of SP-B to the phospholipid monolayers gave positive deviations from additivity of the mean areas in the films. At low protein concentrations (less than 45% amino acid residues which corresponds to 0.5 mol% or 10 weight% SP-B) monolayers of SP-B/DPPC, SP-B/DPPG and SP-B/(DPPC:DPPG) collapsed at surface pressures of about 70 $mN \cdot m^{-1}$, comparable to those of the lipids alone. At higher concentrations of SP-B in the protein-lipid monolayers, kink points appeared in the isotherms at about 40–45 $mN \cdot m^{-1}$, implying possible exclusion of material from the films, hence, changes in the original monolayer compositions. Calculated analyses of the monolayer compositions as a function of surface pressure indicated that nearly pure SP-B, associated with small amounts of phospholipid (2–3 lipid molecules per SP-B dimer), was lost from SP-B/DPPC, SP-B/DPPG, and SP-B/(DPPC:DPPG) films at surface pressures higher than 40–45 $mN \cdot m^{-1}$. The results are consistent with a low effectiveness of SP-B in removing saturated phospholipids, DPPC or DPPG, from the spread SP-B/phospholipid films.

SYMBOLS

A_l^0	surface area per lipid molecule at a given surface pressure in the monolayer of pure lipid
$\bar{A}_l'$	apparent area per lipid molecule in the protein-lipid monolayer
$\bar{A}_l$	partial "residual" (molar) area per lipid molecule in the protein-lipid monolayer
A_{mean}	mean area per "residue" in the protein-lipid film, where "residue" denotes a lipid molecule or a protein amino acid residue
A_{mean}^{id}	mean area per "residue" expected in a protein-lipid film with an ideal mixing of the components
A_r^0	surface area per protein amino acid residue at a given surface pressure in the monolayer of pure protein
$\bar{A}_r$	partial area per protein amino acid residue in the protein-lipid film
E	surface elasticity
N_l	number of lipid molecules in the protein-lipid film determined from the original amount of lipid spread at the interface at $\pi \approx 0$ $mN \cdot m^{-1}$
N_l^{lost}	number of lipid molecules lost from the protein-lipid monolayer during squeeze-out of material at $\pi \geq \pi_{kink}$
N_r	number of protein amino acid residues in the protein-lipid monolayer, determined from the original amount of spread protein at $\pi \approx 0$ $mN \cdot m^{-1}$
N_r^{calc}	number of protein amino acid residues calculated to be present in the protein-lipid film at $\pi > 0$ $mN \cdot m^{-1}$
X_{DPPC}	molar fraction of DPPC in the binary DPPC:DPPG monolayers
X_l	"residual" or molar fraction of lipid molecules in the protein-lipid film, determined from the original amount of spread lipid
X_r	"residual" fraction of protein amino acid residues in the protein-lipid monolayer determined from the original amount of spread protein
X_r^{calc}	"residual" fraction of protein amino acid residues in the protein-lipid monolayer determined from the number of the amino acid residues calculated to be present in the film, N_r^{calc}
X_r^{lost}	"residual" fraction of the protein amino acid residues in the material lost from the protein-lipid monolayer during its compression at $\pi \geq \pi_{kink}$
π	surface pressure, defined as $\pi = \sigma_o - \sigma$, σ and σ_o are the surface tensions at the water-air interface with and without monolayer, respectively
π_{kink}	surface pressure corresponding to the kink point in an isotherm

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Abbreviations used: DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; SP-B, pulmonary surfactant associated protein (M_r 17 400); SP-C, pulmonary surfactant associated protein (M_r 4 186)

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INTRODUCTION

Pulmonary surfactant is a surface active material composed of lipids and proteins which lines the alveolar epithelium and contributes to maintaining the structural stability of the alveolus during respiration (Von Neergaard, 1929). This function is accomplished via the surface tension-reducing properties of the lipid or lipid-protein film at the air-water interface in the alveoli (Clements et al., 1961). Deficiency of

pulmonary surfactant has been shown to cause respiratory distress syndrome (RDS) in premature infants (Avery and Mead, 1959). Over a decade ago a lipid extract of surfactant, now known to contain hydrophobic surfactant-associated proteins SP-B and SP-C, was shown to considerably enhance the ventilatory characteristics of surfactant-deficient lungs when it was administered intratracheally to infants with RSD (Fujiwara et al., 1980). This effect has been confirmed in a large number of subsequent studies.

A possible function for the surfactant proteins is to alter the packing arrangement of lipid bilayer structures in the alveolar hypophase so as to facilitate their adsorption and spreading at the alveolar air-water interface. Thus, SP-B and SP-C when present in phospholipid vesicles, enhanced the adsorption rates of phospholipids to the air-water interface (Hawgood et al., 1987; Yu and Possmayer, 1990). Another potential function for the proteins could be to facilitate the exclusion of lipid from a monolayer under dynamic compression. It is considered that these two functions are possibly important for normal surfactant dynamics in the alveolar space (e.g., Keough, 1992). Also, when present in preformed lipid monolayers, SP-B and SP-C induced insertion of phospholipids from subsurface vesicles into the surface film (Oosterlaken-Dijksterhuis et al., 1991a).

The molecular nature of interaction between the surfactant phospholipids and the hydrophobic proteins in bilayer model systems (vesicles) has been investigated by various experimental methods (Baatz et al., 1990; Elledge and Whitsett, 1989; Pastrana et al., 1991; Shiffer et al., 1993; Simatos et al., 1990; Vandenbusshe et al., 1992). The results of these biophysical studies are consistent with the hydrophobic proteins altering the packing of phospholipid bilayers both through electrostatic and hydrophobic interactions with the lipid.

Surface balance technique was used for studying the monolayer properties of the hydrophobic surfactant-associated proteins and their mixtures with lipids at the air-water interface (Oosterlaken-Dijksterhuis et al., 1991b). An increase in mean molecular area was observed in monolayers consisting of dipalmitoylphosphatidylcholine (DPPC) and SP-B or SP-C. Also, epifluorescent surface balance measurements showed that in spread SP-C/DPPC monolayers the protein decreased the packing efficiency in the lipid array (Perez-Gil et al., 1992).

Surfactant protein SP-B is a homo-dimer of disulphide-linked 79-residue monomers (Johanson et al., 1991). Its sequence, like that of the protein SP-C, is highly conserved in a number of species. The protein is soluble in organic solvents and insoluble in aqueous ones. It has an excess of positively charged side chains. Calculations based on its sequence, and spectral studies of the protein and some of its partial peptides, indicated that there are likely a number of amphipathic helical regions in the protein (Pastrana et al., 1991; Vandenbusshe et al., 1992; Takahashi et al., 1990).

The questions addressed in this work are (i) how does the hydrophobic pulmonary surfactant protein SP-B behave at the air-water interface and (ii) how does it affect the properties of monolayers of two surfactant phospholipids-zwitterionic DPPC and negatively charged DPPG. DPPC is the major phospholipid (about 55% of the total phospholipid) and PG is the major acidic phospholipid (up to about 12% of the total phospholipid) in pulmonary surfactant (Yu et al., 1983).

A study of the interfacial behavior of the lung surfactant proteins alone or mixed with phospholipids will provide insight into their potential roles in the surface related phenomena in alveoli during the respiration cycle. In addition, a study of interactions of the surfactant hydrophobic proteins, SP-B and SP-C, with phospholipids may be relevant to other lipoprotein systems, such as serum lipoproteins, myelin proteolipid and basic proteins, among others. Fundamentals of the interactions are also most likely to be governed by the same forces as exist in membrane systems.

EXPERIMENTAL PROCEDURES

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine was purchased from Sigma Chemical Co. (St. Louis, MO) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) from Avanti Polar Lipids Inc. (Pelham, AL). The lipids were found to be pure by thin-layer chromatography and were used as received.

Protein isolation

Pig lungs were lavaged two times with 0.15 M NaCl and the lavage was centrifuged at 800 × *g* for 10 min. The supernatant was centrifuged at 8000 × *g* for 60 min and the surfactant pellet was obtained. A lipid extract of the pellet was made (Bligh and Dyer, 1959). The extract was purified by successive exclusion column chromatography on Sephadex LH-20 (Pharmacia, Uppsala) in chloroform:methanol 2:1 (v/v), and twice on Sephadex LH-60 (Pharmacia, Uppsala) in chloroform:methanol 1:1 (v/v) containing 5 v% of 0.1 M HCl, in a manner similar to that described by Curstedt et al. (Curstedt et al., 1987). The purity of the proteins was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Laemmli, 1970). The stacking gel was 4% polyacrilamide, 0.1% SDS, 0.125 M Tris, pH 6.8, and the separating gel was 16% polyacrilamide, 0.1% SDS and 0.375 M Tris, pH 8.8. The running buffer was 0.1% SDS, pH 8.3. The samples, dried from chloroform:methanol under nitrogen, were dissolved in 2.5% SDS, 0.0625 M Tris, pH 8.4, containing 0.01% pyronine B and were boiled for 10 min. Gels were subjected to electrophoresis in a Mini-Protean II Slab Cell (Bio-Rad) electrophoresis apparatus at a constant voltage of 200 V for 45 min. Gels were stained with silver stain (New England Nuclear Research Products, Boston, MA). The apparent molecular masses were determined using low molecular weight markers (Bethesda Research Laboratories, Bethesda, MD). Under nonreducing conditions SP-B yielded a major band at about 18 kDa and a minor one at about 29 kDa. SP-C showed one band at about 5 kDa. No cross contamination of the proteins was observed.

Protein was routinely determined by the fluorescamine assay (Udenfriend et al., 1972) which gave similar results to those obtained by quantitative amino acid analysis. In the latter procedure the protein was hydrolyzed for 4 h at 150° with 12 M HCl/trifluoroacetic acid 2:1 (v/v) with 5% mercaptoacetic acid in vacuum (Sarin et al., 1990). Incubation for 8 h gave similar results. Protein estimates were made from the contents of the most readily released stable amino acids (Gly, Ala, Lys) and the published sequence of SP-B (Curstedt et al., 1988). The estimates for the proteins obtained by this procedure were consistent with those obtained under hydrolysis conditions usually employed for the hydrophobic surfactant proteins (e.g., Curstedt et al., 1988). Estimation of the phospholipid content of the protein (Bartlett, 1959) indicated less than 0.5 mol of phospholipid per mol of SP-B, which was the detection limit of the lipid determination.

Monolayer technique

Lipid monolayers were formed from chloroform (for DPPC) or chloroform: methanol 3:1 (v/v) (for DPPG) solutions. Chloroform:methanol 1:1 (v/v) was employed as a spreading solvent for SP-B. Lipid-protein monolayers were spread on the surface from premixed solutions of the components. All monolayers were formed at an initial surface pressure of less than 1 $mN \cdot m^{-1}$. 10 min after spreading the monolayers were compressed in a stepwise fashion at a speed of 12 cm^2/min. The total compression took 30 min. All measurements were done at room temperature (22 ± 1°C) on a Teflon Langmuir trough (22 × 7.8 × 1.5 cm). The trough has been especially constructed for performing monolayer fluorescence microscopy (Nag et al., 1990). To that purpose, the regions in which surface tension and optical observations were made, were confined in two circular compartments (3.2 cm in diameter) connected to the trough area through two canals (0.75 × 1.9 cm). The liquid subphase consisted of 0.15 M NaCl in deionized doubly distilled water. The pH was adjusted to 7 immediately before each experiment with 0.2 M NaOH, and it did not change by more than 0.9 pH units during the time required to obtain a complete surface pressure-area curve. Surface tension was measured using a roughened platinum Wilhelmy plate, in the epifluorescence surface balance (Nag et al., 1990). Surface pressure (π)-molecular area isotherms were constructed for lipid-protein monolayers of various molar ratios of the components. Each curve shown in the paper represents an average result of measurements of two or three separate monolayers.

The initial composition of the lipid-protein monolayers is given by the fraction of amino acid residues of SP-B:

$$X_r = \frac{N_r}{N_r + N_l}, \tag{1}$$

where N_r and N_l are the numbers of the spread amino acid residues of SP-B and lipid molecules, respectively. Values of M_r = 17,400 and 158 amino acid residues per dimer of SP-B were used for the calculations (Curstedt et al., 1988).

The experimental mean areas per "residue," A_{mean} (where "residue" denotes a phospholipid molecule or an amino acid residue of SP-B) in the lipid-protein monolayers were calculated according to (2):

$$A_{mean} = \frac{\text{trough area}}{N_r + N_l}. \tag{2}$$

The mean area per "residue" at a given surface pressure in an ideal binary surface mixture of a protein and a lipid is represented by the additivity rule:

$$A^{id}_{mean} = X_r \cdot A^0_r + X_l \cdot A^0_l, \tag{3}$$

where A^0_r and A^0_l are the areas per protein amino acid residue or lipid molecule in the single-component films at the same surface pressure; X_r and X_l are the mole fractions of the components (protein amino acid residues or lipid molecules) in the mixed monolayer.

When interaction between the components occurs, the mean molecular area A_{mean} in a binary monolayer system is related to the partial "residual" areas of the components by the following equation:

$$A_{mean} = X_r \cdot \bar{A}_r + X_l \cdot \bar{A}_l, \tag{4}$$

where $\bar{A}_r$ is the partial "residual" area per amino acid residue of SP-B and $\bar{A}_l$ is the partial "residual" area per lipid molecule. $\bar{A}_r$ and $\bar{A}_l$ represent the contribution of each component to the total area in the real two-component monolayer. The partial "residual" areas $\bar{A}_r$ and $\bar{A}_l$ were evaluated using the method of intercepts which is usually employed for calculating the partial molar quantities of two-component bulk solutions (e.g., Moore, 1962). The method consists of drawing a tangent to the mean area per "residue" versus monolayer composition curve, $A_{mean}(X_r)$, at a certain composition and determining the intercept made with the A_{mean} axis. The intercept at $X_r = 0$ represents the partial "residual" area per lipid molecule, $\bar{A}_l$, whereas the intercept at $X_r = 1$ gives the partial "residual" area per amino acid residue of SP-B, $\bar{A}_r$. It is noted that for the lipid, the "residual" quantities are equivalent to molar quantities.

RESULTS AND DISCUSSION

Single component SP-B monolayers

SP-B formed stable insoluble monolayers at the air-water interface. The surface pressure versus area per amino acid residue isotherms for different preparations of SP-B were highly reproducible (e.g., the mean area per amino acid residue at 10 $mN \cdot m^{-1}$ for six separate protein preparations was 0.198 ± 0.032 nm^2/amino acid residue (mean ± SD)). Curve 1 in Fig. 1 represents an average isotherm for SP-B. The isotherm displays a plateau region at about 32 $mN \cdot m^{-1}$. As it was pointed out under Experimental Procedures, all experiments were done with a Teflon trough of a special design that contained a measurement area constructed to reduce surface streaming (Nag et al., 1990). Surface pressure versus area measurements performed more recently with a more conventional Langmuir trough without isolated viewing and measuring areas, showed that under the same experimental conditions (pH, subphase, spreading solvent, velocity of compression) spread monolayers of SP-B displayed a plateau region at higher surface pressures of about 40 $mN \cdot m^{-1}$. In general, protein monolayers display high shear viscosities, and therefore, likely, due to viscosity effects the measurements at higher surface pressures were modified by reduced monolayer flow through the canals in the trough. It is worth noting that such a difference was found only for monolayers of pure SP-B, whereas the isotherms for the protein-lipid mixtures and pure phospholipids were negligibly affected. This is not surprising given the fact that the shear viscosities of protein films are two to three orders of magnitude higher

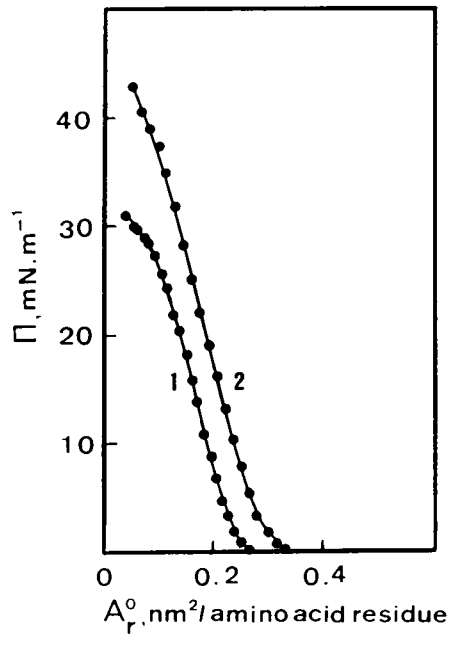

FIGURE 1 Isotherms of surface pressure versus area per amino acid residue for spread monolayers of SP-B, containing less than 0.5 mol of phospholipid per mol of protein (*1*) or 12 mol of DPPC per mol of protein (2). Curves were plotted with the lipid contribution to area on the *x*-axis being ignored.

than the ones of phospholipid films (Colacicco and Scarpelli, 1973; Collacicco et al., 1974).

A $\pi(A_r^0)$ isotherm, identical to the one shown in Fig. 1 (*curve 1*) was measured when the monolayer was formed from chloroform:methanol (2:1, v/v). When methanol was used as a spreading solvent, SP-B gave a more condensed monolayer, with areas per amino acid residue being about 0.04 nm^2/residue lower at all π values. The difference can not be unambiguously interpreted as resulting from solvent-induced conformational changes of the protein because the methanol solubility in the aqueous subphase could compromise the spreading of the protein.

In Table 1 the area occupied by an amino acid residue in the spread films of SP-B (*curve 1*, Fig. 1) was compared with data reported for other amphipathic proteins and polypeptides at the air-water interface (Krebs et al., 1988; Thomas and Ter Minassian-Saraga, 1976). The results show that the characteristic areas per amino acid residue in the spread monolayers of SP-B are consistent with the values found for spread films of other hydrophobic proteins.

The pressure-area characteristics of SP-B found in the present work differ from those previously reported for a porcine SP-B monolayer (Oosterlaken-Dijksterhuis et al., 1991b). We found an area of 0.25 nm^2/amino acid residue corresponding to the lift off in the $\pi(A_r^0)$ curve compared with about 0.75 nm^2/amino acid residue reported previously. The differences in the experimental conditions ($T = 37°C$ in comparison to 22°, 25 mM 4-(hydroxyethyl)-1-piperazine-ethanesulfonic acid as a subphase in comparison to 0.15 M NaCl, compressional velocity of 97.16 cm^2/min as opposed to 12 cm^2/min) could partly account for the observed discrepancy. Some of the difference may be due to various extents of delipidation of SP-B used in the two studies. The amount of 1–2 mol of phospholipid per SP-B molecule reported (Oosterlaken-Dijksterhuis et al., 1991b) represents about 4–8% (w/w). Measurements in this laboratory showed that the extent of delipidation of SP-B and SP-C affected the interfacial properties of the proteins. The isotherm in Fig. 1 (*curve 1*) was measured for preparations of SP-B containing less than 0.5 mol of phospholipid per mol of SP-B. To show the effect of the presence of small amounts of phospholipid on the monolayer of SP-B, DPPC (2, 5, or 12 mol/mol SP-B) was added to the SP-B solution and the isotherms $\pi(A_r^0)$ were measured. In this case the area per amino acid residue of SP-B in the films, A_r^0, were calculated without accounting for the presence of DPPC. As expected, this procedure resulted in an expansion of the $\pi(A_r^0)$ curve for SP-B, e.g., at $\pi = 10$ $mN \cdot m^{-1}$, 2 mol of DPPC gave expansion in the area per amino acid residue of about 4% (result not shown), whereas 12 mol of DPPC resulted in an "expansion" of about 29% (*curve 2*, Fig. 1). A similar effect on the isotherm of SP-B was obtained with 12 mol of DPPG per mol of SP-B.

TABLE 1 Comparison of surface pressure-area characteristics of SP-B to those of other hydrophobic proteins

	Extrapolated area* at $\pi = 0$ $mN \cdot m^{-1}$	Area at lift off in the isotherm
	nm^2/amino acid residue	
Apolipoprotein A-II‡	0.15	0.24
Apolipoprotein A-I‡	0.20	0.28
Myelin proteolipid protein§	0.16–0.17	0.24
SP-B (this paper)	0.22	0.25

* Determined by extrapolation of the linear parts of the surface pressure-area curves to $\pi = 0$.

‡ Krebs et al. (1988).

§ Thomas and Ter Minassian-Saraga (1976).

The results were obtained under different conditions of subphase and spreading solvent. All measurements were done at room temperature.

Mixed monolayers of SP-B and DPPC

DPPC is zwitterionic and forms essentially neutral monolayers over a large range of pH values (Phillips and Chapman, 1968). Fig. 2 shows the surface pressure-mean area per "residue" curves for SP-B/DPPC mixtures of various compositions. For the sake of clarity not all isotherms measured for the lipid-protein mixtures are shown. SP-B/DPPC monolayers of low protein concentrations ($X_r < 0.45$, which corresponds to 0.5 mol% or 10 weight% protein upon an assumption of the dimer form) collapsed at a high surface pressure of about 70 $mN \cdot m^{-1}$, corresponding to the collapse pressure of monolayers of pure DPPC. At higher protein concentrations in the mixed films ($0.45 \leq X_r < 0.80$, or $0.5 \leq$ mol% protein < 2.5) the isotherms exhibited two

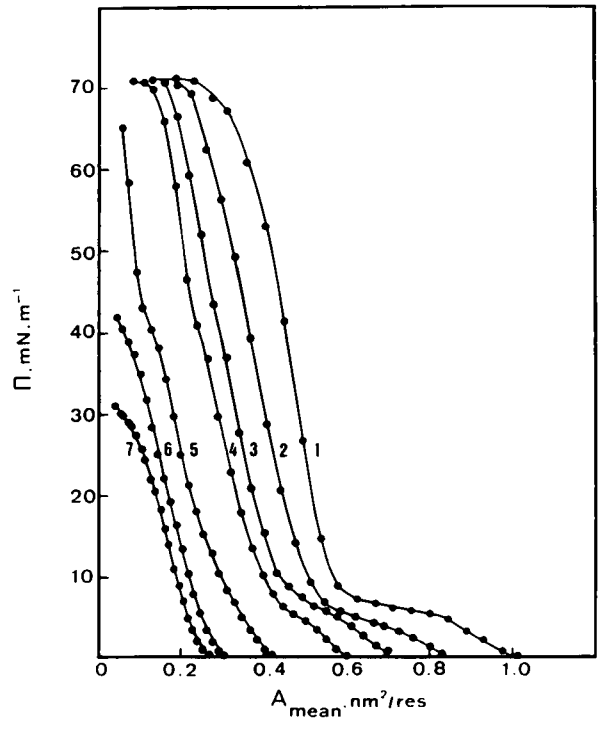

FIGURE 2 Surface pressure π-mean area per "residue," A_{mean}, isotherms for SP-B/DPPC mixtures of various initial compositions X_r: 0.0 (*1*), 0.28 (*2*), 0.45 (*3*), 0.57 (*4*), 0.80 (*5*), 0.92 (*6*), 1.0 (*7*).

collapse points: (i) a kink point at surface pressure of 41 ± 2 $mN \cdot m^{-1}$ independent of the monolayer composition and (ii) a collapse plateau at about 70 $mN \cdot m^{-1}$ corresponding to the collapse pressure of DPPC. This two-step collapse of the binary films is consistent with some separation of the components and suggests that expulsion of either SP-B (the component displaying the lower collapse pressure) or SP-B/lipid units may occur at surface pressures higher than 41 $mN \cdot m^{-1}$. This process would result in changes in the original compositions of the mixed monolayers.

The mean areas per "residue," A_{mean}, in the mixed films were determined from the $\pi(A_{mean})$ curves at certain surface pressures and plotted as a function of the initial monolayer composition in Fig. 3. The broken lines represent the mean area per "residue," A^{id}_{mean}, expected assuming ideal mixing of the components (Eq. 3). The results show that the SP-B/DPPC monolayers exhibit nonideal behavior. This observation is in accord with the expansion effects seen in other binary monolayers of phospholipids and proteins such as hemoglobin and β-casein (Mita, 1989). An interpretation of the expansion effect has been made in terms of hydrophobic interactions between the protein and lipid resulting in perturbation of the lipids and increasing the configurational freedom of their hydrocarbon chains (Mita, 1989). Such an explanation, however, does not exclude possible changes in the conformation of the protein in the mixed films. Our observations are consistent with the recent finding of there being increases in the mean areas per molecule for some mixtures of SP-B or SP-C with DPPC (Oosterlaken-Dijksterhuis et al., 1991b).

Analysis of the $A_{mean}(X_r)$ relationship as a function of the surface pressure (Fig. 3) suggests that, at lower protein concentrations ($X_r \leq 0.57$, equivalent to 0.83 mol% or 17 weight%), the expansion effect of SP-B persists at surface pressures above the collapse pressure for the pure SP-B (*curves b* and *c*). This would be consistent with a presence of the protein in these surface monolayers at the higher pressures. On the other hand, at higher protein concentrations ($X_r \geq 0.80$) at the same surface pressures, the experimental mean areas per "residue" are equal to or lower than those expected for ideal mixing of the components. That finding suggests that either pure SP-B or SP-B associated with some DPPC was lost from the surface. In either case, for films of higher initial protein concentration ($X_r \geq 0.45$), changes from the initial compositions may be expected when the films are compressed to high surface pressures ($\pi \geq 45$ $mN \cdot m^{-1}$).

To evaluate the change in the initial composition of the binary films as a function of the surface pressure the following approach was used. First, for a SP-B/DPPC monolayer of a given composition, the apparent area per DPPC molecule, A'_1, was deduced at different surface pressures by dividing the trough area by the number of the spread lipid molecules only (i.e., assuming $N_r = 0$ in Eq. 2). Curve 1 in Fig. 4 shows a typical result of the $\pi(A'_1)$ relationship (the initial composition of the monolayer was $X_r = 0.45$). Then

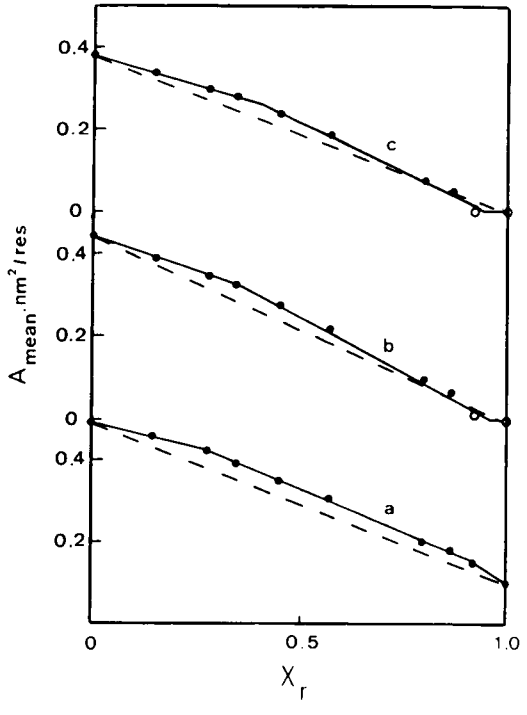

FIGURE 3 Mean area per "residue" (A_{mean}) in the SP-B/DPPC films of initial monolayer composition (X_r) at constant surface pressure: 25 $mN \cdot m^{-1}$ (*a*), 45 $mN \cdot m^{-1}$ (*b*), 55 $mN \cdot m^{-1}$ (*c*). The experimental error is within the size of the symbols (●) which represent average results of at least two separate measurements. The open circles represent extrapolated values of A_{mean} at the given surface pressure.

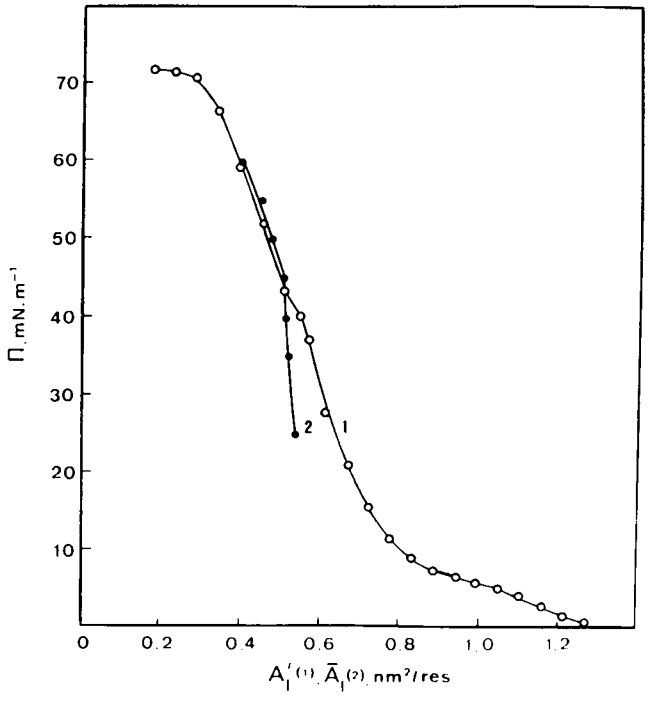

FIGURE 4 Surface pressure as a function of the apparent lipid area $A'_1(1)$ and partial molar lipid area $_1(2)$ for SP-B/DPPC monolayer of initial monolayer composition $X_r = 0.45$.

the partial "residual" areas per lipid molecule, $\bar{A}_l$, and per amino acid residue of SP-B, $\bar{A}_r$, in the same binary mixture were determined from $A_{mean}(X_r)$ plots at a few surface pressures. Curve 2 in Fig. 4 represents the dependence of π on the partial "residual" area of DPPC in the SP-B/DPPC monolayer of composition $X_r = 0.45$. Furthermore, the excess area per DPPC, $\Delta A_l = A'_l - \bar{A}_l$, equal to the difference between the apparent and partial "residual" areas of DPPC was analyzed as a function of surface pressure.

At $\pi < \pi_{kink}$ ($\pi_{kink} = 41 \pm 2$ mN·m^{-1}) $\Delta A_l > 0$, i.e., the apparent lipid area is higher than the partial lipid area. At these pressures the partial "residual" areas per amino acid residue of SP-B were positive which suggested that the protein was present in the film. Therefore, the difference ΔA_l at certain π may be attributed to that part of protein amino acid residues which resides in the surface. Then by dividing ΔA_l by the partial "residual" area of a protein amino acid, $\bar{A}_r$, at the same pressure, the number of amino acid residues of SP-B present in the monolayer, N_r^{calc}, may be determined at different surface pressures. Hence, the compositions, $X_r^{calc} = N_r^{calc}/(N_r^{calc} + N_l)$, of the monolayers could be estimated at different surface pressures. A similar approach was used to determine the changes in the composition of an ideal protein-lipid monolayer (Taneva et al., 1984).

At $\pi \approx \pi_{kink}$ the apparent lipid area is equal to the partial molar lipid area, i.e. $\Delta A_l = 0 \pm 0.005$ nm^2/residue. At these pressures the partial "residual" area of SP-B, $\bar{A}_r$, was zero ($\bar{A}_r = 0 \pm 0.01$ nm^2/residue). These findings suggest that SP-B was no longer present in the monolayer plane and the film area was occupied by the lipid only. The accuracy of determination of the areas ΔA_l and $\bar{A}_r$, however, does not allow reliable differentiation between 0 and 7 w% protein remaining in the films. Therefore, part of the initially spread protein may be present in the mixed films after squeeze-out. As we showed above, at concentrations $X_r < 0.45$, or less than 10 w%, SP-B was not excluded from the mixed films and remained during compression to high pressures.

At $\pi > \pi_{kink}$ the apparent lipid area A'_l is lower than the partial molar lipid area $\bar{A}_l$ and therefore $\Delta A_l < 0$. Though the difference is within the limit of accuracy of determination of the partial "residual" area $\bar{A}_l$, all SP-B/DPPC films of $X_r \geq 0.45$ showed a tendency for ΔA_l to be less than 0 at surface pressures higher than 40–45 mN·m^{-1}. This result suggests that not only SP-B, but also small amounts of DPPC, together with the protein, were removed from the monolayer. By dividing ΔA_l at a certain surface pressure by the partial "residual" lipid area at the same pressure, the number of DPPC molecules lost from the surface, N_l^{lost}, may be deduced (± 1 phospholipid molecule). Based on the fact that at these surface pressures $\bar{A}_r = 0$, one may assume that the whole of the initially spread protein was excluded from the monolayer at surface pressures higher than π_{kink}. Then the relative protein composition of the protein-lipid units lost from the surface, X_r^{lost}, can be approximated using $X_r^{lost} = N_r/(N_r + N_l^{lost})$, where N_r is the number of the initially spread amino acid residues of SP-B.

Using this approach the compositions, X_r^{calc}, of the SP-B/DPPC monolayers were calculated as a function of surface pressure. Table 2 presents the results for a few protein-lipid mixtures of various initial compositions, X_r. The compositions of the protein-lipid units, excluded from the monolayers, X_r^{lost}, are given in the same table. The results in Table 2 indicate that at lower initial protein concentrations ($X_r \leq 0.34$ corresponding to 0.33 mol% protein) changes in the initial compositions of the monolayers did not occur during their compression, i.e., SP-B was not squeezed out from the monolayers even at surface pressures up to about 55–60 mN·m^{-1}. At higher initial protein concentrations ($X_r \geq 0.45$ or 0.51 mol%), exclusion of the initially spread protein was detected at surface pressure of about 45 mN·m^{-1}, corresponding to the kinks in the isotherms. Only small amounts of DPPC, practically independent of the initial concentration of SP-B in the films, were lost from the monolayer together with the protein. The composition of the SP-B/DPPC units, excluded from the monolayers, X_r^{lost}, indicated that pure, or almost pure, SP-B was squeezed out from the monolayer plane which would result in DPPC-enriched films capable of sustaining high surface pressure. This mechanism of refinement of the SP-B/DPPC monolayers was confirmed by the analysis of the surface elasticities $E = -(d\pi/d\ln A_{mean})_T$ as a function of the surface pressure. Surface elasticity was calculated from the equilibrium $\pi(A_{mean})$ isotherms by graphical differentiation

TABLE 2 Calculated composition of SP-B/DPPC monolayers X_r^{calc}, and excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (SP-B:lipid)	Initial (X_r)	Surface pressure (mN·m^{-1})									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	40	45	50	55	25	40	45	50	55
1:898	0.15	0.16	0.14	0.14	0.16	0.15	-	-	-	-	-
1:409	0.28	0.27	0.28	0.27	0.28	0.29	-	-	-	-	-
1:301	0.34	0.32	0.36	0.35	0.36	0.35	-	-	-	-	-
1:195	0.45	0.44	0.41	0	0	0	-	-	0.98	0.98	0.99
									1:3*	1:3*	1:2*
1:119	0.57	0.59	0.60	0	0	0	-	-	0.99	0.99	1.0
									1:1*	1:2*	1:0*
1:39	0.80	0.79	0.80	0	0	0	-	-	0.99	0.99	0.98
									1:1*	1:2*	1:3*

* Calculated molar ratio SP-B:lipid of the excluded phase.

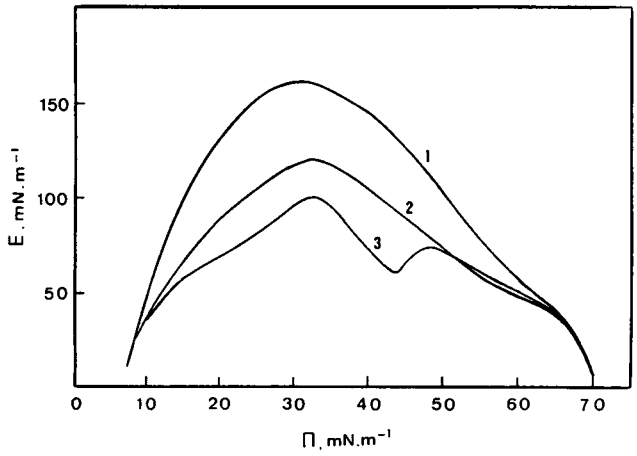

FIGURE 5 Surface elasticity-surface pressure plots for SP-B/DPPC monolayers of various protein concentrations X_r:0.0 (*1*), 0.34 (*2*), 0.45 (*3*).

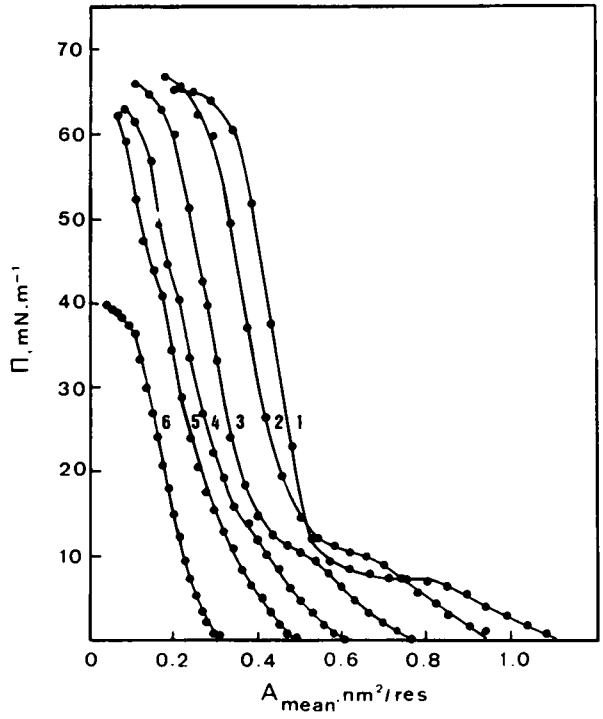

FIGURE 6 $\pi(A_{mean})$ isotherms for binary SP-B/DPPG monolayers of various initial compositions X_r: 0.0 (*1*), 0.25 (*2*), 0.42 (*3*), 0.61 (*4*), 0.74 (*5*), 0.93 (*6*).

and plotted against surface pressure in Fig. 5. At low protein concentrations, e.g., $X_r = 0.34$, where no squeeze-out of protein was suggested by the analysis of $X_r^{calc}(\pi)$ in Table 2, the $E(\pi)$ curves of the pure DPPC and SP-B/DPPC monolayers are similar in shape. At higher surface pressures (about 60 $mN \cdot m^{-1}$) the two curves practically coincide, which suggests that the presence of the protein did not change the rheological characteristics of the DPPC monolayer at these high pressures. For mixed SP-B/DPPC monolayers of $X_r \geq 0.45$ the data for $X_r^{calc}(\pi)$ is consistent with exclusion of SP-B/DPPC unit of approximately 1:2 molar ratio (Table 2). This process is accompanied by a decrease in the surface elasticity of the monolayer with a minimum at about 41 $mN \cdot m^{-1}$. At higher pressures, as a result of the complete exclusion of almost pure protein, the $E(\pi)$ curve of the lipid-enriched film converges with the curve for the pure DPPC monolayer.

Mixed monolayers of SP-B and DPPG

Above pH 4 DPPG forms a fully deprotonated monolayer for subphase ion concentrations higher than 10 mM (Lakhdar-Ghazal et al., 1983). Therefore at pH 7 one could expect electrostatic interactions between the basic SP-B and the acidic lipid in addition to the nonpolar interactions between the lipid hydrocarbon chains and the protein side chains. The isotherms of surface pressure versus mean area per "residue" for SP-B/DPPG monolayers of various initial compositions are shown in Fig. 6. The $\pi(A_{mean})$ curves display features similar to the SP-B/DPPC mixtures of similar compositions. Thus at low initial concentrations of SP-B ($X_r < 0.42$) the monolayers collapsed at surface pressures around 65 $mN \cdot m^{-1}$, corresponding to the collapse pressure of DPPG alone. At higher protein concentrations ($0.42 \leq X_r \leq 0.74$), in addition to the collapse plateaux at 65 $mN \cdot m^{-1}$, kink points at 41 $\pm$ 2 $mN \cdot m^{-1}$ were observed in the isotherms.

The mean area per "residue" in the SP-B/DPPG monolayers at some surface pressures is plotted versus monolayer composition in Fig. 7. Positive deviations of the experimental areas from the additive line are observed, similar to the effect seen in the SP-B/DPPC films.

The change in initial monolayer composition with surface pressure was calculated using the same approach as in the case of SP-B/DPPC monolayers, and the results are summarized in Table 3. At low initial concentrations ($X_r < 0.42$) all SP-B was retained in the monolayer system up to high surface pressures of about 60 $mN \cdot m^{-1}$. An increase in the initial concentration of the protein was accompanied by exclusion of material at $\pi \geq 50$ $mN \cdot m^{-1}$. Similar to the SP-B/DPPC monolayers, almost pure protein was ejected together with small amounts of DPPG (about 2 mol of DPPG per dimer of SP-B). In the SP-B/DPPG monolayers, however, the surface pressure corresponding to the exclusion of SP-B from the lipid monolayer was about 5 $mN \cdot m^{-1}$ higher than in SP-B/DPPC monolayers. This result is also supported by surface elasticity-pressure relations (Fig. 8) where the minima in the $E(\pi)$ curves, corresponding to the exclusion process, are at slightly higher π for SP-B/DPPG monolayers than for SP-B/DPPC ones. As SP-B concentration was increased, the first mixture where exclusion of almost pure SP-B from the films occurred was at $X_r = 0.42$, or 0.46 mol% protein in the film (*curve 3* in Fig. 8), similar to the case of SP-B/DPPC monolayers. In the resulting lipid-enriched monolayers, after SP-B exclusion, elasticity values typical for a monolayer of pure DPPG were seen at $\pi \approx 60$ $mN \cdot m^{-1}$.

The results in Tables 2 and 3 show that in both SP-B/DPPC and SP-B/DPPG mixtures of initial protein concentration $X_r \geq 0.42$ nearly pure SP-B was lost from the surface at

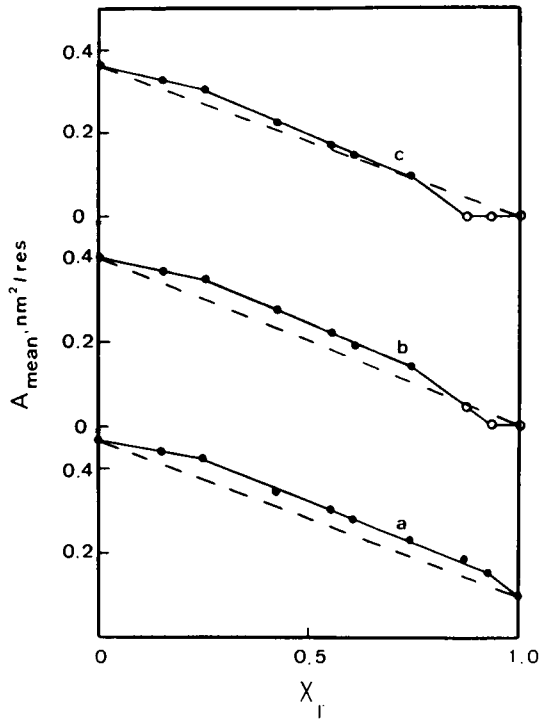

FIGURE 7 Mean area per "residue" versus initial monolayer composition for SP-B/DPPG mixtures at surface pressures of 25 $mN \cdot m^{-1}$ (*a*), 45 $mN \cdot m^{-1}$ (*b*), 55 $mN \cdot m^{-1}$ (*c*).

$\pi \geq 45$–50 $mN \cdot m^{-1}$. Thus SP-B showed poor ability to remove either DPPC or DPPG from the spread SP-B/DPPC or SP-B/DPPG monolayers under conditions of increased film compression.

Comparison of the data for SP-B/DPPC and SP-B/DPPG mixtures revealed substantial similarity in their monolayer behavior which suggests that hydrophobic interactions between the protein and the phospholipid were mainly responsible for the observed deviations from ideal behavior. The slightly higher exclusion pressure for SP-B observed in the mixtures of SP-B with DPPG in comparison to those with DPPC is consistent with stronger interaction of SP-B with DPPG, and could be attributed to electrostatic attraction between the basic protein and the acidic phospholipid.

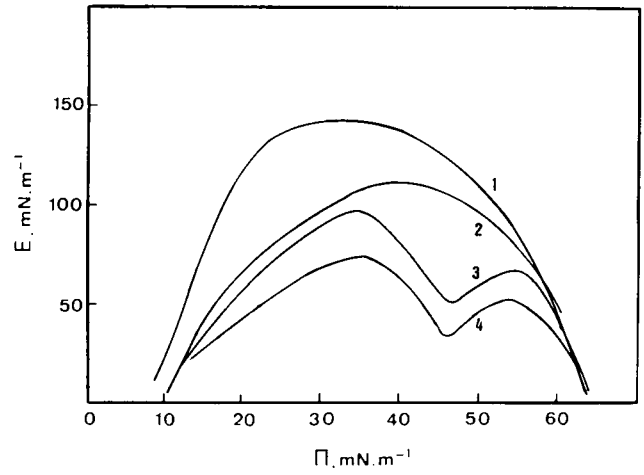

FIGURE 8 $E(\pi)$ relationships for SP-B/DPPG monolayers of various protein concentrations X_r: 0.0 (*1*), 0.25 (*2*), 0.42 (*3*), 0.55 (*4*).

Three-component monolayers of SP-B with DPPC and DPPG

Recent investigations have indicated that SP-B selectively interacts with PG in mixtures containing DPPC and PG. Thus, fluorescence anisotropy studies indicated that in bilayer model membranes of DPPC:DPPG (7:1, mol:mol) SP-B interacted preferentially with DPPG (Baatz et al., 1990). Also, lipid-protein dispersions in a pulsating bubble surfactometer suggested that, in the presence of calcium, SP-B promoted removal of PG molecules from adsorbed monolayers that were composed of DPPC:unsaturated PG (Yu and Possmayer, 1990, 1992). Our measurements on binary spread monolayers of SP-B with DPPC or DPPG gave no indication of a strong specific interaction of SP-B with the anionic phospholipid, though a slightly higher exclusion pressure for SP-B from SP-B/DPPG films than SP-B/DPPC films was detected. In order to study the effects of SP-B on a mixture of DPPC plus DPPG, three-component spread monolayers of SP-B/(DPPC:DPPG) were formed. The molar ratio between the two phospholipids was kept constant at 7:3 (mol:mol) in all ternary protein-lipid mixtures. The composition of the monolayers, X_r, and the mean area per "residue,"

TABLE 3 Calculated composition of SP-B/DPPG monolayers, X_r^{calc}, and excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (SP-B:lipid)	Initial (X_r)	Surface pressure ($mN \cdot m^{-1}$)									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	40	45	50	55	25	40	45	50	55
1:887	0.15	0.15	0.13	0.14	0.16	0.15	-	-	-	-	-
1:475	0.25	0.26	0.23	0.24	0.25	0.26	-	-	-	-	-
1:218	0.42	0.42	0.41	0.40	0	0	-	-	-	1.0	0.97
										1:0*	1:4*
1:103	0.61	0.59	0.60	0.60	0	0	-	-	-	0.99	0.99
										1:2*	1:2*
1:56	0.74	0.74	0.73	0.71	0	0	-	-	-	0.99	0.99
										1:1*	1:1*

* Calculated molar ratio SP-B:lipid of the excluded phase.

A_{mean}, were determined using Eqs. 1–4, where N_1 represents the total number of spread DPPC and DPPG molecules.

First, the isotherms of surface pressure versus mean area per molecule in the binary spread monolayers of DPPC with DPPG in various molar ratios were measured in the absence of protein (Fig. 9). The mean areas per molecule in the DPPC/DPPG films were determined at certain surface pressures and plotted versus the mole fraction of DPPC, X_{DPPC}, in Fig. 10. The broken lines represent the area expected from ideal mixing according to the additivity rule. In the whole range of concentrations and surface pressures studied, the plots of the mean area per molecule versus monolayer composition showed nonideal mixing of DPPC and DPPG. Similar results have already been reported for PC/PG couples of various acyl chain lengths (Mashak et al., 1982; Boonman et al., 1987), where a marked tendency for miscibility of PC and PG in monolayers has been shown (Mashak et al., 1982).

Furthermore, the $\pi(A_{mean})$ isotherms of the ternary monolayers of SP-B with DPPC:DPPG (7:3, mol:mol) were measured at various protein-lipid ratios (Fig. 11). The curves for SP-B/(DPPC:DPPG) monolayers were similar to those obtained for the binary SP-B/DPPC and SP-B/DPPG films. Monolayers of low protein concentrations, e.g., $X_r = 0.27$ or 0.23 mol% protein (*curve 2* in Fig. 11) collapsed at pressures similar to the collapse pressure of the monolayer of the DPPC:DPPG mixture without protein (*curve 1* in Fig. 11). For monolayers of compositions $0.42 \leq X_r \leq 0.74$, or $0.46 \leq$ mol% protein ≤ 1.85, kink points at 41 ± 2 mN·m^{-1} were detected in the isotherms in addition to the plateaux at about

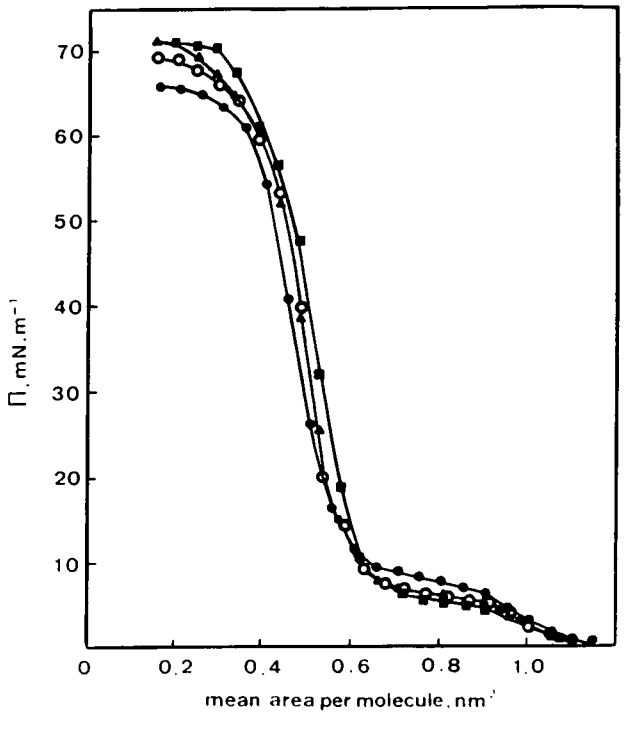

FIGURE 9 Surface pressure-mean area per molecule curves for monolayers of DPPC with DPPG. Compositions of films are expressed as mole fractions of DPPC, X_{DPPC}: 0.25 (●), 0.50 (o), 0.70 (▲), 0.87 (■).

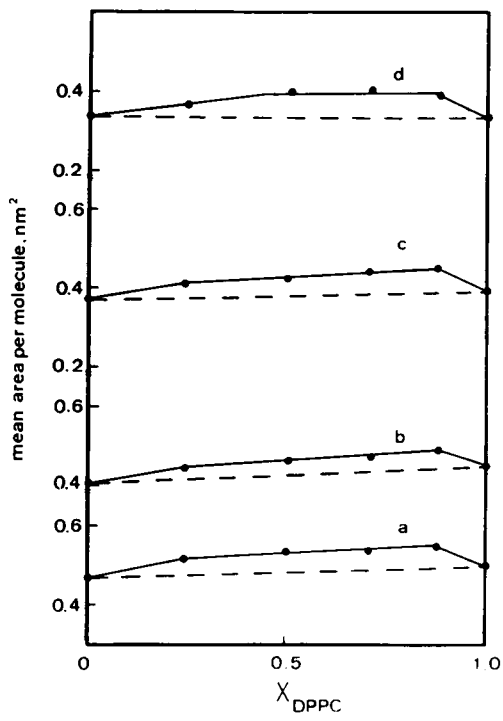

FIGURE 10 Mean area per molecule in DPPC:DPPG monolayers versus the mole fraction of DPPC at constant surface pressure of 25 mN·m^{-1} (*a*), 45 mN·m^{-1} (*b*), 55 mN·m^{-1} (*c*), 60 mN·m^{-1} (*d*).

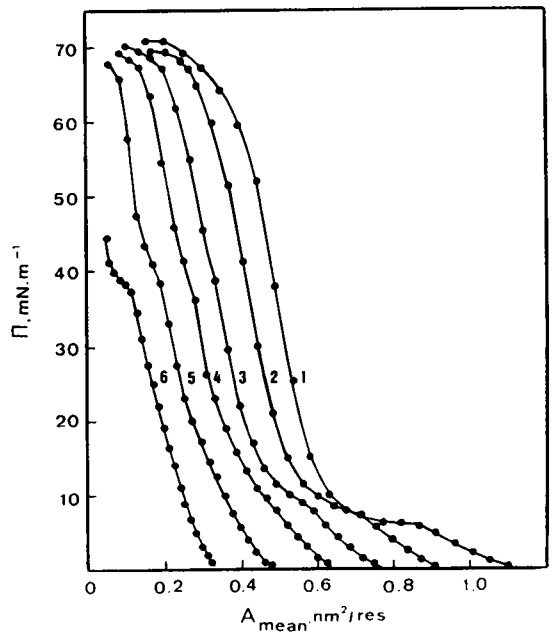

FIGURE 11 $\pi(A_{mean})$ isotherms for ternary SP-B/(DPPC:DPPG) monolayers of various initial protein concentrations, X_r:0.0 (*1*), 0.27 (*2*), 0.42 (*3*), 0.60 (*4*), 0.74 (*5*), 0.92 (*6*).

70 mN·m^{-1}. Positive deviations from linearity were observed in the mean area per "residue" versus monolayer composition diagrams in Fig. 12, similar to the ones seen in SP-B/DPPC and SP-B/DPPG films. The compositions of the ternary SP-B/(DPPC:DPPG) films, X_r^{calc}, were determined as a function

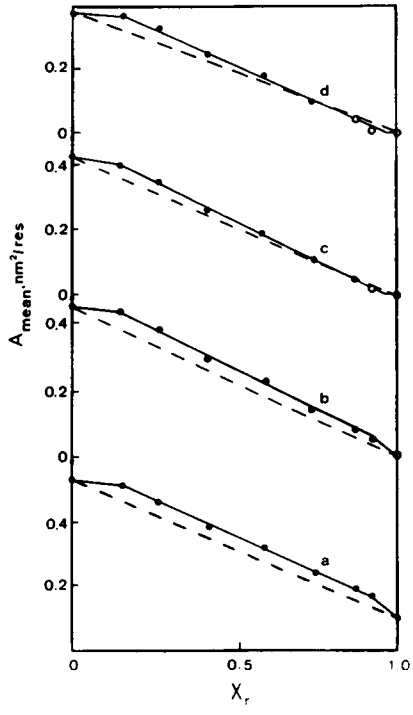

FIGURE 12 Mean area per "residue" in the ternary SP-B/(DPPC:DPPG) films versus initial protein concentration at constant surface pressure of 25 $mN \cdot m^{-1}$ (*a*), 45 $mN \cdot m^{-1}$ (*b*), 55 $mN \cdot m^{-1}$ (*c*), 60 $mN \cdot m^{-1}$ (*d*).

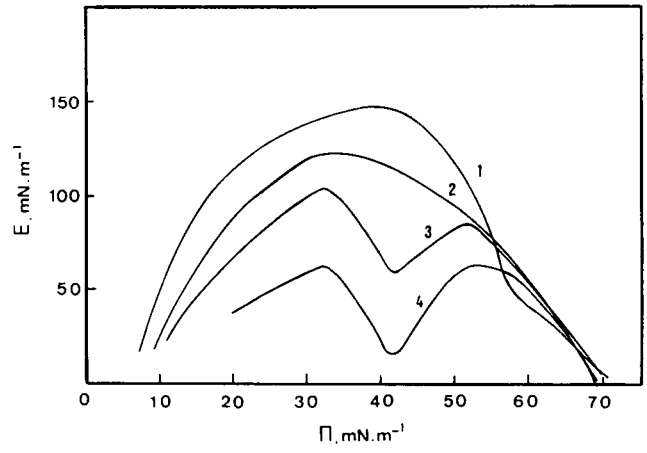

FIGURE 13 Surface elasticity-surface pressure plots for SP-B/(DPPC: DPPG) monolayers of various protein concentrations X_r: 0.0 (*1*), 0.27 (*2*), 0.42 (*3*), 0.75 (*4*).

of surface pressure using the approach already described. The results are summarized in Table 4. The process of squeeze-out of material from the ternary mixtures commenced at $X_r \geq 0.42$, similar to the SP-B/DPPC and SP-B/DPPG mixtures. Nearly pure SP-B, associated with a small amount of phospholipid (about 3 mol of lipid per mol of SP-B), was lost from the monolayer at surface pressures of about 50 $mN \cdot m^{-1}$, similar to the SP-B/DPPG system. The amount of lipid excluded from the ternary films with SP-B is low, as it was for the binary films (Tables 2 and 3). The elasticity versus surface pressure plots for the ternary monolayers (Fig. 13) resembled those for the individual binary SP-B/lipid monolayers containing the same amounts of protein (Figs. 5 and 8).

The data from the surface pressure measurements on the ternary SP-B/(DPPC:DPPG) monolayers are insufficient to answer the question of whether SP-B selectively removes either of the phospholipids from the films. The results in Table 4 show that the exclusion pressure of SP-B/(DPPC: DPPG) units from the ternary films of $X_r \geq 0.42$ corresponds to the pressure where squeeze-out of SP-B/DPPG complexes from the binary SP-B/DPPG monolayers occurred. This might be considered an indication that SP-B removes preferentially DPPG from the lipid mixture. However, a close inspection of $E(\pi)$ curves in Fig. 13 showed that this might not be the case. Curve 2 in Fig. 13 represents the $E(\pi)$ dependence for SP-B/(DPPC:DPPG) mixture of $X_r = 0.27$. This composition corresponds to 436 mol of lipid (DPPC: DPPG, 7:3, mol:mol) per mol of SP-B, or 131 mol of DPPG only per mol of SP-B. In other words, if SP-B preferentially interacted with DPPG in the mixture, SP-B/DPPG units might be expected to be leaving the surface during compression of the film at $\pi > 45$ $mN \cdot m^{-1}$, because, SP-B, associated with DPPG was squeezed out from the binary SP-B/DPPG films of similar protein-lipid ratios (Table 3). The $\pi(E)$ curve for the ternary mixture of that composition

TABLE 4 Calculated composition of SP-B/(DPPC:DPPG) monolayers, X_r^{calc}, and excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (SP-B:lipid)	Initial (X_r)	Surface pressure ($mN \cdot m^{-1}$)									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	40	45	50	55	25	40	45	50	55
1:870	0.15	0.15	0.15	0.15	0.15	0.15	-	-	-	-	-
1:218	0.42	0.41	0.40	0.40	0	0	-	-	-	0.97 1:4*	0.97 1:4*
1:106	0.60	0.59	0.59	0.61	0	0	-	-	-	0.99 1:1*	0.97 1:4*
1:53	0.74	0.75	0.74	0.75	0	0	-	-	-	0.98 1:3*	0.97 1:4*

* Calculated molar ratio SP-B:lipid of the excluded phase.

(*curve 2*, Fig. 13) showed no minimum and suggests that no loss of material occurred during monolayer compression. Therefore, more likely SP-B was evenly distributed in the DPPC:DPPG matrix without specific interaction with the anionic phospholipid, at least to the point of causing it to be selectively eliminated.

SUMMARY

In the spread monolayers of SP-B with DPPC, DPPG, and DPPC:DPPG (7:3, mol:mol) expansions in the mean area per "residue" were detected that were consistent with nonideal protein-lipid interactions in the films. Qualitatively similar effects were observed in the binary mixtures of SP-B with DPPC and DPPG, suggesting that predominantly nonpolar interactions were responsible for the nonideal behavior of the monolayers. Given the net positive charge of SP-B, the findings that the effects on DPPC and DPPG were quite similar is somewhat surprising. It indicates the importance of hydrophobic forces in lipid-protein interactions even in the presence of potential electrostatic effects. The preponderance of hydrophobic interactions over electrostatic ones could have important implications for interaction of other amphipathic proteins with bilayers and monolayers.

The results of the static surface pressure-area measurements showed that at low initial protein concentrations in the protein-lipid films ($X_r < 0.45$, which corresponds to about 10 weight% or 0.5 mol% SP-B) SP-B was retained in the monolayers up to surface pressures that are likely to be relevant to the lateral pressure at the alveolar air-water interface (60–70 $mN \cdot m^{-1}$) (Schürch, 1982). At these concentrations the presence of the protein in the monolayer did not seem to affect the elasticity values that were characteristic of the pure phospholipid monolayers at 60–65 $mN \cdot m^{-1}$. At initial concentrations of $X_r \geq 0.45$, SP-B associated with small amounts of phospholipid was excluded from the interface at $\pi > 40$ $mN \cdot m^{-1}$ for SP-B/DPPC and $\pi > 45$ $mN \cdot m^{-1}$ for SP-B/DPPG and SP-B/(DPPC:DPPG, 7:3) monolayers. When it was excluded, SP-B did not remove much lipid from the film and it did not show selective effects in promoting the squeeze-out of one phospholipid over the other. The compositions of the protein-lipid units lost from the films at high surface pressures were similar for the three SP-B/lipid monolayer systems studied. They consisted primarily of protein plus a small amount of lipid (about 2–3 lipid molecules per SP-B dimer).

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