Pulmonary Surfactant Proteins SP-B and SP-C in Spread Monolayers at the Air-Water Interface: III. Proteins SP-B Plus SP-C With Phospholipids in Spread Monolayers

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ABSTRACT Spread binary monolayers of surfactant-associated proteins SP-B and SP-C were formed at the air-water interface. Surface pressure measurements showed no interactions between the hydrophobic proteins. The effects of a mixture of SP-B plus SP-C (2:1, w/w) on the properties of monolayers of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), and DPPC:DPPG (7:3, mol:mol) were studied. During compression of ternary and quaternary films, containing less than 0.4 mol% or 5 weight% total protein, the proteins were not squeezed out and appeared to remain associated with the film until collapse at surface pressures of about 65–70 mN·m^{-1}. At initial concentrations of total protein of about 0.9 mol% or 10 weight%, exclusion of protein-lipid complexes was observed at 40–50 mN·m^{-1}. Larger amounts of phospholipid were removed by proteins from (SP-B:SP-C)/DPPG films than from (SP-B:SP-C)/DPPC ones. Separate squeeze-out of SP-B (or SP-B plus DPPC) at about 40 mN·m^{-1}, followed by exclusion of SP-C (or SP-C plus DPPC) at about 50 mN·m^{-1}, was observed in (SP-B:SP-C)/DPPC films. This led to a conclusion that there was independent behavior of SP-B and SP-C in (SP-B:SP-C)/DPPC monolayers. The quaternary (SP-B:SP-C)/(DPPC:DPPG) films showed qualitatively similar process of squeeze-out of the proteins. In the ternary mixtures of SP-B plus SP-C with DPPG separate exclusion of SP-B was not detected; rather, the data was consistent with exclusion of a (SP-B:SP-C)/DPPG complex at about 50 mN·m^{-1}. The results imply possible interactions between SP-B and SP-C and the acidic phospholipid.

INTRODUCTION

Experiments in which either of the pulmonary surfactant-associated proteins SP-B or SP-C, or a combination of the two, was added to DPPC or DPPC plus other lipid revealed that these proteins facilitate adsorption and spreading of phospholipids from vesicles in the subphase to the air-water interface (Curstedt et al., 1987; Yu et al., 1987). Some experiments utilizing pulsating bubble surfactometers to monitor the surface properties of lipid-protein dispersions suggested that SP-B and SP-C together enhanced phospholipid adsorption more than either protein alone (Mathialagan and Possmayer, 1990; Takahashi et al., 1990; Yu and Possmayer, 1988). Due to the nature of the measurements it was not ascertained whether the effects were synergistic or additive. Possible interactions between the proteins might account for the combined effect of SP-B and SP-C on phospholipid surface activity (Yu and Possmayer, 1988).

In this work surface balance technique was used to study the potential interaction between SP-B and SP-C in spread monolayers of the proteins without lipids, and the effect of a combination of SP-B and SP-C (2:1, w/w) on properties of monolayers of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and a mixture of DPPC:DPPG (7:3, mol/mol).

MATERIALS AND METHODS

Protein isolation and purification were described in detail in the first part of this series. The purity of the proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16% gel). 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 under nonreducing and reducing conditions. The phospholipid content of the proteins was less than 0.5 mol of phospholipid per mol of SP-B and 0.05 mol of phospholipid per mol of SP-C, which were the detection limits of the lipid determination (Bartlett, 1959).

DPPC from Sigma Chemical Co. (St. Louis, MO) and DPPG from Avanti Polar Lipids Inc. (Pelham, AL) were used. They were determined to be pure by thin-layer chromatography, and were used as received.

Monolayers were spread from organic solvent solutions: chloroform: methanol (1:1, v/v) for SP-B:SP-C monolayers; chloroform for DPPC; and chloroform:methanol (3:1, v/v) for DPPG. Mixed films were formed by spreading of premixed solutions of the components. The experimental equipment and conditions were identical to those described in the first paper in this series.

The compositions of the binary monolayers of SP-B plus SP-C are expressed by the mole fraction of SP-B, $X_{sp\text{-}b}$:

$$X_{sp\text{-}b} = N_{sp\text{-}b}/(N_{sp\text{-}b} + N_{sp\text{-}c}) \quad (1)$$

where $N_{sp\text{-}b}$ and $N_{sp\text{-}c}$ represent the number of spread molecules of SP-B (M_r 17,400) and SP-C (M_r 4,186).

In all monolayers composed of SP-B:SP-C plus phospholipids the ratio between the two proteins was held constant at 2:1 w/w, corresponding to $X_{sp\text{-}b} = 0.34$. Surface pressure measurements were performed on (SP-B:SP-C)/lipid monolayers where the ratio between the proteins was 1:1 (w/w). The results, not included in this paper, showed qualitatively similar properties of the lipid-protein monolayers with the effects being proportional to the relative amounts of the individual proteins. Studies using different experimental methods for isolation of the hydrophobic proteins have indicated

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All abbreviations and symbols are as defined in the first paper of this series.

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various ratios between the two hydrophobic proteins in the natural pulmonary surfactant and lipid extracts of surfactant (Curstedt et al., 1987; Mathialagan and Possmayer, 1990; Takahashi et al., 1990). A ratio of 2:1 (w/w) between SP-B and SP-C is consistent with some of the results suggesting a mass enrichment of SP-B (Hawgood et al., 1987; Mathialagan and Possmayer, 1990; Takahashi et al., 1990).

The initial composition of the (SP-B:SP-C)/lipid monolayers is given by the fraction of the protein amino acid residues, X_r:

$$X_r = N_r/(N_r + N_l) \quad (2)$$

where N_r is the total number of the spread protein amino acid residues (69.7% SP-B and 30.3% SP-C amino acids) and N_l is the total number of the spread lipid molecules.

The experimental mean area per molecule in the binary SP-B:SP-C monolayers was calculated by dividing the trough area by the total number of the spread protein molecules. In the (SP-B:SP-C)/lipid films, the mean area per "residue," A_{mean}, where "residue" denotes lipid molecule or protein amino acid residue, was deduced from the following expression:

$$A_{mean} = \text{trough area}/(N_r + N_l). \quad (3)$$

The partial areas per protein amino acid residue ($\bar{A}_r$) or phospholipid molecule ($\bar{A}_l$) in the (SP-B:SP-C)/lipid monolayers were determined from the plots of the mean area per "residue" versus monolayer composition using the methods described in the first paper of this series. $\bar{A}_r$ represents the contribution of an average protein amino acid residue to the monolayer area in the mixed monolayers, and it does not distinguish between SP-B and SP-C residues.

RESULTS AND DISCUSSION

Spread binary monolayers of SP-B and SP-C

The isotherms of surface pressure versus area per amino acid residue for the single component monolayers of SP-B and SP-C were discussed in detail in the accompanying papers. In Fig. 1 isotherms for the proteins are plotted on the basis of area per molecule (SP-B (*curve 1*) and SP-C (*curve 6*)). The difference in the areas at the lift-off pressures of the curves (approximately 40 nm^2/molecule for SP-B and 6 nm^2/molecule for SP-C) reflects the greater size of the SP-B (158 amino acid residues) than SP-C (35 amino acid residues). In Fig. 1 isotherms for some mixtures of SP-B and SP-C are plotted. The mean areas per protein molecule in these binary films were determined from the isotherms at certain surface pressures, and they were plotted versus monolayer composition $X_{sp\text{-}b}$ in Fig. 2. The binary films of SP-B and SP-C showed additivity of the mean areas per molecule, i.e., they displayed properties consistent with random interaction between the proteins in the monolayers. The observed behavior in Fig. 2, however, may represent either ideal mixing or total immiscibility of the components. It was difficult to experimentally distinguish between these possibilities using the collapse pressures of the films, since the isotherms of the two proteins showed similar collapse pressures of about 32–35 $mN \cdot m^{-1}$ and so did their binary mixtures. Complete immiscibility seems, however, to be an unlikely cause of the observed behavior. It is worth noticing that the mixing properties of the proteins in the spread films depended on the extent of their delipidation. Additive behavior of the mean area per molecule in the SP-B:SP-C monolayers was observed only when extensively delipidated proteins were used (containing less than 0.5 mol of phospholipid per mol of SP-B and 0.05 mol of phospholipid per mol of SP-C). When partially delipidated proteins were used in the experiments (e.g., proteins containing $\geq$ 0.5 mol of phospholipid per mol of SP-B and $\geq$ 1.4 mol of phospholipid/mol of SP-C), an expansion in the mean area per molecule was observed in the mixed films. An commensurable expansion was observed when small amounts of DPPG (about 3 mol/mol of protein) were added to the mixtures of delipidated SP-B and SP-C. These experiments showed that the determination of the intrinsic interfacial properties of SP-B, SP-C and their mixtures required the use of extensively delipidated proteins.

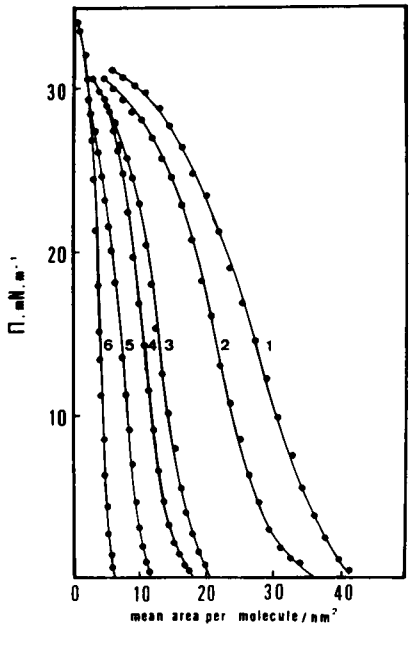

FIGURE 1 Surface pressure-area per molecule isotherms of SP-B, SP-C, and their mixed monolayers of compositions $X_{sp\text{-}b}$: 1.0 (*1*), 0.76 (*2*), 0.43 (*3*), 0.34 (*4*), 0.19 (*5*), 0.0 (*6*)

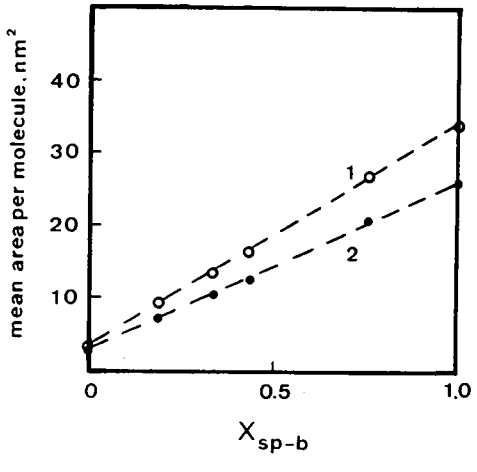

FIGURE 2 Mean area per molecule in the SP-B:SP-C monolayers as a function of mole fraction of SP-B at a surface pressure of 5 $mN \cdot m^{-1}$ (*1*) and 15 $mN \cdot m^{-1}$ (*2*).

Ternary monolayers of SP-B:SP-C with DPPC

Fig. 3 shows the isotherms of surface pressure versus mean area per "residue" for the monolayers of a mixture of SP-B:SP-C (2:1 w/w) and DPPC. Films which contained total protein of $X_r < 0.42$, corresponding to 0.93 mol% or 10 weight%, showed a single collapse at about 70 $mN \cdot m^{-1}$. This result suggests that the two proteins were retained in the monolayer up to surface pressures corresponding to the collapse pressure of DPPC, where the lipid-protein film collapsed as one phase. Inspection of the $\pi(A_{mean})$ isotherms for monolayers of $X_r \geq 0.42$, e.g., curves 3 and 4 in Fig. 3, showed a change in the slope of the isotherms at $\pi \approx 40$ $mN \cdot m^{-1}$ and a second kink point at $\pi \approx 50$ $mN \cdot m^{-1}$, followed by the collapse plateau at 70 $mN \cdot m^{-1}$. These findings were confirmed by the dependence of surface elasticity (E) of the films on surface pressure (Fig. 4). For monolayers of initial composition $X_r \geq 0.42$ discontinuities in the $E(\pi)$ plot at $\pi \approx 40$ $mN \cdot m^{-1}$ and $\pi \approx 50$ $mN \cdot m^{-1}$ were observed, consistent with changes in the physical state of the monolayer. Previous measurements on binary SP-B/DPPC films showed that exclusion of SP-B associated with a small amount of DPPC occurred at surface pressures of about 40 $mN \cdot m^{-1}$ (Fig. 5, first of this series of papers). Exclusion of SP-C/DPPC units at 50 $mN \cdot m^{-1}$ was detected in binary SP-C/DPPC films (Fig. 3, second of this series of papers). The minima in the $E(\pi)$ plots for the ternary (SP-B:SP-C)/DPPC films, therefore, correspond to the pressures where exclusion of the two proteins from the individual binary mixtures with DPPC occurred. This result suggests that during compression of the ternary films, squeeze-out of SP-B, or SP-B plus DPPC, commenced at $\pi \approx 40$ $mN \cdot m^{-1}$, followed by exclusion of SP-C, or SP-C plus DPPC, at $\pi \approx 50$ $mN \cdot m^{-1}$. This would be consistent with independent behavior of SP-B and SP-C in the DPPC films where each protein acted as an individual species in a manner similar to its behavior in the respective binary film with DPPC.

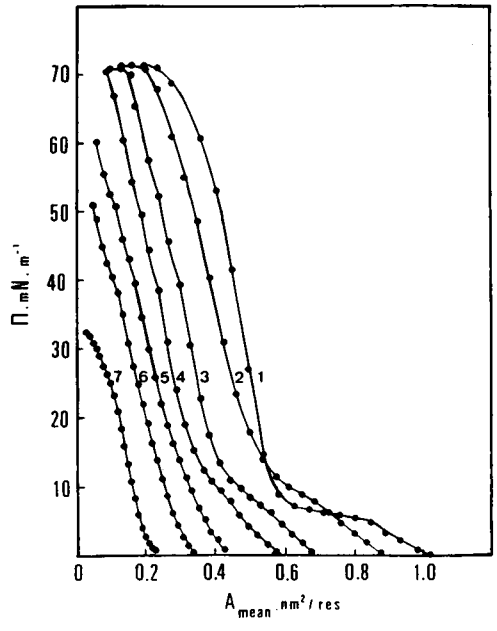

FIGURE 3 Isotherms of surface pressure versus area per "residue" of ternary (SP-B:SP-C)/DPPC monolayers of various initial compositions, X_r: 0.0 (*1*), 0.25 (*2*), 0.42 (*3*), 0.57 (*4*), 0.73 (*5*), 0.87 (*6*), 1.0 (*7*). The ratio SP-B:SP-C in all mixtures was 2:1 (w/w).

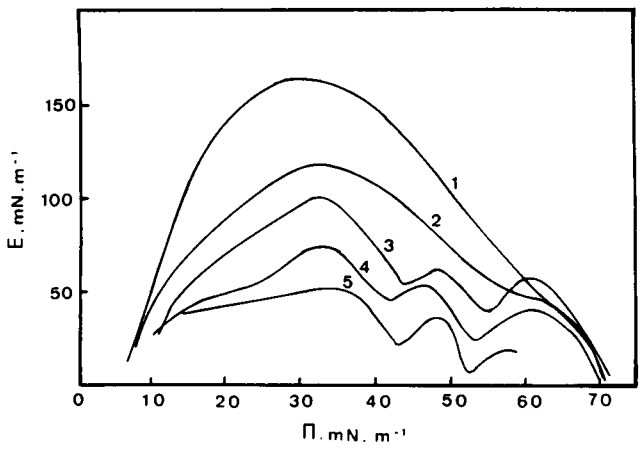

FIGURE 4 Surface elasticity-surface pressure plots for (SP-B:SP-C)/DPPC monolayers of initial composition X_r: 0.0 (*1*), 0.25 (*2*), 0.42 (*3*), 0.57 (*4*), 0.73 (*5*).

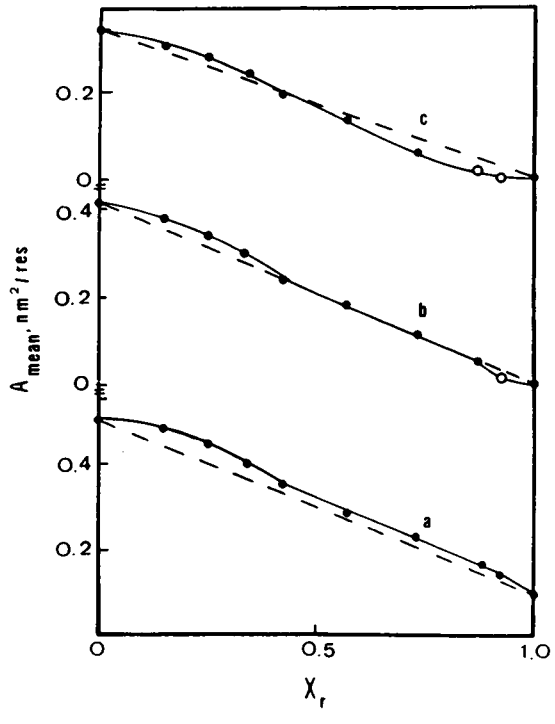

FIGURE 5 Mean area per "residue" in the (SP-B:SP-C)/DPPC monolayers versus their initial composition at constant surface pressure: 25 $mN \cdot m^{-1}$ (*a*), 50 $mN \cdot m^{-1}$ (*b*), 60 $mN \cdot m^{-1}$ (*c*). The full circles represent average results of at least two experiments. The open circles represent extrapolated values of A_{mean} at the given surface pressure.

The mean area per "residue" in the ternary (SP-B:SP-C)/DPPC films was determined at selected surface pressures and plotted versus initial monolayer composition in Fig. 5. At low

surface pressures, e.g., $\pi = 25$ mN·m^{-1}, the $A_{mean}(X_r)$ curve for the (SP-B:SP-C)/DPPC films seems to combine the features of the $A_{mean}(X_r)$ plots for the binary films of SP-B/DPPC (Fig. 3, first paper in series) and SP-C/DPPC (Fig. 4, second paper in series) at the same surface pressure. This suggests that SP-B and SP-C may have independent expansion effects on the DPPC monolayer. To check this possibility the following approach was used. The difference between the experimental mean area per "residue" in a mixed film (*full circles* in Fig. 5) and the area per "residue" in the case of ideal mixing of the components (*dashed lines* in Fig. 5) was defined as a monolayer expansion, $A_{mean} - A^{id}_{mean}$. It was determined from the $A_{mean}(X_r)$ diagram at 25 mN·m^{-1} (Fig. 5 *a*) and plotted against the initial monolayer composition in Fig. 6 (*curve 1*). On the other hand, assuming that SP-B and SP-C in the ternary films with DPPC exerted independent expansion effects on the phospholipid, the expansion of the ternary film at 25 mN·m^{-1} was calculated by adding up the expansions for the binary SP-B/DPPC (Fig. 3 *a*, first paper of series) and SP-C/DPPC (Fig. 4 *a*, second paper of series) films at the same pressure. In this case, 69.7% of the amino acid residues in the (SP-B:SP-C)/DPPC films were assume to contribute to the expansion of the ternary film as SP-B amino acid residues and 30.3% as SP-C residues. The resulting calculated values of the monolayer expansion were compared to the experimental values in Fig. 6. Within experimental error, the two curves are sufficiently close to conclude that SP-B and SP-C have additive effects in the three-component monolayers with DPPC. The partial areas of the protein amino acid residues and lipid molecule in the ternary films were determined from the plots of the mean area per "residue" versus initial monolayer composition in Fig. 5. They were used for evaluation of the changes in the compositions of the monolayers during their compression (first paper of series), and the results are summarized in Table 1. The initial composition of the ternary monolayers of $X_r \leq 0.25$, corresponding to 0.42 mol% or 4.8 weight% protein, was not changed with increasing surface pressure. At higher initial concentrations of the protein $X_r \geq$ 0.42, equivalent to 0.93 mol% or 10 weight%, the $E(\pi)$ data were consistent with a two-step process of squeeze-out of the proteins (Fig. 4). It commenced at $\pi > 40$ mN·m^{-1}, followed by separation of a second phase at $\pi > 50$ mN·m^{-1} before the final collapse at $\pi \approx 70$ mN/m^{-1}. The data in Table 1 suggest that at $\pi > 45$ mN·m^{-1} nearly pure protein (likely SP-B) was squeezed out. Exclusion of protein/DPPC (likely SP-C/DPPC) followed at $\pi \approx 55$ mN·m^{-1}. At this surface pressure the amounts of DPPC removed from the (SP-B:SP-C)/DPPC films together with the proteins were comparable to the lipid lost from the binary SP-C/DPPC films at the same pressure (Table 1, second paper of this series).

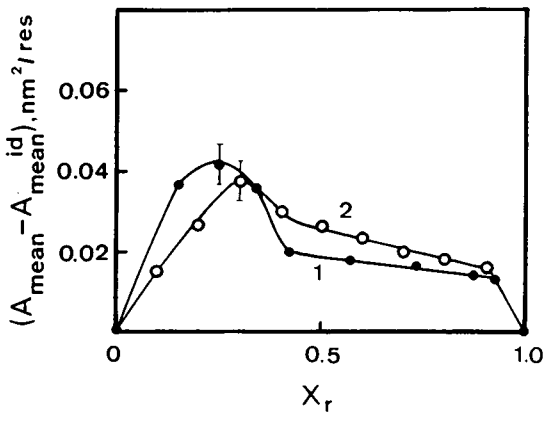

FIGURE 6 Expansion in the (SP-B:SP-C)/DPPC monolayers as a function of their composition at $\pi = 25$ mN·m^{-1} experimental (*1*), calculated (*2*).

Ternary monolayers of SP-B:SP-C plus DPPG

The $\pi(A_{mean})$ isotherms for (SP-B:SP-C)/DPPG monolayers are shown in Fig. 7. Ternary monolayers of initial protein concentration $X_r = 0.16$ collapsed at π of about 65 mN·m^{-1}. The curves for monolayers of higher initial protein concentration $0.25 \leq X_r \leq 0.57$ exhibited a second collapse point at $\pi \approx 50$ mN·m^{-1}. Monolayers of $X_r = 0.74$ showed one collapse plateau at about 50 mN·m^{-1}. These observations were confirmed by the plots of surface elasticity, E, versus surface pressure in Fig. 8. The $E(\pi)$ diagrams for monolayers of $X_r \geq 0.25$ display one minimum at $\pi \approx 50$ mN·m^{-1}. The $E(\pi)$ data for the ternary films of (SP-B:SP-C)/DPPG are consistent with one-step exclusion of protein (or protein-lipid units) from the monolayers, as opposed to the (SP-B:SP-C)/DPPC films where a two-step exclusion process was detected (Fig. 4). Our previous measurements on binary films of either SP-B or SP-C plus DPPG showed that exclusion of SP-B/DPPG units from the SP-B/DPPG films occurred at $\pi \approx 45$ mN·m^{-1} (Fig. 8, first paper of series), whereas SP-C/DPPG complexes were squeezed out from the SP-C/DPPG films at surface pressures of about 50 mN·m^{-1} (Fig. 6, second paper of series). The results for the ternary films of (SP-B:SP-C) plus DPPG suggest that SP-B was not separately squeezed out from the films at the exclusion pressure determined for the binary SP-B/DPPG films. Rather, the two proteins appeared to be associated in the DPPG environment causing their collective squeeze-out at $\pi \approx 50$ mN·m^{-1}, which corresponds to the exclusion pressure of SP-C from binary SP-C/DPPG films.

Analysis of the mean area per "residue," A_{mean}, as a function of initial monolayer composition X_r and surface pressure (Fig. 9) showed expansion of the monolayers in the whole range of protein concentrations when surface pressure was lower than 50 mN·m^{-1} (π_{kink}). At $\pi \geq \pi_{kink}$, for films containing low amounts of protein, positive deviations of the experimental mean areas per "residue" from ideal behavior was observed up to $\pi \approx 60$ mN·m^{-1}, consistent with a presence of protein in the films. At higher initial concentrations of protein in the monolayers the data suggests that exclusion of (SP-B:SP-C)/DPPG units occurred. From the $A_{mean}(X_r)$ plots the expansion of the (SP-B:SP-C)/DPPG films, A_{mean}-A^{id}_{mean}, was determined at 25 mN·m^{-1} and plotted as a function of initial monolayer composition in Fig. 10

TABLE 1 Calculated compositions of the (SP-B:SP-C)/DPPC monolayers, X_r^{calc}, and the excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (protein:lipid)	Initial (X_r)	Surface pressure ($mN \cdot m^{-1}$)									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	45	50	55	60	25	45	50	55	60
1:422	0.15	0.15	0.15	0.15	0.15	0.15	-	-	-	-	-
1:234	0.25	0.24	0.21	0.24	0.24	0.24	-	-	-	-	-
1:106	0.42	0.41	0.40	0	0	0	-	-	0.99	0.90	0.87
									1:1*	1:8*	1:11*
1:58	0.57	0.56	0.53	0	0	0	-	-	1.0	0.91	0.87
									1:0*	1:7*	1:11*
1:28	0.73	0.74	0.73	0	0	0	-	-	1.0	0.90	0.87
									1:0*	1:8*	1:11*

* Calculated protein:lipid molar ratio of the excluded phase.

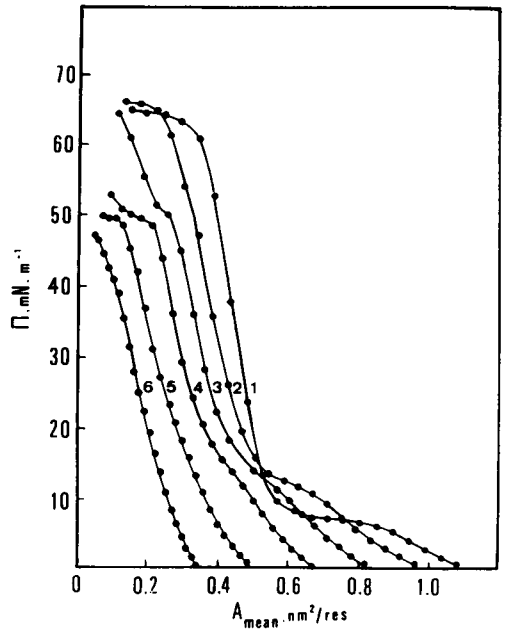

FIGURE 7 Surface pressure-area per "residue" curves of (SP-B:SP-C)/DPPG films of various initial compositions X_r: 0.0 (*1*), 0.25 (*2*), 0.43 (*3*), 0.57 (*4*), 0.74 (*5*), 0.87 (*6*). The ratio between SP-B and SP-C in all mixtures was 2:1 (w/w).

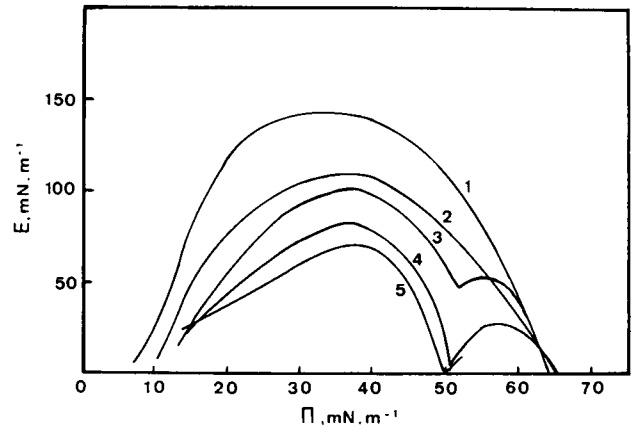

FIGURE 8 Surface elasticity-pressure plots for (SP-B:SP-C)/DPPG monolayers of initial composition X_r: 0.0 (*1*), 0.16 (*2*), 0.25 (*3*), 0.43 (*4*), 0.57 (*5*).

(*curve 1*). On the other hand, the expansion in the films at the same pressure was calculated, assuming that 69.7% of the amino acid residues in the ternary mixtures had an expansion effect on DPPG, the same as the one experimentally determined for the binary SP-B/DPPG monolayers of the same initial composition and surface pressure (Fig. 7 *a*, first paper of series). Similarly, the remaining 30.3% of the amino acid residues were assumed to behave as SP-C residues (Fig. 7 *a*, second paper of this series). The result of this calculation is shown in Fig. 10 (*curve 2*). In the whole range of protein concentrations the experimental values of monolayer expansion are higher than the calculated ones based on the assumption of additive effects of SP-B and SP-C on DPPG in the ternary films. Also, the two curves show different concentration dependencies, which suggests that SP-B and SP-C together possibly have a different mechanism of interaction with DPPG compared to those when the proteins are separately present in binary films with DPPG.

In a manner similar to that described in the first paper of this series, the calculated compositions, X_r^{calc}, of the (SP-B:SP-C)/DPPG films were determined as a function of surface pressure. Also, the compositions of the protein-lipid units, X_r^{lost}, lost from the monolayers during their compression were calculated and the results are shown in Table 2. Similar to what happened in the (SP-B:SP-C)/DPPC films, the protein-lipid complexes removed from the surface were enriched in the protein component. In the ternary films with DPPG, however, SP-B plus SP-C removed considerably higher amount of phospholipid, consistent with a stronger association of the combined proteins with DPPG than with DPPC. A comparison of the amount of DPPG removed by SP-B and SP-C from the ternary films, with the amount of DPPG removed from either SP-B/DPPG (Table 3, first paper of series) or SP-C/DPPG (Table 2, second paper of series) shows that at higher initial protein concentrations the two proteins acted synergistically to remove DPPG from the ternary films.

The surface pressure measurements for the ternary films of SP-B:SP-C with phospholipid showed enhanced interac-

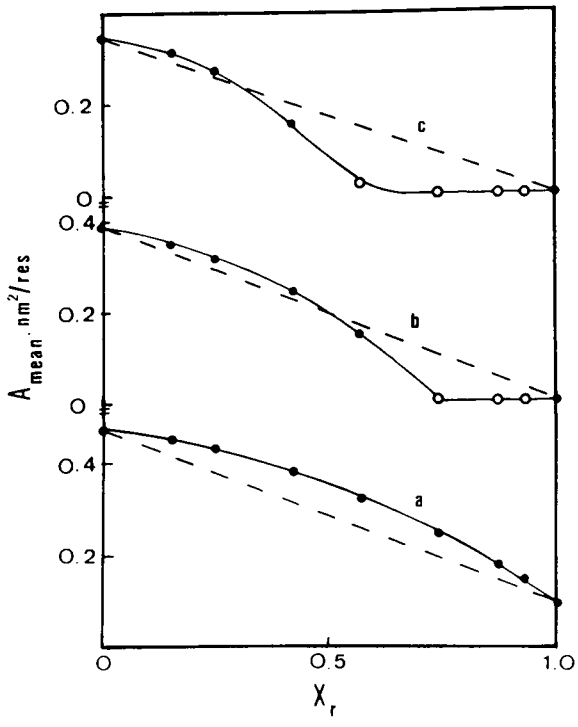

FIGURE 9 Mean area per "residue" in the (SP-B:SP-C)/DPPG films as a function of the initial composition at constant surface pressure: 25 $mN \cdot m^{-1}$ (*a*), 50 $mN \cdot m^{-1}$ (*b*), 60 $mN \cdot m^{-1}$ (*c*).

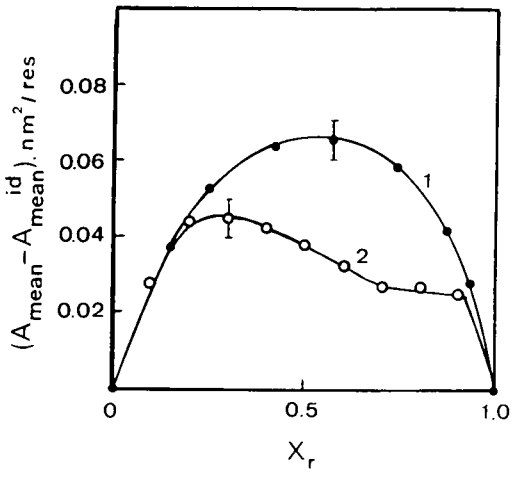

FIGURE 10 Expansion in the (SP-B:SP-C)/DPPG films at π = 25 $mN \cdot m^{-1}$ versus monolayer composition: experimental (*1*), calculated (*2*).

tion of the proteins with DPPG in comparison with DPPC, and this resulted in a higher expansion in the ternary films with DPPG than with DPPC. It also caused removal of higher amounts of phospholipid from (SP-B:SP-C)/DPPG films than from (SP-B:SP-C)/DPPC films of the same initial compositions. Higher efficiency of interaction of a combination of SP-B and SP-C with DPPG (or egg PG) vesicles than with vesicles of DPPC has been reported (Shiffer et al., 1988). In that work, surface pressure measurements showed that the addition of a combination of SP-B and SP-C to preformed phospholipid vesicles promoted the adsorption of the negatively charged phospholipids, but negligibly affected DPPC adsorption.

Quaternary monolayers of (SP-B:SP-C)/(DPPC:DPPG)

The lipid extract of surfactant contains a spectrum of phospholipids plus the hydrophobic proteins SP-B and SP-C (Yu et al., 1983). In an attempt to mimic somewhat more closely the composition of natural pulmonary surfactant and to approximate the lipid components of some artificial surfactant preparations, four-component spread monolayers consisting of SP-B:SP-C (2:1 w/w) and DPPC:DPPG (7:3 mol/mol) were studied. Various ratios between the "protein" (SP-B:SP-C) and the "lipid" (DPPC:DPPG) were used in the multicomponent films. The $\pi(A_{mean})$ isotherms are shown in Fig. 11, where A_{mean} is the mean area per "residue" calculated by Eq. 3 ("residue" denotes lipid molecule or amino acid residue). The surface elasticity, E, of the monolayers was determined from the $\pi(A_{mean})$ curves and plotted versus surface pressure in Fig. 12. The results for the mixed films of $X_r \leq 0.42$ suggest that during compression of the monolayers there was no separation of the components and they collapsed as one phase at high surface pressures. At higher protein concentrations, e.g. $X_r = 0.57$, curve 3 in Fig. 12, the $E(\pi)$ plots are consistent with a three-step collapse of the monolayers, with loss of material at $\pi \approx 40$ $mN \cdot m^{-1}$ and $\pi \approx 50$ $mN \cdot m^{-1}$ preceding the complete collapse of the monolayer at $\pi \approx 70$ $mN \cdot m^{-1}$.

A quantitative interpretation of the data for the multicomponent monolayers was not possible; however, the results for the ternary SP-B/(DPPC:DPPG) (first paper of series), SP-C/(DPPC:DPPG) (second paper of series), and (SP-B:SP-C)/lipid monolayers can give some qualitative suggestions about the protein-lipid complexes which are squeezed out during film compression. Thus, one may expect that, for monolayers of $X_r \geq 0.57$ (equivalent to 0.57 mol% SP-B and 1.11 mol% SP-C, or 11.4 weight% SP-B and 5.3 weight% SP-C) at $\pi \approx 40$ $mN \cdot m^{-1}$, removal of some SP-B associated with small amounts of DPPC and DPPG commences (see Fig. 13, first paper of series). At higher surface pressures of $\pi \geq 50$ $mN \cdot m^{-1}$ SP-C/(DPPC:DPPG) complexes are possibly excluded from the monolayer (see Fig. 9, second paper of series). As it was shown earlier in this work (Fig. 8), a surface pressure of 50 $mN \cdot m^{-1}$ corresponds to the squeeze-out of a complex of (SP-B:SP-C)/DPPG, so it is likely that this kind of lipid-protein formation is also being ejected at 50 $mN \cdot m^{-1}$ from the four-component films. Three-step collapse was already observed in ternary (SP-B:SP-C)/DPPC monolayers, consistent with independent behavior of SP-B and SP-C in the ternary films. The results for the quaternary monolayers also imply that at higher initial protein concentrations, ($X_r > 0.42$, corresponding to 0.32 mol% SP-B and 0.63 mol% SP-C, or 6.8 weight% SP-B and 3.2 weight% SP-C) there is separation of SP-B and SP-C resulting in a two-step exclusion of the proteins from the lipid film. This

TABLE 2 Calculated compositions of the (SP-B:SP-C)/DPPG monolayers, X_r^{calc}, and the excluded phases, X_r^{lost}, as a function of surface pressure

Initial molar ratio (protein:lipid)	Initial (X_r)	Surface Pressure ($mN \cdot m^{-1}$)									
		Film (X_r^{calc})					Excluded phase (X_r^{lost})				
		25	45	50	55	60	25	45	50	55	60
1:411	0.16	0.15	0.16	0.16	0.16	0.17	-	-	-	-	-
1:229	0.25	0.25	0.25	0.24	0	0	-	-	-	0.97	0.79
										1:2*	1:20*
1:103	0.43	0.42	0.42	0	0	0	-	-	0.93	0.69	0.65
									1:5*	1:34*	1:41*
1:57	0.57	0.52	0.58	0	0	0	-	-	0.77	0.65	0.60
									1:27*	1:41*	1:50*
1:27	0.74	0.74	0.73	0	0	0	-	-	0.24	0.74	0.74
									1:27*	1:27*	1:27*

* Calculated protein:lipid molar ratio of the excluded phase.

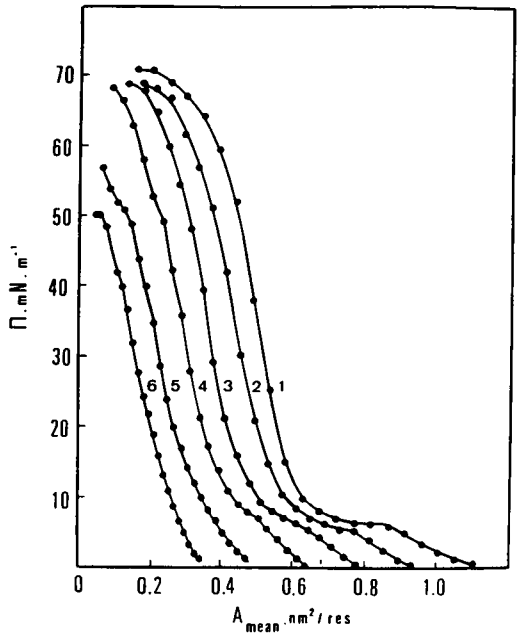

FIGURE 11 Surface pressure-area per "residue" curves for (SP-B:SP-C)/(DPPC:DPPG) monolayers. The composition is expressed by the residual fraction of the protein X_r: 0.0 (*1*), 0.27 (*2*), 0.42 (*3*), 0.57 (*4*), 0.74 (*5*), 0.87 (*6*). In all mixtures the SP-B:SP-C ratio was 2:1 (w/w) and DPPC:DPPG ratio was 7:3 (mol/mol).

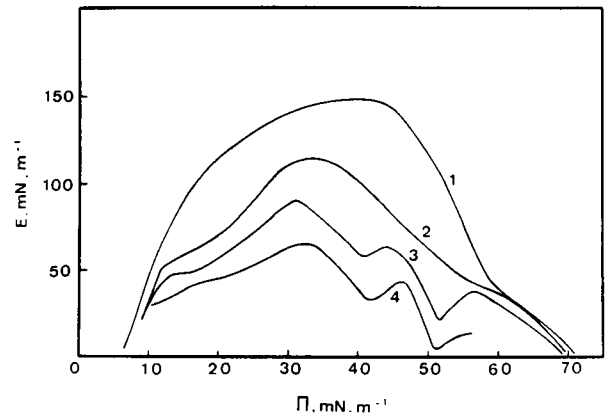

FIGURE 12 Surface elasticity of the quaternary (SP-B:SP-C)/(DPPC:DPPG) monolayers as a function of the surface pressure. The composition of the films X_r: 0.0 (*1*), 0.42 (*2*), 0.57 (*3*), 0.74 (*4*).

observation suggests that in a multicomponent film, such as the initial monolayer at the alveolar-air interface likely is, the two hydrophobic proteins may not have identical roles in surface-related phenomena such as decreasing surface tension, adsorption, or refining the lipid film. The idea of differential interfacial functions of SP-B and SP-C in multicomponent films is consistent with recent measurements with the pulsating bubble machine which suggested that SP-B has a major function in removal of unsaturated phospholipids (Mathialagan and Possmayer, 1990) or phosphatidylglycerol (Yu and Possmayer, 1990) from the surfactant lipid monolayer, leading to an enrichment of DPPC.

SUMMARY DISCUSSION AND CONCLUSIONS

The exact role of the pulmonary proteins in the formation and function of the monomolecular film at the alveolar-air interface is not completely understood. It is not known whether the hydrophobic surfactant proteins SP-B and SP-C are incorporated into the phospholipid monolayer, or the manner in which they enhance the adsorption of phospholipids to the alveolar-air interface or whether they influence desorption of phospholipid from the surface film (Keough, 1992).

In this series of three papers we have studied the interfacial properties of the hydrophobic surfactant-associated proteins SP-B and SP-C in their individual protein monolayers, in combinations without lipids, and in mixtures with the major surfactant phospholipids, DPPC and DPPG. We note that a substantial amount of phosphatidylglycerols in pulmonary surfactant are unsaturated, although the proportions of saturated and unsaturated PG reported vary throughout the literature. These studies were performed to examine the possible contribution of electrostatic effects between the positively charged SP-B or SP-C and acidic phospholipids to the overall lipid-protein interactions. DPPG and DPPC were

chosen to eliminate any effects arising from differences in the length and degree of unsaturation of the acyl chains of the phospholipids. The measurements were conducted at 22°C, while the surfactant functions at 37°C in mammals, although not in airbreathing poikilotherms. In this temperature range both DPPC and DPPG are below their gel to liquid crystalline transition temperature (T_c = 41°C). While there may be some quantitative differences between measures of their interactions with the hydrophobic proteins at 22 and 37°C, the major qualitative factors influencing their properties are likely to be the same at the two temperatures.

The results of the surface pressure measurements, in terms of mean area in the spread protein-lipid films, revealed that the presence of the hydrophobic proteins, SP-B, SP-C, or a mixture of SP-B:SP-C (2:1 w/w), leads to a concentration-dependent expansion of phospholipid monolayers containing either DPPC, DPPG, or a mixture DPPC:DPPG (7:3 mol/mol). In most mixtures studied, maximal perturbation by the hydrophobic proteins of the phospholipid monolayer packing was observed at approximately $X_r \approx 0.25$, equivalent to about 5 weight% protein. In the (SP-B:SP-C)/DPPG films, it corresponded to $X_r \approx 0.50$ or about 17 weight% protein.

Expansion in the mean areas in the mixed films composed of about 5 weight% surfactant hydrophobic protein (SP-B, SP-C, or SP-B:SP-C (2:1, w/w)) and phospholipid was observed in the whole range of studied pressures $0 < \pi \approx 60$ $mN \cdot m^{-1}$ (Figs. 3, 7, and 12 first paper of series, Figs. 4 and 7, second paper of series, Figs. 5 and 9, this paper). This result might have relevance to gas exchange in the lungs since expanded monolayers have been shown to exhibit little resistance to the adsorption of gases compared to condensed ("solid") films which exhibit greater resistance to the gas transport (Birdi, 1989). This would be especially relevant in situations where there was no steady-state concentration gradient of gases from the alveoli to the cells or capillaries.

The collapse behavior of the lipid-hydrophobic surfactant protein monolayers was found to be concentration-dependent. When lipid-protein monolayers containing less than 4 weight% hydrophobic protein (SP-B, SP-C or SP-B:SP-C) were compressed, no segregation and exclusion of the protein at lower pressures, near the collapse pressures of the protein alone, was observed. At these lower protein concentrations, due to hydrophobic and electrostatic (DPPG) interactions with the phospholipid, SP-B, and SP-C appeared to be retained in the monolayers up to surface pressures corresponding to the collapse pressure of the phospholipid (about 70 $mN \cdot m^{-1}$). At this point the lipid-protein mixtures collapsed as one phase. This suggests that the hydrophobic proteins may still be present in the monolayer at the alveolar-air interface at the high surface pressures associated with lung deflation (60–70 $mN \cdot m^{-1}$ (Schürch, 1982)). We note that the way in which the proteins are oriented in the interface, if they are present, could be different in the natural surfactant than in the solvent-spread system used here. Extensive adsorption studies will be required to determine if the hydrophobic proteins can adsorb to the interface in the natural system.

The experimental observation that low amounts of SP-B or SP-C or a mixture of them collapsed together with the lipid component, rather than being squeezed out at lower pressures, could be related to the ability of the proteins to promote the adsorption and spreading of phospholipids to the air-water interface (Curstedt et al., 1987; Yu et al., 1987). A general interpretation of the collapse for solid-type monolayers, such as those of fatty acids and cholesterol, is that when the film becomes excessively compressed, it breaks to give double-layered platelets resting on the monolayer. Because of the lack of contact between the water surface and the polar groups of the molecules in the collapse structures, the latter can not provide a reservoir to replenish molecules lost from the monolayer (Ries, 1979; Ries and Swift, 1987; Ries and Swift, 1989). Thus, the film collapse, in this case, is a rather irreversible process. Although direct experimental evidence for the collapse mechanism of DPPC is not available, the poor respreadability of the monolayer after dynamic compression past collapse is consistent with the mechanism proposed for monolayers of more simple amphipathic molecules. But if proteins were present in the collapse phase, respreading might be enhanced by their presence.

Electron micrographs of collapsed films of some polypeptides and polymers, such as valinomycin and poly(vinyl acetate) did not conform to the above mechanism of monolayer collapse (Ries and Swift, 1989). Rather, the experimental and theoretical studies were consistent with a predominantly reversible displacement of segments of protein from the interface and negligible irreversible material loss due to desorption (MacRitchie, 1977; MacRitchie, 1981). In agreement with this mechanism, measurements in this laboratory of cyclic surface pressure-area curves for SP-B and SP-C films showed that at the high surface pressures the fourth compression isotherm for the monolayers compressed past their collapse practically superimposed with the first one (unpublished data), i.e., no irreversible lost of molecules occurred during collapse of the protein monolayers.

Bearing in mind these considerations one may speculate that the hydrophobic surfactant-associated proteins, which co-collapse with the lipid in the lipid-protein monolayers of $X_r < 0.25$, corresponding to about 5 weight% hydrophobic protein, may alter the mode of phospholipid collapse, yielding enhanced respreadability of the collapsed phase upon subsequent expansion through a reversible exchange between the monolayer and the collapse phase. The reproducible hysteresis in the surface pressure-trough area cycles for spread monolayers of lipid extract surfactant are consistent with a formation of collapse structures which remain attached to the monolayer and facilitate reinsertion of material during monolayer expansion. Interestingly, freeze fracture replicas of the foam from lipid extract surfactant showed the presence of structures in the bulk phase attached to bubble surfaces, whereas DPPC foam did not show any formations directly associated with the interface (Sen et al., 1988).

The surface pressure-area curves for the lipid-hydrophobic protein monolayers of higher protein concentrations (>5 w%) exhibited kink point(s) at lower pressure(s) in addition to their collapse at high pressures typical of those of the phospholipid monolayers alone (Figs. 2, 6, and 11, first paper of series, Figs. 2, 5, and 8, second paper of series, Figs. 3, 7, and 11, this paper). The new collapse state(s) are consistent with expulsion of protein that is associated with some amount of phospholipid. When used separately in the monolayer the proteins carried only small amount of lipids with them as they left the monolayer. SP-C was found to be more efficient in the process of phospholipid removal than SP-B. SP-B and SP-C together gave indications of a combined function in the ternary films with DPPG. This resulted in removal of larger amounts of DPPG from the ternary films than from the binary films of either of the proteins with DPPG (Table 3, first paper of series, Table 2, second paper of series, Table 2, this paper). Though the effect of film refinement was observed at protein concentrations higher than those reported for the natural lung surfactant, one may speculate that possibly this is one of the mechanisms of lipid (DPPC) enrichment of the monolayer at the alveolar surface.

The exclusion pressures for the SP-B/lipid (about 40 $mN \cdot m^{-1}$) and SP-C/lipid (about 50 $mN \cdot m^{-1}$) complexes fall in the interval of pressures corresponding to the plateau region of the dynamic surface pressure-trough area isotherms for spread monolayers of surfactant (Keough, 1984). The difference in the pressures of squeeze-out of the two proteins from their individual films with phospholipids suggests that SP-B and SP-C may have differential roles in the events at the alveolar-air interface during the breathing cycle.

A further investigation on the effects of Ca^{2+} and unsaturated phospholipids on the behavior of the lipid-hydrophobic surfactant protein monolayers, both in equilibrium and dynamic conditions, is worthwhile in order to obtain a better understanding of the properties of the multicomponent film at the alveolar-air interface.

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