Cholesterol Modifies the Properties of Surface Films of Dipalmitoylphosphatidylcholine plus Pulmonary Surfactant-Associated Protein B or C Spread or Adsorbed at the Air−Water Interface[†]

Svetla Taneva[‡] and Kevin M. W. Keough*[,§]

Department of Biochemistry and Discipline of Pediatrics, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

Received September 18, 1996[⊗]

ABSTRACT: Cholesterol is a substantial component of pulmonary surfactant (∼8 wt % or ∼14 mol % of surfactant lipids). This study investigated the effect of cholesterol on the way in which hydrophobic SP-B and SP-C modulated the adsorption of lipid into the air−water interface and their respreading from collapsed phase produced on overcompression of the surface film. The properties of binary spread monolayers of SP-B or SP-C plus cholesterol (CH) were consistent with miscibility between the hydrophobic proteins and the sterol. Results from surface pressure versus area measurements at 23 °C on spread monolayers of dipalmitoylphosphatidylcholine (DPPC) plus SP-B in the presence of 8 wt % cholesterol implied that CH did not significantly affect the properties of the films of SP-B/(DPPC/CH) compared to those of binary SP-B/DPPC monolayers. In contrast, CH appeared to enhance the mixing of SP-C with DPPC/CH in ternary SP-C/(DPPC/CH) films compared to the miscibility of SP-C with DPPC in the SP-C/DPPC films. It is estimated that about 10 wt % SP-C might remain in the SP-C/(DPPC/CH) monolayers compressed to high surface pressures of about 72 mN/m, whereas SP-C at concentrations of ≥5 wt % was squeezed out at $\pi \approx 50$ mN/m from SP-C/DPPC films without cholesterol. Cholesterol reduced the stability of the films of SP-B/(DPPC/CH) and SP-C/(DPPC/CH) when they had been compressed to $\pi \approx 72$ mN/m, in contrast to films of SP-B/DPPC and SP-C/DPPC which exhibited a relatively slow relaxation from the collapse pressure of 72 mN/m. Dynamic cyclic compression beyond collapse of SP-B/(DPPC/CH) and SP-C/(DPPC/CH) monolayers showed that cholesterol diminished their postcollapse respreading compared to the respreading of the protein/DPPC films without cholesterol. Cholesterol, at 8 wt %, inhibited the rate of adsorption to the air-water interface at 35 °C of aqueous dispersions of DPPC containing 2.5 or 5 wt % SP-B or SP-C. The results suggest that cholesterol has an apparent negative influence on the surfactant surface properties, which are generally considered to be important in surfactant function, although increasing protein concentrations can counteract some of the negative influences.

Cholesterol is a constituent of natural pulmonary surfactant (King & Clements, 1972) and of some potential artificial and synthetic surfactants (Tanaka et al., 1986). It comprises up to about 8 wt % of the lipid components of natural pulmonary surfactant (King & Clements, 1972; Suzuki, 1982; Yu et al., 1983). Functional importance for cholesterol in pulmonary surfactant has been suggested because of its ability to modify the gel to liquid crystalline transition temperature of water dispersions of phospholipids, in particular DPPC which is major component of surfactant (Notter & Morrow, 1975). A putative role for cholesterol, among the various non-DPPC constituents of pulmonary surfactant, is to fluidize the acyl chains of the mixture so that, at body temperature, surfactant can adsorb into the interface and respread from collapse phase(s) in a facile fashion (Keough, 1992). In vitro measurements at 23 °C have shown that cholesterol does not substantially enhance respreading of monolayers of DPPC/CH (9:1 mole:mole) compressed beyond collapse (Notter et al., 1980a), but that particular lipid mixture is likely to be in a fairly ordered phase at this temperature (Vist & Davis, 1990). In the same study, films of DPPC/CH (9:1 mole:mole) exhibited better respreadability at 37 °C, but they did not attain very high surface pressures during maximal compression. This would be consistent with the films being in the fluid state at this temperature. Likewise, in experiments with reconstituted mixtures of surfactant phospholipids plus SP-A and SP-B, the addition of cholesterol (8 wt %) impaired the ability of the films to attain minimum surface tension near 0 mN/m at maximum compression (Suzuki, 1982). Recently, cholesterol has been shown to destabilize films of lipid extracts of bovine pulmonary surfactant which had been compressed to very low surface tensions in a pulsating bubble surfactometer (Yu

[†] This work was supported by the Medical Research Council of Canada.

* To whom the correspondence should be addressed. Phone: (709) 737-2530. Fax: (709) 737-2552. E-mail: kkeough@morgan.ucs.mun.ca.
[‡] Department of Biochemistry.
[§] Dicipline of Pediatrics.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; SP-A, pulmonary surfactant-associated protein A; SP-B, pulmonary surfactant-associated protein B; SP-C, pulmonary surfactant-associated protein C; CH, cholesterol; X_r, mole fraction of protein amino acid residues in the protein/lipid monolayers determined from the amounts of protein and lipid initially-spread in the monolayers. $X_r = N_r/(N_r + N_{DPPC} + N_{CH})$, where N_r, N_{DPPC}, and N_{CH} are the numbers of amino acid residues of protein and molecules of DPPC and CH, respectively, that are spread initially in each monolayer.

S0006-2960(96)02354-9 CCC: $14.00

& Possmayer, 1994). These biophysical studies have suggested that cholesterol in amounts comparable to those in natural surfactant might be detrimental to the ability of films of lipid extracts and some reconstituted surfactant mixtures to decrease surface tension to very low values during dynamic compression and to the stability of the films compressed to near-zero surface tension. In the multicomponent system of the natural pulmonary surfactant, however, some specific interactions may be operating so that cholesterol does not interfere with these abilities in surfactant (Suzuki, 1982; Yu & Possmayer, 1993, 1994).

To determine a possible role for cholesterol in the interfacial properties of surfactant phospholipid/protein complexes, we studied the influence of cholesterol on interactions of DPPC with hydrophobic SP-B and SP-C in monolayers at the air−water interface. Specifically, we examined the effect of cholesterol on the ability of SP-B and SP-C to enhance the respreading of DPPC/protein monolayers compressed beyond collapse. The influence of cholesterol on the adsorption kinetics of aqueous dispersions of SP-B/DPPC and SP-C/DPPC was assessed from measurements of surface pressure versus time adsorption isotherms. In addition, the interfacial interactions of cholesterol with SP-B or SP-C were characterized as a necessary step for understanding the effect of the sterol on the properties of the ternary films of DPPC/CH plus hydrophobic surfactant proteins. Monolayers of cholesterol plus hydrophobic protein also constitute well-defined models for studying cholesterol−protein interactions in biological membranes, where existence of such interactions has been suggested (London, 1974; Giraud et al., 1976; Surewicz et al., 1986).

EXPERIMENTAL PROCEDURES

Materials. DPPC and cholesterol, purchased from Sigma Chemical Co. (St. Louis, MO), were found to be pure by thin-layer chromatography and were used without further purification. Sodium chloride, chloroform, methanol, hydrochloric acid, 1-butanol, and trifluoroacetic acid were ACS grade or higher and were purchased from Fisher Scientific Co. (Ottawa, ON). Water was deionized and doubly distilled in glass, the second distillation being from a dilute potassium permanganate solution.

Purification of SP-B and SP-C. SP-B and SP-C were isolated from porcine lung lavage. Pig lungs were washed two times with 0.15 M NaCl, and 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. It was extracted with distilled *n*-butanol (Haagsman et al., 1987). The butanol phase was dried by rotary evaporation. SP-B and SP-C were obtained by gel exclusion chromatography on Sephadex LH-60 using chloroform/methanol (1:1 v:v) containing either 2% by volume 0.1 M HCl (SP-B) or 2.5% by volume 0.1 M trifluoroacetic acid (SP-C). SP-B and SP-C were stored in chloroform/methanol (1:1 v:v). Under non-reducing conditions on SDS−polyacrylamide gel electrophoresis (16% gels) (Laemmli, 1970), SP-B yielded a band at about 18 kDa and SP-C showed a band at about 5 kDa after visualization with silver stain (Daiichi Pure Chemicals Co., Ltd., Tokyo).

Analytical Methods. Concentrations of the solutions of SP-B and SP-C were determined using the fluorescamine assay method of Undenfriend et al. (1972), a procedure which we have verified with quantitative amino acid analysis (Sarin et al., 1990). Analysis of phosphorus in the protein preparations (Bartlett, 1959; Keough & Kariel, 1987) showed less than 0.5 mol of phospholipid per mole of SP-B (dimer) or SP-C (monomer). DPPC or cholesterol was dissolved in chloroform; the concentrations of DPPC solutions were determined by phosphorus assay (Bartlett, 1959; Keough & Kariel, 1987), whereas cholesterol concentrations were determined gravimetrically.

Surface Pressure versus Area Measurements on Spread Monolayers. Measurements were performed in a Langmuir trough which employed a continuous Teflon ribbon barrier (Applied Imaging, Dukesway Team Valley, Gateshead, Tyne and Wear, England). Surface tension was measured by the Wilhelmy plate method, using a platinum plate roughened by scouring with emery paper. Lipid/protein mixtures for spreading were prepared by premixing of stock solutions of the components. The subphase was 0.15 M NaCl; the pH was adjusted to 7.0 immediately before each experiment with 0.1 M NaOH, and it did not change by more than 1 pH unit during the surface pressure versus area measurements. With DPPC being zwitteronic, its isotherms did not change over this pH range. Isotherms of the protein spread at pH 5.8 were essentially identical with those spread initially at pH 7.0. The subphase temperature was 22−24 °C.

Ten minutes after their formation by application of small drops of the spreading solutions to the aqueous surface by the use of microsyringe (Hamilton Co., Reno, NV), monolayers were continuously compressed at a rate of 40 cm^2/min. The total compression between a maximal area of 500 cm^2 and a minimal area of 100 cm^2 took 10 min.

Dynamic cyclic surface pressure versus area measurements were performed under two initial spreading conditions (Notter et al., 1980a,b): (i) "surface dilute" conditions, where the initial spreading of the lipid/protein monolayers gave rise to relatively low surface pressures (π < 10 mN/m); and (ii) "surface excess" conditions, where the initial spreading of surface active material, which was in excess to that required for monolayer coverage, generated a π of about 45−48 mN/m. The film area was continuously reduced and enlarged four times.

All isotherms were repeated two or three times, except for a few cases (noted in Tables 1 and 2) where isotherms of the same mixtures had been obtained before (Taneva & Keough, 1994) and good agreement with previous results was obtained. The isotherms shown are representative and not average. The reproducibility was such that the range of values for area per residue was ±0.01 nm^2/residue and within the size of the symbols in Figures 4 and 8.

The compositions of the spread protein/lipid monolayers were given by the weight percent of protein and by the mole fraction of protein amino acid residues, X_r. In all experiments, the ratio between DPPC and cholesterol was held constant at 6:1 mole:mole or 11:1 w:w, and the properties of the monolayers were examined as a function of the protein concentration. The mean area in the spread lipid/protein films, A_{mean} [=trough area/($N_r + N_{DPPC} + N_{CH}$)], was defined as mean area per "residue", where residue denoted an amino acid residue of the protein or a molecule of DPPC or cholesterol. For all calculations, molecular weights of 17 400 for SP-B (158 amino acid residues, dimer form) and 4186 for SP-C (35 amino acid residues, dipalmitoylated) were used (Curstedt et al., 1990).

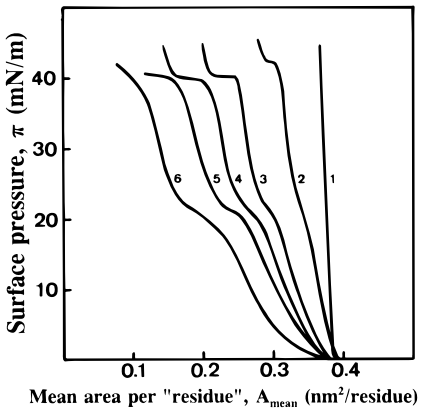

FIGURE 1: Isotherms of surface pressure versus mean area per residue for monolayers of cholesterol, SP-B, and their mixtures. The initial concentrations of SP-B in the monolayers were as follows: $X_r = 0.0$ (1), $X_r = 0.29$ or 10 wt % (2), $X_r = 0.49$ or 21 wt % (3), $X_r = 0.62$ or 31 wt % (4), $X_r = 0.79$ or 51 wt % (5), and $X_r = 1.0$ wt % (6).

Surface Pressure versus Time Measurements on Adsorbed Films. Measurements of adsorption kinetics of aqueous dispersions of DPPC plus SP-B or SP-C in the presence or absence of cholesterol were performed in a Teflon dish ($r = 1.15$ cm) with a subphase volume of 5 mL. At time zero, desired volumes of lipid/protein dispersions were injected below the surface of the subphase through an injection septum and the surface tension was measured as a function of time using the Wilhelmy plate method and a Computer Controlled Transducer Readout, TSAR 1 (Tech-Ser, Inc., Torrance, CA). The subphase (0.15 M NaCl) was stirred continuously with a Teflon-coated stirring bar and a magnetic stirrer to minimize diffusion resistance. The subphase temperature was 35 ± 1 °C.

To prepare the samples, chloroform solutions of DPPC or DPPC/CH (6:1 moles:moles or 11:1 w:w) were mixed with solutions of SP-B or SP-C in chloroform/methanol (1:1 v:v). Solvents were evaporated under nitrogen at room temperature. The lipid/protein films were hydrated with 0.15 M NaCl for 30 min at 50 °C; the suspensions were vortexed intermittently. Under these experimental conditions, multilamellar liposomes are formed spontaneously (Bangham et al., 1965).

RESULTS AND DISCUSSION

Monolayers of Cholesterol plus SP-B or SP-C

Figure 1 shows the compressional isotherms for monolayers of SP-B, cholesterol, and their mixtures. A characteristic kink at about 20 mN/m and a maximum surface pressure of about 42 mN/m were typical for isotherms of SP-B alone (curve 6 in Figure 1) (Taneva & Keough, 1995). The area at liftoff pressure of the isotherm, approximately 0.37 nm^2/(amino acid residue), was about 50% larger than that previously reported by this laboratory for SP-B monolayers formed under similar experimental conditions (Taneva & Keough, 1994b, 1995). The difference in the monolayer areas for the monolayers of SP-B in the present study and those reported before possibly was due to two different methods of preparation of lipid extract surfactant; 1-butanol (Haagsman et al., 1987) was used in this study, instead of chloroform/methanol (Bligh & Dyer, 1959) employed in the previous papers (Taneva & Keough, 1994b, 1995). Measurements in this laboratory suggest that the method of extraction of surfactant pellet with butanol affects some of the interfacial properties of SP-B and SP-C alone, or mixed with DPPC, possibly through modification of the chemical and secondary structures of the proteins (results to be reported separately).

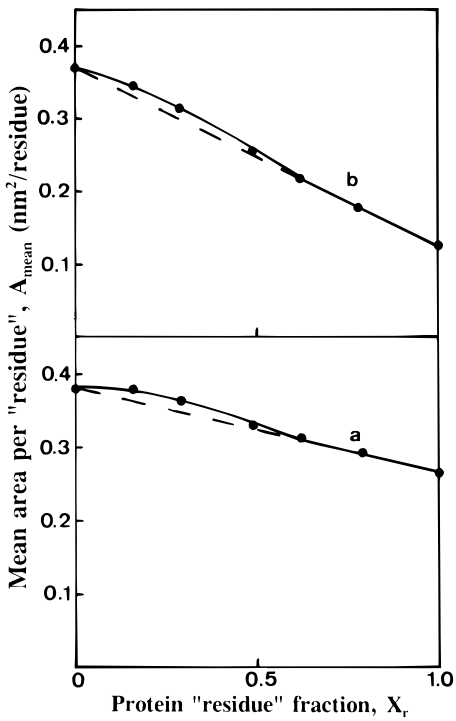

FIGURE 2: Mean area per residue at constant surface pressure versus initial protein concentration for SP-B/CH monolayers. The surface pressure π in millinewtons per meter was 10 (a) and 35 (b).

The isotherm for the monolayers of cholesterol (curve 1 in Figure 1) was in good agreement with those reported in the literature (Ries & Swift, 1989). The area per molecule of cholesterol obtained by extrapolation of the isotherm to zero surface pressure gave a value of 0.39 nm^2/molecule, and a dynamic collapse pressure of 44 mN/m was measured in the films. Each of the isotherms for the monolayers of SP-B/CH (curves 2−5 in Figure 1) displayed a kink at about 20 mN/m which likely arose from intrinsic properties of the protein in the mixed films. At maximum compression of the mixed films, dynamic collapse pressures of about 45 mN/m were attained. At surface pressures of about 40 mN/m, plateau regions were seen in the isotherms for the SP-B/CH monolayers, the plateau lengths being proportional to the percentage of SP-B in the monolayers. This suggested that SP-B was predominantly separated from the monolayers at a π corresponding to the plateau pressures; i.e., demixing of the components, at least in their collapse phases, possibly occurred. The diagrams of the mean area per residue versus protein concentration given by the residue fraction of SP-B (Figure 2) showed positive deviations from additivity for SP-B/CH monolayers containing <21 wt % SP-B, or $X_r < 0.49$. The appearance of the $A_{mean}(X_r)$ plots suggested miscibility with nonideal mixing of SP-B and cholesterol in the monolayers of lower protein concentrations. If for a two-component system a parameter such as area per residue or molecule shows a dependence which is a linear combination of the values of that parameter for the pure component multiplied by the mole fraction of the component for each component present, then one assumes that both components

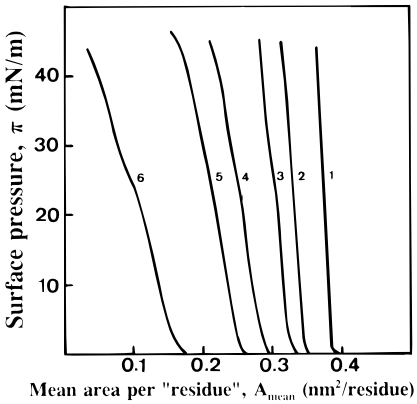

FIGURE 3: Surface pressure versus area curves for SP-C/CH monolayers for various initial protein concentrations: $X_r = 0.0$ (1), $X_r = 0.15$ or 5 wt % (2), $X_r = 0.28$ or 10 wt % (3), $X_r = 0.47$ or 21 wt % (4), $X_r = 0.60$ or 31 wt % (5), and $X_r = 1.0$ wt % (6).

have no influence on one another. This happens when there is totally ideal mixing (miscibility but no interaction energies between components) or total demixing of components. When deviations of the parameters from a straight line relationship (Figure 2) occur, they suggest that the components are miscible and that they mutually influence one abother to change the intrinsic value of the parameter (e.g., area per molecule). At higher concentrations, ≥31 wt % SP-B or an X_r of ≥0.62, the behavior was consistent with either ideal mixing or complete demixing of the components. The observation of two collapse pressures in the SP-B/CH monolayers (Figure 1) which suggested that SP-B was collapsing separately from the lipid was consistent with the interpretation that there was demixing of protein and lipid at high protein concentrations. Surface pressure versus area measurements on monolayers of cholesterol plus SP-B or SP-C of one particular concentration (16.7 wt % protein) have shown that the mean areas per molecule in the films were hardly increased compared to the values calculated by the additivity law (Oosterlaken-Dijksterhuis et al., 1991b).

Isotherms for SP-C, cholesterol, and their mixtures are plotted in Figure 3. Similar to the observations for the films of SP-B, SP-C which was purified from butanol extracts of surfactant showed higher molecular areas in the spread monolayers (curve 6 in Figure 3), compared to SP-C isolated from chloroform/methanol extracts (Taneva & Keough, 1994c, 1995). One dynamic collapse pressure at about 45 mN/m was observed in the SP-C/sterol monolayers (curves 2−5 in Figure 3), and this pressure corresponded to the collapse pressures of the monolayers of cholesterol (curve 1 in Figure 3) and SP-C (curve 6 in Figure 3). The $A_{mean}(X_r)$ plots for the SP-C/CH monolayers obeyed the additivity law in the whole range of protein concentrations studied (Figure 4). As noted above, two interpretations of the additivity of the mean monolayer areas are possible. SP-C and cholesterol formed ideal (random) solutions, or SP-C and cholesterol were essentially mutually insoluble and occupied separate regions in a heterogeneous monolayer. Additional microscopic observations of the monolayer structures are necessary to asses whether complete immiscibility or ideal mixing occurs in SP-C/CH monolayers. The ability of SP-C and CH to cocollapse in the mixed monolayers, however, suggested that bidimensional miscibility of the components possibly accounted for the additivity of the $A_{mean}(X_r)$ diagrams.

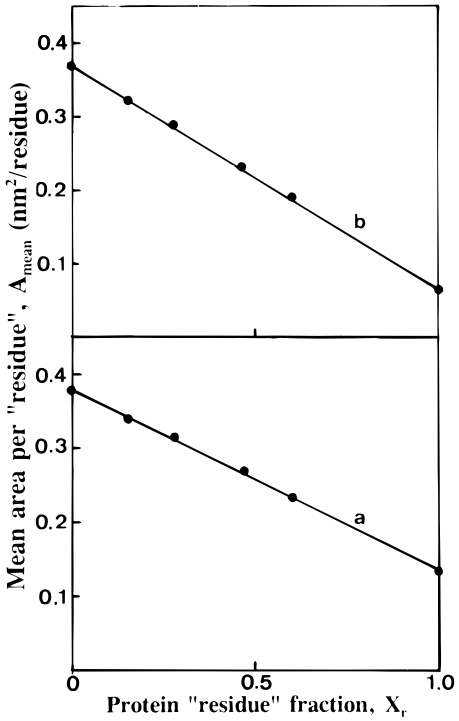

FIGURE 4: Mean area per "residue" in the SP-C/CH monolayers versus initial protein concentration at constant surface pressure, π, mN/m: 10 (a) and 35 (b).

Comparison of the properties of the monolayers of SP-B/CH to those of SP-C/CH indicated that the two proteins had somewhat different affinities for cholesterol in the films. Interactions of cholesterol with proteins, or polypeptides, could occur through hydrogen bonds between the hydroxyl groups of cholesterol and the peptide linkages or the side chains of proteins, or both, van der Waals forces between the nonpolar moieties of the molecules (Eley & Hedge, 1956; Colacicco, 1973; London et al., 1974; Schubert & Marie, 1982), and steric accommodation or space filling (Baglioni et al., 1986). Differences in the primary and secondary structures of SP-B and SP-C might determine different capacities of the proteins to form interfacial hydrogen bonds with cholesterol. SP-B, with a higher amount of polar amino acid residues (Curstedt et al., 1990; Johansson et al., 1991) and a smaller proportion of α-helix than SP-C (Pastrana et al., 1991; Vandenbussche et al., 1992a,b; Pastrana-Rios et al., 1995), is probably more prone to form hydrogen bonds with CH than is SP-C. The character of α-helices of SP-B and SP-C, amphipathic versus hydrophobic (Takahashi et al., 1990), also would affect potential interfacial interactions of the proteins with cholesterol. The two palmitoyl chains of porcine SP-C might modify the interactions of that protein with cholesterol.

Monolayers of DPPC/CH plus SP-B

The $\pi(A_{mean})$ isotherms of SP-B/(DPPC/CH) monolayers are shown in Figure 5. The ratio between DPPC and cholesterol (6:1 mole:mole or 11:1 w:w) was held constant in each ternary film, and the properties of the monolayers were studied as a function of protein concentration. Curve 1 in Figure 5 shows the isotherm for the films of DPPC/CH without protein. Similar to observations by other researchers (Shah & Schulman, 1967; Ghosh & Tinoco, 1972; Müller-Landau & Cadenhead, 1979), the experimental mean mo-

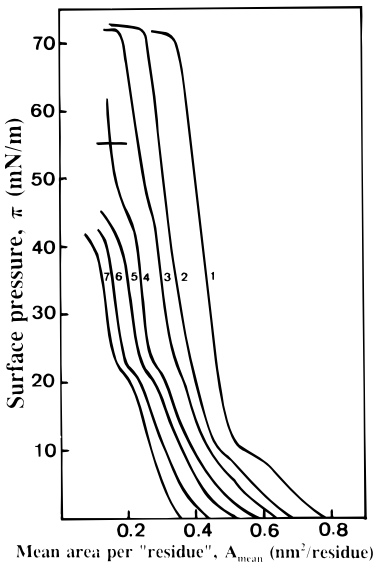

FIGURE 5: Surface pressure versus mean area per residue isotherms for monolayers of SP-B plus DPPC/CH (6:1 moles:moles or 11:1 w:w). The initial concentration of protein in the films was as follows: $X_r = 0.0$ (1), $X_r = 0.25$ or 5 wt % (2), $X_r = 0.41$ or 10 wt % (3), $X_r = 0.56$ or 17 wt % (4), $X_r = 0.73$ or 30 wt % (5), $X_r = 0.86$ or 50 wt % (6), $X_r = 1.0$ (7). The horizontal bar shows the equilibrium surface pressure to which the collapse surface pressures (curves 1−3) relaxed when the monolayers were held at a constant area.

lecular areas in DPPC/CH monolayers at a π of <30 mN/m were smaller that those calculated from the weighed sums of the molecular area of each component at a given surface pressure (data not shown). Cholesterol does not condense DPPC monolayers at higher surface pressures at 22 °C; however, at 37 °C, the monolayers of DPPC are still in a fluid state at a π of >30 mN/m (Albrecht et al., 1978), and cholesterol might be expected to produce a "condensing effect" on DPPC at the higher surface pressures. The condensing effect of cholesterol has been interpreted in terms of specific interactions (Demel et al., 1967; Tajima & Gershfeld, 1978; Müller-Landau & Cadenhead, 1979) or in terms of a specific mechanism of packing of the components which allows insertion of cholesterol into the molecular cavities of the phospholipid monolayer (Shah & Schulman, 1967). During compression of the DPPC/CH monolayers (curve 1 in Figure 5), a dynamic collapse pressure of about 71 mN/m was attained. A similar collapse pressure has also been found at 21 °C by Snik et al. (1978) for DPPC/CH monolayers containing 10 mol % cholesterol. Notter et al. (1980c) have reported a lower dynamic collapse pressure for films of DPPC/CH, with 10 mol % cholesterol, spread at low initial surface concentration at 25 °C. Besides the slight difference in temperature, a number of factors, such as the compression rate, spreading solvent, and initial surface concentration, could determine dynamic collapse surface pressures achieved by mixed films (Notter et al., 1980c).

At the end of compression of the DPPC/CH films, the dynamic postcollapse surface pressure was monitored as a function of time while films were held at a constant area. The surface pressure for DPPC/CH monolayers (curve 1 in Figure 5) decreased and reached an equilibrium value of about 53−54 mN/m in about 600 s (horizontal bar in Figure 5). In contrast to this observation, surface pressure in monolayers of DPPC which had been compressed to the point of collapse at a π of ≈72 mN/m did not change during an interval of about 1200 s. Similar stable dynamic collapse surface pressures for DPPC films have been measured by Watkins (1968) and Tabak et al. (1977). The ability of cholesterol to increase the relaxation of dynamic postcollapse surface pressure in DPPC films has been reported by Notter et al. (1980c). It is worth noting that, opposite of the effect of cholesterol (8 wt % or 14 mol %), SP-B (up to 17 wt % or 0.8 mol %) and SP-C (up to 10 wt % or 1.9 mol %) did not affect the stability of the protein/DPPC monolayers compressed beyond collapse at 72 mN/m (unpublished data).

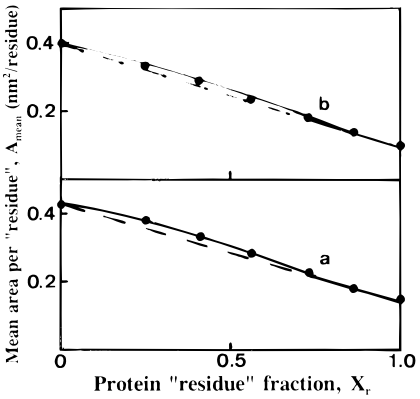

FIGURE 6: Mean area per residue in the SP-B/(DPPC/CH) (6:1, moles:moles or 11:1 w:w) monolayers versus initial protein concentration. The surface pressure π in millinewton meter was 25 (a) and 45 (b).

Compressional isotherms for monolayers of DPPC/CH which contained various amounts of SP-B are shown in curves 2−6 in Figure 5. The isotherm for SP-B/(DPPC/CH) monolayers of low protein concentrations, e.g., 5 wt % or 0.2 mol % SP-B (curve 2 in Figure 5), displayed one collapse pressure of about 72 mN/m; i.e., the films did not show obvious squeeze-out of the less stable components of the monolayers, SP-B and cholesterol, which have dynamic collapse surface pressures of about 42 and 44 mN/m (curves 6 and 1 in Figure 1). In the presence of higher amounts of protein, e.g., 10 wt % or 0.4 mol % SP-B, an inflection in the isotherm was observed at a π of ≈45 mN/m (curve 3 in Figure 5). Films of SP-B/DPPC without cholesterol which contained the same amounts of protein (≥10 wt %) displayed similar discontinuities at a π of ≈45 mN/m, and this pressure was associated with squeeze-out of SP-B in the SP-B/DPPC monolayers (Taneva & Keough, 1994b, 1995). When monolayers of SP-B/(DPPC/CH) containing low amounts of protein were compressed to 72 mN/m (curves 2 and 3 in Figure 5) and the surface area was held constant, the surface pressure relaxed to its equilibrium value of about 54 mN/m over a period of 600 s (horizontal bar in Figure 5). As mentioned above, the collapse pressure of 72 mN/m for SP-B/DPPC films in the absence of cholesterol remained constant over an interval of approximately 900 s (unpublished data).

At maximum compression of SP-B/(DPPC/CH) monolayers which contained ≥30 wt % or ≥1.7 mol % SP-B (curves 5 and 6 in Figure 5), maximal surface pressures of about 45 mN/m were attained.

Figure 6 shows the behavior of the mean area per residue at constant surface pressure in the SP-B/(DPPC/CH) films as a function of the mole fraction of the amino acid residues of SP-B. There were positive deviations from the additivity rule, consistent with interactions between SP-B and DPPC/

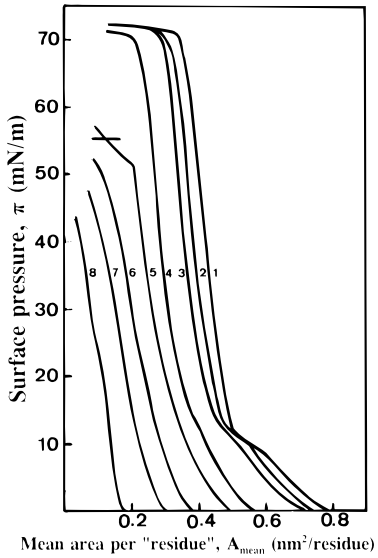

FIGURE 7: Surface pressure versus area isotherms for monolayers of SP-C plus DPPC/CH (6:1, moles:moles). The initial composition of the films was as follows: $X_r = 0.0$ (1), $X_r = 0.13$ or 2.5 wt % (2), $X_r = 0.23$ or 5 wt % (3), $X_r = 0.39$ or 10 wt % (4), $X_r = 0.54$ or 17 wt % (5), $X_r = 0.71$ or 30 wt % (6), $X_r = 0.85$ or 50 wt % (7), and $X_r = 1.0$ wt % (8). The horizontal bar shows the equilibrium surface pressure to which the collapse surface pressures (curves 1−4) relaxed when the films were held at a constant surface area.

CH. Since the $A_{mean}(X_r)$ plots for SP-B/DPPC monolayers in the absence of cholesterol showed similar nonideal behavior (Taneva & Keough, 1994b, 1995), it appeared that cholesterol at this concentration (DPPC/CH, 6:1 moles:moles or 11:1 w:w) did not significantly affect the interactions between SP-B and DPPC in the ternary SP-B/(DPPC/CH) monolayers. It is worth noting that 10 wt % or 0.4 mol % SP-B in the films of DPPC/CH did not affect the ability of cholesterol to produce a condensation effect on the phospholipid monolayer in its liquid state. This conclusion was based on observations of strong negative deviations from the additivity law in $A_{mean}(X_{CH})$ diagrams which were obtained by plotting the mean area per residue in CH/(DPPC/SP-B) monolayers (DPPC/SP-B, 9:1 w:w) versus the mole fraction of cholesterol at a π of <30 mN/m (data not shown).

Monolayers of DPPC/CH plus SP-C

The isotherms for monolayers of SP-C/(DPPC/CH) (DPPC/CH, 6:1 mole:mole or 11:1 w:w) containing various amounts of protein are plotted in Figure 7. Films which contained ≤10 wt % or ≤1.8 mol % SP-C showed a single collapse pressure at about 72 mN/m (curves 2−4 in Figure 7). Our previous measurements on SP-C/DPPC monolayers without cholesterol were consistent with exclusion of SP-C at a π of ≈50 mN/m from the SP-C/DPPC films which contained protein concentrations above 5 wt % SP-C (Taneva & Keough, 1994c, 1995). The lack of apparent squeeze-out of SP-C from the SP-C/(DPPC/CH) films which contained 10 wt % protein was consistent with a stronger association of SP-C with DPPC in the presence of cholesterol than with DPPC alone. As a result, SP-C up to 10 wt % did not appear to be separately removed from the films at a π of ≈50 mN/m, but it cocollapsed with DPPC/CH at a π of ≈72 mN/m. The collapse pressures for the SP-C/(DPPC/CH) monolayers containing 2.5−10 wt % SP-C (curves 2−4 in Figure 7) were not stable, and they relaxed to a π of ≈55 mN/m in about 600 s (horizontal bar in Figure 7). In the absence of cholesterol, the collapse pressures at about 72 mN/m in the SP-C/DPPC monolayers of similar protein concentrations were stable up to about 900 s. SP-C/(DPPC/CH) films containing ≥17 wt % or ≥3.2 mol % SP-C showed an apparent collapse at a π of ≈50 mN/m.

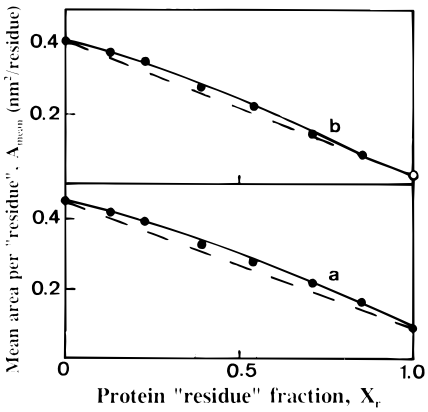

FIGURE 8: Mean area per residue in the SP-C/(DPPC/CH) (6:1 moles:moles or 11:1 w:w) monolayers as a function of initial protein concentration at a constant surface pressure π in millinewtons per meter of 25 (a) and 45 (b).

The plots of the mean area per residue in SP-C/(DPPC/CH) monolayers at a constant surface pressure as a function of initial protein concentration (Figure 8) showed positive deviations from the additivity law in the whole range of protein concentrations studied. In the absence of cholesterol, $A_{mean}(X_r)$ plots for SP-C/DPPC films showed nonideal behavior only for relatively low protein concentrations (X_r < 0.61 or <20 wt % SP-C), whereas an additivity of mean areas was seen at higher protein concentrations (Taneva & Keough, 1994c, 1995). As discussed earlier, self-association of SP-C possibly occurred in the SP-C/DPPC monolayers when protein concentrations were high, and this led to demixing of the protein and phospholipid (Taneva & Keough, 1994c). The ability of SP-C to cause an expansion in the ternary SP-C/(DPPC/CH) monolayers over the whole range of protein concentrations (Figure 8) implied that cholesterol modified the protein−phospholipid interactions in the ternary films in comparison to the interactions of SP-C with DPPC in the binary SP-C/DPPC monolayers without cholesterol. Association between SP-C and cholesterol in the three-component films possibly prevented SP-C from self-aggregation in the monolayers, and this led to a better miscibility of SP-C with DPPC/CH than with DPPC alone. A putative preferential interaction of SP-C with cholesterol or cholesterol-rich phases in the DPPC/CH monolayers is consistent with the property of SP-C and cholesterol to mix randomly in their binary monolayers (Figure 4).

It is worth noting that at low pressures (π < 30 mN/m) cholesterol produced a condensing effect in the films of DPPC alone as in well as in those of DPPC containing SP-B or SP-C (data not shown). Therefore, one can assume that at low concentrations neither protein significantly changed the interactions between DPPC and CH in the ternary monolayers compared to those which occurred in the DPPC/CH films without protein. Given that assumption, the surface pressure versus composition phase diagram for mixtures of DPPC plus cholesterol (Albrecht et al., 1981) could be used as a basis for speculation about possible structures of the ternary protein/(DPPC/CH) monolayers. According to the phase diagram of DPPC/CH monolayers, at surface pressures

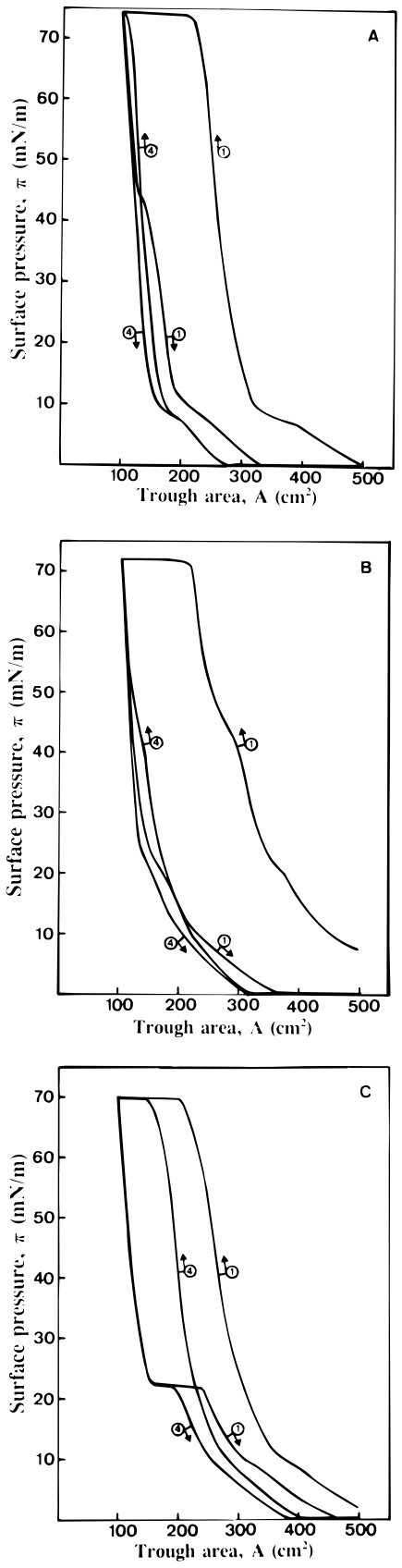

FIGURE 9: Cyclic compression−expansion isotherms for monolayers of DPPC/CH (6:1 moles:moles or 11:1 w:w) (A), DPPC/CH plus 10 wt % SP-B or $X_r = 0.41$ (B), and DPPC/CH plus 10 wt % SP-C or $X_r = 0.39$ (C) formed at initial surface dilute concentrations. Cycles 1 and 4 are shown. The time of the compression−expansion cycle was 240 s, and the relative change in monolayer area was 80%.

above that of the transition from the liquid-expanded to the liquid-condensed state, phases containing nearly pure DPPC and a mixture of DPPC/CH coexist in DPPC/CH monolayers of the composition used in the present study, 8 wt % or 14 mol % cholesterol (Albrecht et al., 1981). In the ternary SP-C/(DPPC/CH) monolayers, SP-C quite possibly had a stronger affinity for microdomains of DPPC/CH than for those of DPPC alone. Since cholesterol did not exert substantial effects on the properties of SP-B/(DPPC/CH) monolayers compared to those of SP-B/DPPC films, one might speculate that SP-B did not interact selectively with any of the lipid compositional microdomains in those ternary monolayers.

Effect of Cholesterol on Respreading Properties of Monolayers of DPPC plus SP-B or SP-C Compressed Beyond Collapse

Initial Surface Dilute Condition. In a series of experiments, monolayers of DPPC/CH (6:1 mole:mole or 11:1 w:w) or DPPC/CH plus either SP-B or SP-C were formed at a relatively low initial surface pressure (<10 mN/m), and they were cyclically compressed and expanded four times. In each cycle, films were compressed beyond the collapse point of about 72 mN/m, and then they were expanded to the initial monolayer area. Figure 9A shows the first and the fourth compression−expansion cycles for monolayers of DPPC/CH. The fourth compression curve in Figure 9A was displaced to lower areas compared to the first one, suggesting loss of molecules when the monolayers were compressed beyond collapse; i.e., there was low capability of DPPC/CH to respread from its collapse phase(s). As a measure of the respreading ability of the monolayers that had been compressed beyond collapse, we used the ratios of the collapse plateau lengths of the successive cycles (Notter et al., 1981a). The values for the collapse plateau length ratios for DPPC and DPPC/CH monolayers, recorded in Table 1, were essentially the same as those previously reported (Taneva & Keough, 1994a), and they indicated that the respreading for DPPC/CH monolayers was not enhanced over the respreading of DPPC alone. Similarly, Snik et al. (1978) have shown that, under surface dilute conditions, cholesterol at 10 and 20 mol % was not capable of modifying the collapse and respreading characteristics of DPPC.

To examine if the hydrophobic pulmonary surfactant proteins influenced the respreading of DPPC/CH monolayers after the compression beyond collapse, measurements were performed on monolayers of DPPC/CH plus increasing amounts of SP-B or SP-C. Representative data for protein/(DPPC/CH) films which contained 10 wt % SP-B or SP-C are shown in panels B and C of Figure 9. The absence of a collapse plateau in the fourth cycles for the films of DPPC/CH (Figure 9A) as well as those of SP-B/(DPPC/CH) (Figure 9B) suggested that SP-B did not improve the respreading of the films of SP-B/(DPPC/CH) compared to that of DPPC/CH. Table 1 also indicates that the addition of SP-B, up to 10 wt % or 0.4 mol %, or SP-C, up to 5 wt % or 0.9 mol %, to the DPPC/CH monolayers did not significantly affect the respreading of the films. At sufficiently high concentrations, 10 wt % or 1.8 mol %, SP-C, in contrast to SP-B, enhanced the respreading of SP-C/(DPPC/CH) films over that of DPPC/CH monolayers. At this protein concentration, one collapse phase was formed at a π of $\approx$72 mN/m which

Table 1: Effect of Cholesterol on Respreading of Monolayers of DPPC plus SP-B or SP-C Formed at Initial Surface Dilute Conditions[a]

monolayer	protein concentration wt%	protein concentration X_r[b]	initial spreading surface pressure π (mN/m)	collapse plateau length ratios cycle *n*:1 2:1	collapse plateau length ratios cycle *n*:1 4:1
DPPC			1.2	0.44	0.30
DPPC/CH[c]			0.5	0.36	0.13
SP-B/(DPPC/CH)[c]	2.5	0.14	0.72	0.45	0.19
SP-B/(DPPC/CH)[c]	10.0	0.41	7.4	0.23 ± 0.030[d]	0.09 ± 0.03[d]
SP-B/DPPC	2.5	0.15	3.8	96	0.90
SP-B/DPPC	5.0	0.26	5.4	0.95	0.92
SP-B/DPPC	10.0	0.43	9.8	0.94	0.91
SP-C/(DPPC/CH)[c]	2.5	0.13	1.9	0.41	0.23
SP-C/(DPPC/CH)[c]	5.0	0.23	3.1	0.43	0.20
SP-C/(DPPC/CH)[c]	10.0	0.39	2.7	0.82 ± 0.04[d]	0.63 ± 0.03[d]
SP-C/DPPC	2.5	0.14	2.2	0.92	0.78
SP-C/DPPC	5.0	0.25	3.2	0.95	0.88
SP-C/DPPC	10.0	0.41	3.3	0.90	0.80

[a] Each monolayer contained an identical amount of DPPC, corresponding to 0.97 nm^2 per molecule of DPPC at the start of compression. In each experiment, for each successive cycle, a maximum surface pressure of 72 ± 1 mN/m was attained at the end of compression. [b] Mole fraction of amino acid residues of protein in the mixed films. [c] The ratio between DPPC and cholesterol was 6:1 mole:mole or 11:1 w:w. [d] The values represent $\pm$SD ($n = 3$). All films containing cholesterol were studied at least twice, and the ranges found were on the same orders noted for the SD reported. Other films were studied once because the values were consistent with those obtained in a more elaborate previous study (Taneva & Keough, 1994).

exhibited a relatively good ability to respread after collapse (Figure 9C).

In Table 1, the collapse plateau lengths ratios for the protein/(DPPC/CH) monolayers were compared with corresponding values for protein/DPPC films, without cholesterol, which contained identical levels of protein. The ratios for the protein/DPPC films were very similar to those reported previously (Taneva & Keough, 1994a), and they were consistent with a better respreading of the monolayers of DPPC plus hydrophobic protein compared to that of DPPC alone. From the results in Table 1, the addition of cholesterol to the protein/DPPC films appeared to decrease substantially the beneficial effects of SP-B and SP-C on respreading of the protein/lipid monolayers. This property of cholesterol of inhibiting postcollapse respreading might be related to its ability to affect the stability of surface pressure of the protein/lipid monolayers compressed past collapse. As discussed above, cholesterol increased the relaxation of dynamic postcollapse surface pressure of the DPPC/CH and SP-B/(DPPC/CH) or SP-C/(DPPC/CH) films compared to those of their counterparts without cholesterol. The characteristic times of surface pressure relaxations to the equilibrium values were on the order of 600 s, i.e., comparable to the time taken for the compression-expansion cycle in Figure 9A−C (about 240 s). It appears that in the presence of cholesterol the respreading of the films from collapse phase(s) likely was influenced by cholesterol affecting the kinetics of collapse of the monolayers. Cholesterol-induced fast collapse of the films of protein/(DPPC/CH), or the instability of the films compressed to collapse, apparently led to a decline in the respreading abilities of the collapse phases of the monolayers.

Initial Surface Excess Conditions. Figure 10A shows the first and fourth compression−expansion isotherms for monolayers of DPPC/CH formed under initial surface excess conditions. Similar measurements were carried out with DPPC/CH plus SP-B or SP-C (representative data for monolayers containing 10 wt % protein are shown in panels B and C of Figure 10). The absence of reproducible hysteresis in the successive cyclic isotherms for the surface excess films of DPPC/CH (Figure 10A) was consistent with a poor respreading of the collapse phase(s) formed at maximum compression. The ratios of the collapse plateau lengths for DPPC/CH films, recorded in Tables 1 and 2, indicated that, independently of the initial spreading conditions, DPPC/CH monolayers compressed past collapse did not easily respread. The addition of up to 10 wt % or 0.4 mol % SP-B did not improve the respreading ability of the ternary surface excess films of SP-B/(DPPC/CH), whereas at all concentrations studied, SP-C marginally enhanced the respreading of the SP-C/(DPPC/CH) monolayers compared to DPPC/CH alone. In contrast, in the absence of cholesterol, SP-B or SP-C promoted the respreading of DPPC monolayers formed under the same initial conditions (Table 2). Therefore, cholesterol appeared to inhibit the favorable impact of SP-B and SP-C on respreading of the excess protein/DPPC monolayers.

It is worth noting that different kinetic processes for collapse and respreading presumably may operate in the films with and without cholesterol. In the films of DPPC and DPPC plus hydrophobic protein, in the absence of cholesterol, stable collapse pressures of about 72 mN/m were observed at least on the time scale of the dynamic compression−expansion of the monolayers; i.e., real collapse phases with equilibrium surface pressure of about 72 mN/m existed. In this case, the estimation of the collapse plateau length ratios seemed to provide a measure for the intrinsic ability of the collapse phases to respread during expansion of the films. Factors which affected the structures of both the monolayers and collapse phases, i.e., initial spreading pressure, pressure at the end of expansion for each cycle, as well as the concentration of the respreading−promoting agent, possibly determined postcollapse respreading of the lipid/protein films without cholesterol (Taneva & Keough, 1994a). In the presence of cholesterol, however, under the experimental conditions employed in the present study, the collapse phase formed at 72 mN/m was transient and it rapidly converted to another surface phase which was at equilibrium at a surface pressure of about 55 mN/m. In this case, both the kinetics of collapse and respreading seemed to determine the respreading abilities of the films.

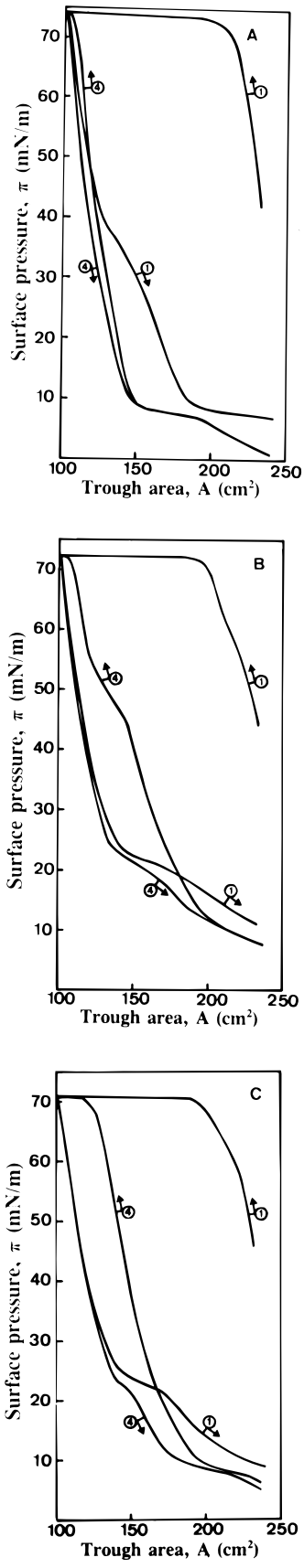

FIGURE 10: Cyclic compression−expansion behavior of surface excess films of DPPC/CH (6:1 moles:moles or 11:1 w:w) (A), DPPC/CH plus 10 wt % SP-B or $X_r = 0.41$ (B), and DPPC/CH plus 10 wt % SP-C or $X_r = 0.39$ (C). Cycles 1 and 4 are shown. The time for compression−expansion was 30 s, and the relative change in monolayer area was 57%.

Adsorption of Aqueous Dispersions of DPPC plus SP-B or SP-C in the Presence and Absence of Cholesterol

Multilamellar liposomes of DPPC/CH (6:1 moles:moles, or 11:1 w:w) did not adsorb to the air−water interface at 35 °C (curve a in Figure 11). A similar adsorption isotherm was observed for DPPC dispersions without cholesterol (data not shown). SP-B dramatically enhanced the rate of adsorption of the lipid dispersions in the presence (curves b, d, and f in Figure 11) or absence of cholesterol (curves c, e, and g in Figure 11), the effect of SP-B being concentration-dependent. These observations were consistent with previous reports on the effects of the hydrophobic pulmonary surfactant proteins on surface active properties of some phospholipid mixtures (Notter et al., 1987; Oosterlaken-Dijksterhuis et al., 1991a). The adsorption isotherms for the dispersions of SP-B/(DPPC/CH) (curves b, d, and f in Figure 11) and those of SP-B/DPPC of similar protein concentrations (curves c, e, and g in Figure 11) suggested that the protein/lipid dispersions adsorbed to surface pressures of about the same final magnitude whether cholesterol was present. At comparable protein levels, however, for films which contained 2.5 or 5 wt % SP-B, the adsorption process was significantly slower in the presence of cholesterol (compare curve b with c and curve d with e). Cholesterol exerted a similar inhibitory effect on the adsorption rates of DPPC/CH mixtures reconstituted with low concentrations of SP-C (Figure 12); the adsorption rate of SP-C/(DPPC/CH) dispersions which contained 5 wt % or 0.9 mol % SP-C was retarded by cholesterol (compare curves c and d in Figure 12), whereas the sterol completely suppressed the adsorption of SP-C/(DPPC/CH) mixtures which contained 2.5 wt % or 0.4 mol % SP-C (compare curves a and b in Figure 12) in the period studied.

In the presence of 10 wt % or 0.4 mol % SP-B, cholesterol did not affect substantially the adsorption rate of SP-B/(DPPC/CH) dispersions compared to those of SP-B/DPPC (compare curves f and g in Figure 11). At 10 wt % or 1.8 mol % SP-C, faster adsorption was observed in the protein/lipid mixtures which contained cholesterol than in the SP-C/DPPC dispersions without cholesterol (compare curves e and f in Figure 12).

The results from the adsorption experiments indicated that cholesterol inhibited the rate of surface film formation for multilamellar liposomes of DPPC which contained either SP-B or SP-C and cholesterol in amounts relevant to those determined for natural lung surfactant, 2.5 and 8 wt %, respectively. Our findings suggested that SP-B and SP-C when present at 10 wt % overcame the negative effect of cholesterol on the adsorption rate of the protein/(DPPC/CH) dispersions.

CONCLUSIONS

The hydrophobic pulmonary surfactant proteins appeared to interact differentially with cholesterol in the spread binary protein/sterol monolayers. SP-B displayed a limited miscibility with cholesterol in the SP-B/CH monolayers and possibly formed a collapse phase separate from that of cholesterol. The properties of the monolayers of SP-C/CH were consistent with the behavior of ideal solutions, and the protein and sterol showed an apparent cocollapse, implying miscibility of their collapse phases. Cholesterol did not significantly affect the interactions in the spread monolayers

Table 2: Effect of Cholesterol on Properties of Monolayers of DPPC plus SP-B or SP-C Formed at Initial Surface Excess Conditions[a]

monolayer	protein concentration wt%	protein concentration X_r[b]	initial spreading surface pressure π (mN/m)	collapse plateau length ratios cycle *n*:1 2:1	collapse plateau length ratios cycle *n*:1 4:1
DPPC			47.0	0.40	0.19
DPPC/CH[c]			47.0	0.36	0.11
SP-B/(DPPC/CH)[c]	2.5	0.14	46.2	0.30	0.12
SP-B/(DPPC/CH)[c]	5.0	0.25	45.0	0.26	0.09
SP-B/(DPPC/CH)[c]	10.0	0.41	44.4	0.30 ± 0.01[d]	0.10 ± 0.01[d]
SP-B/DPPC	2.5	0.15	45.7	0.57	0.50
SP-B/DPPC	5.0	0.26	45.5	0.55	0.43
SP-B/DPPC	10.0	0.43	45.0	0.51	0.43
SP-B/DPPC	10.0	0.43	45.0	0.51	0.39
SP-C/(DPPC/CH)[c]	2.5	0.13	44.6	0.42	0.25
SP-C/(DPPC/CH)[c]	5.0	0.23	46.3	0.43	0.22
SP-C/(DPPC/CH)[c]	10.0	0.39	45.8	0.42 ± 0.04[d]	0.21 ± 0.04[d]
SP-C/DPPC	2.5	0.14	48.2	0.56	0.35
SP-C/DPPC	5.0	0.25	48.0	0.72	0.62
SP-C/DPPC	10.0	0.41	46.3	0.67	0.66

[a] The initial surface pressure corresponded to 0.33 nm^2 per molecule of DPPC. In each experiment, a maximum surface pressure of 72 ± 1 mN/m was attained at the end of compression. [b] Mole fraction of amino acid residues of protein. [c] The ratio between DPPC and cholesterol was 6:1 moles:moles or 11:1 w:w. [d] The values represent mean ±SD ($n = 3$). See also footnote *d* in Table 1.

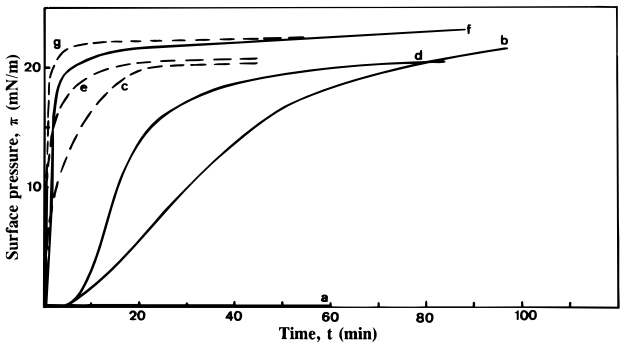

FIGURE 11: Adsorption isotherms for aqueous dispersions of DPPC/CH (a), DPPC/CH plus 2.5 wt % SP-B (b), DPPC plus 2.5 wt % SP-B (c), DPPC/CH plus 5 wt % SP-B (d), DPPC plus 5 wt % SP-B (e), DPPC/CH plus 10 wt % SP-B (f), and DPPC plus 10 wt % SP-B (g). Isotherms were the average of at least two experiments; the range of values did not exceed 1−2 mN/m. The subphase was 0.15 M NaCl (pH 5.6); the final concentration of DPPC was 0.06 mg/mL. In all mixtures containing cholesterol, the DPPC/CH ratio was 6:1 mole:mole or 11:1 w:w.

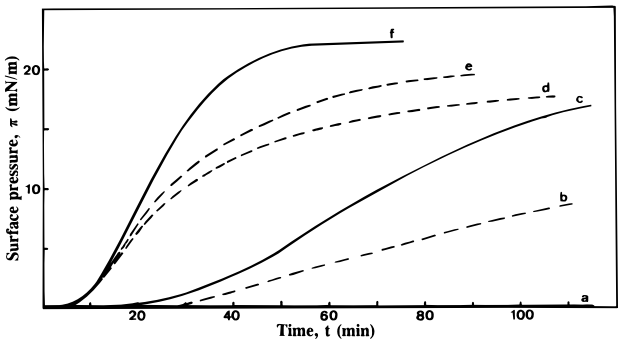

FIGURE 12: Adsorption isotherms of DPPC/CH plus 2.5 wt % SP-C (a), DPPC plus 2.5 wt % SP-C (b), DPPC/CH plus 5 wt % SP-C (c), DPPC plus 5 wt % SP-C (d), DPPC plus 10 wt % SP-C (e), and DPPC/CH plus 10 wt % SP-C (f). Isotherms were the average of at least two experiments; the range of values did not exceed 1−2 mN/m. The subphase was 0.15 M NaCl (pH 5.6); the final concentration of DPPC was 0.06 mg/mL. The DPPC/CH ratio was 6:1 mole:mole or 11:1 w:w.

of SP-B/(DPPC/CH) compared to those in SP-B/DPPC films, as was suggested by the absence of an effect of cholesterol on either the exclusion pressure of SP-B (about 45 mN/m) or the $A_{mean}(X_r)$ diagrams for the protein/lipid monolayers. Cholesterol seemed to strengthen the interactions of SP-C with lipid in DPPC/CH compared to SP-C interaction with DPPC alone in that SP-C was more difficult to squeeze out from the monolayers of DPPC/CH than from DPPC and the protein was better accommodated in the SP-C/(DPPC/CH) films than in those of SP-C/DPPC.

The presence of cholesterol in the SP-B/(DPPC/CH) and SP-C/(DPPC/CH) monolayers spread under dilute or excess initial conditions inhibited the postcollapse respreading of the films compared to the respreading of protein/lipid films without cholesterol; cholesterol practically abolished the beneficial impacts of low concentrations of SP-B and SP-C on the respreadability of the films. At sufficiently high concentrations, e.g., 10 wt %, SP-C appeared to counterbalance the negative effect of cholesterol on the postcollapse respreading of dilute monolayers of SP-C/(DPPC/CH). The property of cholesterol to diminish the postcollapse respreading of both dilute and excess monolayers of SP-B/(DPPC/CH) and SP-C/(DPPC/CH) seemed to correlate with its decreasing of the stability of the surface films compressed to collapse (72 mN/m).

Evaluation of the adsorption behavior of DPPC plus hydrophobic surfactant proteins in the presence or absence of cholesterol demonstrated an inhibitory effect of cholesterol on the adsorption rates for those protein/(DPPC/CH) mixtures which contained low protein levels (2.5 and 5.0 wt %). At higher concentrations of SP-B or SP-C, e.g., 10 wt %, the unfavorable impact of cholesterol on the adsorption rate of the protein/(DPPC/CH) dispersions was suppressed, and cholesterol appeared to act cooperatively with SP-C to accelerate the adsorption process of SP-C/(DPPC/CH) dispersions.

This study presents evidence that cholesterol was detrimental to the interfacial properties of reconstituted mixtures of DPPC plus hydrophobic surfactant protein deemed desirable in pulmonary surfactant in situ. It destabilized the surface pressure of monolayers compressed to a collapse regime at 72 mN/m, impaired the postcollapse respreading of the films, and inhibited the rate of formation of surface films from liposomes in the subphase. The hydrophobic surfactant proteins, in amounts comparable to those in natural surfactant, could not overcome the negative impact of cholesterol on these interfacial properties of the protein/

(DPPC/CH) films. An increase in the concentration of the protein counteracted the unfavorable impact of cholesterol on the postcollapse respreading and adsorption rates of the protein/(DPPC/CH) monolayers, although concentrations of protein greater than those associated with natural surfactant appeared to be required for this beneficial effect. The studies on surface films were conducted at 23 °C, a temperature relevant to surfactant in many poikilothermic animals. In the case of mammalian surfactants, it might be expected that the negative influence of cholesterol on desirable surfactant functions might be somewhat exacerbated at 37 °C. The relevance of these results to a potential role of cholesterol in the interfacial behavior of lung surfactant in vivo remains to be fully established, but they suggest that the presence of cholesterol is not likely "beneficial" to the adsorption and respreading properties of natural surfactant. It has been suggested that in the multicomponent lung surfactant there might exist natural "antidotes" for putative negative effects of cholesterol on surface active properties of the protein/lipid surfactant complexes (Suzuki, 1982; Yu & Possmayer, 1993, 1994). The mechanisms of such antidotes are not clear, but hypothetical roles have been attributed to the protein constituents and the heterogenicity of the surface active material (Suzuki, 1982; Yu & Possmayer, 1993, 1994). The results in the present study also suggest roles for the hydrophobic proteins in counteracting some of the inhibitory effects of cholesterol on the surface activity of surfactant.

REFERENCES

Albrecht, O., Gruler, H., & Sackman, E. (1978) *J. Phys. (Paris) 39*, 301−313.

Albrecht, O., Gruler, H., & Sackmann, E. (1981) *J. Colloid Interface Sci. 79*, 319−338.

Baglioni, P., Dei, L., Ferroni, E., & Gabrielli, G. (1986) *J. Colloid Interface Sci. 109*, 109−114.

Bangham, A. D., Standish, M. M., & Watkins, J. C. (1965) *J. Mol. Biol. 13*, 238−252.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.

Colacicco, G. (1973) in *Biological horizons in surface science* (Prince, L. M., & Sears, D. F., Eds.) pp 247−288, Academic Press, New York.

Curstedt, T., Johansson, J., Persson, P., Eklund, A., Robertson, B., Lövenadler, B., & Jörnvall, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2985−2989.

Demel, R. A., Van Deenen, L. L. M., & Pethica, B. A. (1967) *Biochim. Biophys. Acta 135*, 11−19.

Eley, D. D., & Hedge, D. G. (1956) *Faraday Discuss. Chem. Soc. 21*, 221−228.

Ghosh, D., & Tinoco, J. (1972) *Biochim. Biophys. Acta 226*, 41−49.

Giraud, F., Claret, M., & Garay, R. (1976) *Nature 264*, 646−648.

Haagsman, H. P., Hawgood, S., Sargeant, T., Buckley, D., White, T. R., Drickamer, K., & Benson, B. J. (1987) *J. Biol. Chem. 262*, 13877−13880.

Johansson, J., Curstedt, T., & Jörnvall, H. (1991) *Biochemistry 30*, 6917−6921.

Keough, K. M. W. (1992) in *Pulmonary surfactant: From molecular biology to chemical practice* (Robertson, B., Van Golde, L. M., & Batenburg, J. J., Eds.) pp 109−164, Elsevier, Amsterdam.

Keough, K. M. W., & Kariel, N. (1987) *Biochim. Biophys. Acta 902*, 11−18.

King, R. J., & Clements, J. A. (1972) *Am. J. Physiol. 223*, 715−726.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

London, Y., Demel, R. A., Geurts Van Kessel, W. S. M., Zahler, P., & Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 332*, 69−84.

Mita, T. (1989) *Bull. Chem. Soc. Jpn. 62*, 2299−2306.

Müller-Landau, F., & Cadenhead, D. A. (1979) *Chem. Phys. Lipids 25*, 315−328.

Notter, R. H., & Morrow, P. E. (1975) *Ann. Biomed. Eng. 3*, 119−159.

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

Notter, R. H., Holcomb, S., & Mavis, R. D. (1980b) *Chem. Phys. Lipids 27*, 305−319.

Notter, R. H., Tabak, S. A., Holcomb, S., & Mavis, R. D. (1980c) *J. Colloid Interface Sci. 74*, 370−377.

Notter, R. H., Shapiro, D. L., Ohning, B., & Whitsett, J. A. (1987) *Chem. Phys. Lipids 44*, 1−17.

Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., van Golde, L. M. G., & Demel, R. A. (1991a) *Biochemistry 30*, 8276−8281.

Oosterlaken-Dijksterhuis, M. A., Haagsman, H. P., van Golde, L. M. G., & Demel, R. A. (1991b) *Biochemistry 30*, 10965−10971.

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

Pastrana-Rios, B., Taneva, S., Keough, K. M. W., Mautone, A. J., & Mendelson, R. (1995) *Biophys. J 69*, 2531−2540.

Sarin, V. K., Gupta, S., Leung, T. K., Taylor, V. E., Ohning, B. L., Whitsett, J. A., & Fox, J. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2633−2637.

Schubert, D., & Marie, H. (1982) *Biochim. Biophys. Acta 684*, 75−82.

Shah, D. O., & Schulman, J. H. (1967) *J. Lipid Res. 8*, 215−226.

Suzuki, Y. (1982) *J. Lipid Res. 23*, 62−69.

Snik, A. F. M., Kruger, A. J., & Joos, P. (1978) *J. Colloid Interface Sci. 66*, 435−439.

Tabak, S. A., Notter, R. H., Ultman, J. S., & Dinh, S. M. (1977) *J. Colloid Int. Sci. 60*, 117−125.

Tajima, K., & Gershfeld, N. L. (1978) *Biophys. J. 22*, 489−500.

Takahashi, A., Waring, A. J., Amirkhanian, J., Fan, B., & Taeusch, H. W. (1990) *Biochim. Biophys. Acta 1044*, 43−49.

Tanaka, Y., Takei, T., Aiba, T., Masuda, K., Kiuchi, A., & Fujiwara, T. (1986) *J. Lipid Res. 27*, 475−485.

Taneva, S., & Keough, K. M. W. (1994a) *Biochemistry 33*, 14660−14670.

Taneva, S., & Keough, K. M. W. (1994b) *Biophys. J. 66*, 1137−1148.

Taneva, S., & Keough, K. M. W. (1994c) *Biophys. J. 66*, 1149−1157.

Taneva, S., & Keough, K. M. W. (1995) *Biochim. Biophys. Acta 1236*, 185−195.

Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Loimgrukes, W., & Weigele, M. (1972) *Science 178*, 871−872.

Vandenbussche, G., Clercx, A., Clercx, M., Curstedt, T., Johansson, J., Jörnvall, H., & Ruysschaert, J.-M. (1992a) *Biochemistry 31*, 9169−9176.

Vandenbussche, G., Clercx, A., Curstedt, T., Johansson, J., Jörnvall, H., & Ruysschaert, J.-M. (1992b) *Eur. J. Biochem. 203*, 201−209.

Vist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451−464.

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

Yu, S. H., & Possmayer, F. (1993) Biochim. *Biophys. Acta 1167*, 264−271.

Yu, S. H., & Possmayer, F. (1994) *Biochim. Biophys. Acta 1211*, 350−358.

Yu, S. H., Smith, N., Harding, P. G. R., & Possmayer, F. (1983) *Lipids 18*, 522−529.

BI9623542