# Interactions of the Low Molecular Weight Group of Surfactant-Associated Proteins (SP 5–18) with Pulmonary Surfactant Lipids†

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ABSTRACT: The interaction of the low molecular weight group of surfactant-associated proteins, SP 5–18, with the major phospholipids of pulmonary surfactant was studied by fluorescence measurements of liposomal permeability and fusion, morphological studies, and surface activity measurements. The ability of SP 5–18 to increase the permeability of large unilamellar lipid vesicles was enhanced by the presence of negatively charged phospholipid. The permeability of these vesicles increased as the protein concentration was raised and the pH was lowered. SP 5–18 also induced leakage from liposomes made both from a synthetic surfactant lipid mixture and from lipids separated from SP 5–18 during its purification from canine sources. When SP 5–18 was added to egg phosphatidylglycerol liposomes, the population of liposomes which became permeable leaked all encapsulated contents, while the remaining liposomes did not leak at all. The extent of leakage was higher in the presence of 3 mM calcium. SP 5–18 also induced lipid mixing between two populations of egg phosphatidylglycerol liposomes in the presence of 3 mM calcium, as monitored by resonance energy transfer between two different fluorescent lipid probes, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine. Negative-staining electron microscopy showed that the addition of SP 5–18 and 3 mM calcium produced vesicles twice the size of control egg phosphatidylglycerol liposomes. In addition, surface balance measurements revealed that the adsorption of liposomal lipids to an air/water interface was enhanced by the presence of SP 5–18, negatively charged phospholipids, and 3 mM calcium. These observations suggest a similar lipid dependence for the interactions observed in the fluorescence and adsorption experiments.

Pulmonary surface active material (SAM)[1] lowers the surface tension at the air/water interface in the lung (Schürch et al., 1976), thus allowing lower inflation pressures and preventing lung collapse (Goerke & Clements, 1986). It is a lipid/protein complex, generated in type II cells of the lung and stored in lamellar bodies prior to being secreted into the alveolar lumen (Woodside & Dalton, 1958; Buckingham & Avery, 1962). The lipids present in this complex are rich in dipalmitoylphosphatidylcholine (DPPC) and unsaturated PC and contain phosphatidylglycerol (PG) and cholesterol in addition to other trace lipids (King & Clements, 1985). Once in the alveolar subphase, the lamellar body contents are converted in whole or in part into a lattice-like structure called tubular myelin (Weibel et al., 1966; Gil & Reiss, 1973; Paul et al., 1977; Hassett et al., 1977; Sanderson & Vatter, 1977; Williams, 1977, 1978; Sanders et al., 1980) in a process that requires calcium ions (Benson et al., 1984). When the alveolar surface is significantly expanded, as during an inspiration to total lung capacity, the surfactant adsorbs to the air/water interface and spreads rapidly. It is not known whether all lipid and protein components of the tubular myelin or other forms participate in this process. On compression of this film during expiration, the negatively charged and unsaturated lipids as well as the surfactant-associated proteins, if present, are probably squeezed out of the monolayer, leaving a nearly pure DPPC film possessing a stiffness which strongly resists further decrease in area.

Two groups of surfactant-associated proteins have been described (King, 1982). The higher molecular weight peptides will be collectively referred to as SP 28–36, because, in addition to the major peptide of $M_r$ 32 000, the purified protein preparation also contains two minor peptides of $M_r$ 28 000 and 36 000. The lower molecular weight group of peptides will be referred to as SP 5–18 because peptides with apparent $M_r$ of 5000, 8000, and 18 000 are seen when the SP 5–18 is electrophoresed under nonreducing conditions with sodium dodecyl sulfate (Hawgood et al., 1987).

The low molecular weight group of surfactant-associated proteins, SP 5–18, has not been studied in as much detail as the SP 28–36 group. The amino acid composition (Whitsett et al., 1985; Suzuki, 1982; King et al., 1973, 1977; Phizackerley et al., 1979; Takahashi & Fujiwara, 1986) and sequence analysis of these peptides (Hawgood et al., 1987), as well as the lack of immunologic cross-reactivity between SP 5–18 and SP 28–36 (Suzuki et al., 1986; Yu & Possmayer, 1986;

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[1] Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; CF, carboxyfluorescein; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DPX, *N*,*N*′-(*p*-phenylenedimethylene)bis(pyridinium bromide); EDTA, ethylenediaminetetraacetic acid; egg PC, egg phosphatidylcholine; egg PG, egg phosphatidylglycerol; LUV, large unilamellar vesicles; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RH-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; SAM, pulmonary surface active material; SP 5–18, surfactant proteins of molecular mass 5–18 kDa; SP 28–36, surfactant proteins of molecular mass 28–36 kDa; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Whitsett et al., 1986), indicate that these two groups of proteins are different. Recent studies show that there are at least two distinct hydrophobic proteins within the SP 5–18 group (Hawgood et al., 1987). The larger protein, SP-18, has an apparent $M_r$ of 18 000 when electrophoresed under nonreducing conditions with sodium dodecyl sulfate and a molecular weight under reducing conditions of approximately 5000–8000. The cDNA and $NH_2$-terminal sequence analyses of SP-18 indicate that it is cleaved posttranslationally from a larger precursor protein of approximately 42 kDa. The amino acid sequence of SP-18 is relatively hydrophobic as well as rich in cysteine residues (approximately 7% of the total residues) and positively charged amino acids such as lysine and arginine (approximately 3 and 5% of the total residues, respectively). Purified SP-18 has been shown to promote the formation of phospholipid surface films; film formation is further enhanced in the presence of SP 28–36 and calcium (Hawgood et al., 1987). Two smaller peptides, SP-5 and SP-8, have also been identified within this group. These peptides have molecular weights in the range of 5000–8000 when electrophoresed under nonreducing conditions with sodium dodecyl sulfate and a molecular weight under reducing conditions of approximately 5000. Comparison of the $NH_2$-terminal amino acid sequence of these two proteins shows them to be identical but clearly distinct from the sequence of the SP-18 (Hawgood et al., 1987). The cDNA and $NH_2$-terminal sequence analyses of SP-5 and SP-8 indicate that both of these proteins are cleaved posttranslationally from a larger precursor protein of approximately 22 kDa. Both SP-5 and SP-8 contain an extremely hydrophobic region of 23 consecutive hydrophobic residues. In addition, each peptide contains two positively charged amino acids (one lysine and one arginine residue). The 22-kDa protein is also different from the 42-kDa precursor protein of the SP-18 (Warr et al., 1987). Since complete immunocytochemical studies have not been performed, the exact location of any of the SP 5–18 peptides is not known. The events which generate SP-5, -8, or -18 from their precursor protein molecules are also not known. Although most of the functional studies on SP 5–18 have been performed with the entire group, not individually purified peptides, the group has been shown to enhance lipid surface activity (Suzuki, 1982; Takahashi & Fujiwara, 1986; Yu & Possmayer, 1986, Tanaka et al., 1983; Hawgood et al., 1987) and to promote the uptake of lipids by isolated type II cells (Claypool et al., 1984).

Possible roles for both groups of surfactant-associated proteins may include (1) carrying lipids to lamellar bodies and aiding in their packaging, (2) expanding secreted lamellar body contents into tubular myelin, (3) enhancing the adsorption of surfactant lipids to the air/water interface, (4) facilitating the removal of non-DPPC surfactant lipids from the air/water interface, and (5) promoting the readsorption of surfactant components back into type II cells. All these putative functions concern the interaction of the proteins with lipid moieties.

Here we report our studies on the interactions of SP 5–18 with the major phospholipids of pulmonary surfactant. These interactions were monitored by fluorescence measurements of liposomal permeability and fusion, morphological studies, and surface activity measurements. Preliminary accounts of our findings have been published (Shiffer et al., 1986, 1987).

## Experimental Procedures

*Materials.* Egg phosphatidylcholine (egg PC), phosphatidylglycerol derived by base exchange from egg PC (egg PG), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), and bovine brain phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterol (recrystallized) was from Applied Sciences (State College, PA). Lipids were found to be chromatographically pure by thin-layer chromatography, except for PS, which was repurified by thin-layer chromatography (Touchstone et al., 1980). Thin-layer chromatographic plates were from Whatman (type LK 5D, Clifton, NJ). Carboxyfluorescein (CF) (purchased as chromatographically purified), 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS), *N,N'*-(*p*-phenylenedimethylene)bis(pyridinium bromide) (DPX), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (RH-PE) were from Molecular Probes, Inc. (Eugene, OR). Fluorescamine, L-histidine, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (Tes), and ethylenediaminetetraacetic acid (EDTA) were from Sigman (St. Louis, MO). Sephadex LH-20 and Sephadex G-75 were from Pharmacia (Piscataway, NJ). Chloroform and methanol (Mallinckrodt, Inc., Paris, KY) were redistilled prior to use, and butanol (Mallinckrodt, Inc., Paris, KY) was used as supplied. Water was double distilled from glass.

*Preparation and Analysis of Surface Active Material.* SAM was isolated from the lung lavage fluid of adult dogs according to the procedure of Hawgood et al. (1985). Briefly, the lungs from exsanguinated dogs were removed, degassed, and lavaged 3 times at 4 °C with 1000 mL of 5 mM Tris-HCl–100 mM NaCl, pH 7.4 (lavage buffer), per lavage. The pooled lung washings were spun at $150g_{av}$ for 20 min (Sorvall RC 2-B, I. Sorvall, Inc., Norwalk, CT) to remove cellular material. The supernatant was then centrifuged at $20000g_{av}$ for 15 h (Beckman L3-40, Beckman Instruments, Inc., Palo Alto, CA) with a type 15 rotor, and the resulting pellet (approximately 90–92% of the total phospholipid from the supernatant in the previous step) was dispersed in lavage buffer containing 1.64 M NaBr. After equilibration for 1 h, this suspension was spun at $100000g_{av}$ for 4 h (Beckman L5-50B) in a SW28 rotor (Beckman). The pellicle was resuspended in lavage buffer and centrifuged at $100000g_{av}$ for 1 h twice, and then the final pellet was resuspended in double-distilled water and used as purified SAM All procedures, including the lavage, were performed at 4 °C, and the isolated material was stored at −15 °C. Lipid concentrations were calculated from phosphorus (Bartlett, 1959) and cholesterol (Franey & Amador, 1968) contents. For protein determination (Lowry et al., 1951), 1% sodium dodecyl sulfate (Bio-Rad, Richmond, CA) was added to all samples, and bovine serum albumin was used as the standard. A phospholipid/protein ratio (w/w) of about 7.5 was observed.

The lipids and proteins were isolated from purified SAM as described previously (Hawgood et al., 1987). In order to separate surfactant lipids from SP 5–18, purified SAM was resuspended in 2 mL of double-distilled water (approximately 10–15 mg of phospholipid/mL), injected into 100 mL of rapidly stirred 1-butanol, and then stirred at room temperature for 30 min. After centrifugation at $10000g_{av}$ for 20 min (Sorvall RC 2-B), the supernatant (butanol phase containing surfactant lipids plus SP 5–18) was dried under vacuum at 42 °C and then dissolved in chloroform/methanol/0.1 N HCl (1/1/0.05 v/v/v). After centrifugation at $10000g_{av}$ for 10 min, the chloroform/methanol-soluble fraction was chromatographed on Sephadex LH-20 with the same acidified chloroform/methanol mixture. The eluted fractions (0.5 mL) were monitored for protein by the fluorescamine assay (Bohlen et al., 1973) and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis utilizing silver stained gels and for inorganic

phosphorus (Bartlett, 1959) and cholesterol (Franey & Amador, 1968). SP 5–18 was eluted in the void volume fractions, closely followed, with only minimal overlap, by a second peak containing phospholipid and then a third peak containing neutral lipids and free fatty acids. Fractions containing protein but no detectable phospholipid were pooled and used as SP 5–18. All SP 5–18 protein concentrations were determined by fluorescamine analysis (Bohlen et al., 1973). A phospholipid/SP 5–18 ratio of 10/1 (w/w) was used in most of the experiments. This ratio was based on our estimates of the amount of SP 5–18 in extracellular surfactant (phospholipid/SP 5–18, 100/1 w/w), as determined by a Lowry protein assay (Lowry et al., 1951), and on the finding that our SP 5–18 fluorescamine values were consistently 10 times higher than the protein concentrations obtained in our Lowry assay.

*Preparation of Liposomes.* Large unilamellar liposomes (LUV, 100–200 nm in diameter) were prepared by reverse-phase evaporation from organic solvents according to the procedure of Szoka and Papahadjopoulos (1978), with some modifications (Düzgüneş et al., 1983). Liposomal phospholipid concentrations were determined by phosphorus analysis (Bartlett, 1959). Composition of mixed-lipid liposomes was assessed by thin-layer chromatography (Touchstone et al., 1980) and phosphorus analysis (Bartlett, 1959). Vesicle size was determined by dynamic light scattering (Day et al., 1977) on a Coulter Model N4 submicron particle size analyzer.

*Measurements of Liposomal Leakage.* The interaction of SP 5–18 with phospholipid vesicles caused a disruption in the permeability barrier of the lipid bilayer. This increased permeability was monitored by measurement of the leakage of fluorescent probes encapsulated in the phospholipid vesicles. The release of these fluorophores into the medium resulted in an increase in fluorescence.

A fluorescent probe, ANTS, and its collisional quencher, DPX, were encapsulated inside LUV. The DPX was present in an amount (45 mM) high enough to quench the ANTS fluorescence by collisional quenching. The fluorescence signal directly indicated the amount of leakage which had occurred because the dequenching reaction was fast enough (less than 2 s) to allow for a continuous measurement of leakage (Ellens et al., 1984). Fluorescence was monitored in a Fluorolog 2 spectrofluorometer (SPEX Industries, Inc., Edison, NJ). Excitation was at 360 nm, and emission was measured through a Schott GG-435 cut-off filter (Melles Griot, Irvine, CA). The residual fluorescence of the liposomes, containing 45 mM DPX and 12.5 mM ANTS, was taken as 0% release. Maximal fluorescence (corrected for residual fluorescence), obtained after disruption of the liposomes with the detergent $C_{12}E_8$ (Calbiochem, San Diego, CA), was taken as 100% release. The relative fluorescence measured at any time represented the percent of leakage. Liposomes were suspended in 2 mL of 120 mM NaCl, 2 mM L-histidine, 2 mM Tes, and 1 mM EDTA, pH 7.4, at a final concentration of 10 μg/mL, and the base-line fluorescence was recorded. SP 5–18 dissolved in methanol (1%, final concentration) was injected into the cuvette with constant stirring, and the fluoresence was recorded for a total of 5 min. In control experiments, the same amount of methanol was injected. All experiments were conducted at 37 °C.

To determine whether the liposomal leakage process was a graded release or an all-or-none event, various concentrations of ANTS and DPX or of carboxyfluorescein (CF) were encapsulated within egg PG LUV (Weinstein et al., 1981). The fluorescence of each of these preparations of vesicles was determined before and after detergent ($C_{12}E_8$) lysis, and from this ratio a percent fluorescence value for each liposomal fluorophore concentration was obtained (experimental quench curve). Next, egg PG LUV (containing either 12.5 mM ANTS and 45 mM DPX or 50 mM CF) and SP 5–18 were incubated for 10 min at 37 °C, allowing leakage of some ANTS/DPX or CF to take place. After 10 min, the reaction mixture was applied to a Sephadex G-75 column (5.5 × 0.8 cm), and the vesicles, now separated from free dye, were tested for their pre-to-post detergent fluorescence ratio. These values were then used with the experimental quench curves to estimate liposomal fluorophore concentrations. If leakage were an all-or-none event, then there should have been no dilution of the intraliposomal probe. Hence, the concentration of the probe would remain high with maximal self-quenching (i.e., 50 mM CF), producing a low percent fluorescence value upon lysis. Partial leakage would dilute the intraliposomal probe concentration, allowing less self-quenching and producing higher calculated percent fluorescence values after lysis. Excitation was at 430 nm for CF or at 360 nm for ANTS. Emission was measured through a Corning 3-68 cut-off filter for CF (Corning Glassworks, Corning, NY) or a Schott GG-435 cut-off filter for ANTS.

*Measurements of Lipid Mixing.* Lipid mixing was monitored by resonance energy transfer (Struck et al., 1981). Egg PG LUV with no probes (5 μg/mL) and egg PG LUV containing 0.6 mol % *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and 0.6 mol % *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (RH-PE) (5 μg/mL) were mixed together in the cuvette; the fluorescence intensity of this mixture was taken as 0% mixing. The fluorescence level corresponding to complete mixing of lipids between labeled and unlabeled LUV was established from egg PG LUV that contained 0.3 mol % of each probe at the same total lipid concentration in the reaction mixture (10 μg/mL). Buffer and the probe-containing vesicles were placed in a stirred cuvette, and SP 5–18 (1 μg/mL, final concentration) was injected into the cuvette at time = 0. In this assay, the NBD emission was measured, and lipid mixing was registered as an increase in fluorescence from the NBD probe due to a decreased energy transfer between NBD-PE and RH-PE as the two probes were diluted. All experiments were conducted at 37 °C. Excitation was at 450 nm, fluorescence emission was measured at 520 nm, and 90° light scattering was measured at 450 nm with a 03FIV004 band-pass filter (Melles Griot).

*Negative-Staining Electron Microscopy of Liposomes.* An aliquot of SP 5–18 in methanol (4 μg/mL, final concentration) was injected into a suspension of egg PG LUV (40 μg/mL, final concentration) with continued stirring at 37 °C. After 5 min, a drop of this mixture was applied to a 300-mesh copper grid filmed with Parlodion and carbon coated. After 1 h, most of the liquid was drawn off, and a drop of 2% phosphotungstic acid, pH 6.5, was applied. The grid was blotted to remove excess stain and allowed to dry at room temperature. Control experiments were performed with methanol alone with and without 3 mM $Ca^{2+}$. The grids were examined in a Siemens Elmiskop 101 electron microscope.

*Measurements of Surface Activity.* Surface film pressure ($\pi$) was measured with a platinum Wilhelmy dipping plate (Hildebran et al., 1979) for experiments which lasted 1 h. A Teflon chamber (surface area 3.1 $cm^2$, final subphase volume 3.0 mL) was placed in a temperature-controlled chamber at 37 °C after being scrubbed with phosphate-free detergent and rinsed exhaustively in tap and distilled water. The surface of the subphase buffer (120 mM NaCl, 2 mM L-histidine, 2 mM

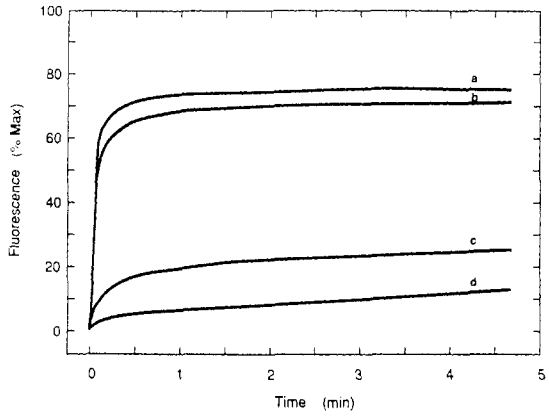


FIGURE 1: Effect of phospholipid species on SP 5–18 induced leakage of ANTS/DPX from single lipid component liposomes. Buffer (120 mM NaCl, 2 mM L-histidine, 2 mM Tes, 1 mM EDTA, pH 7.4) and the ANTS/DPX-containing LUV (10 μg/mL, final concentration) were placed in a stirred cuvette, and SP 5–18 (1 μg/mL, final concentration) was injected at time = 0. Leakages of ANTS/DPX without SP 5–18 were negligible over this period. All experiments were conducted at 37 °C. The liposomes (and final leakages) were (a) egg PG LUV (72.5 ± 3.3), (b) DPPG LUV (69.3 ± 1.5), (c) egg PC LUV (27.8 ± 1.1), and (d) DPPC LUV (15.5 ± 1.7). Values are the mean ± SEM of four experiments and represent the amount of fluorescent probe released after 5 min.

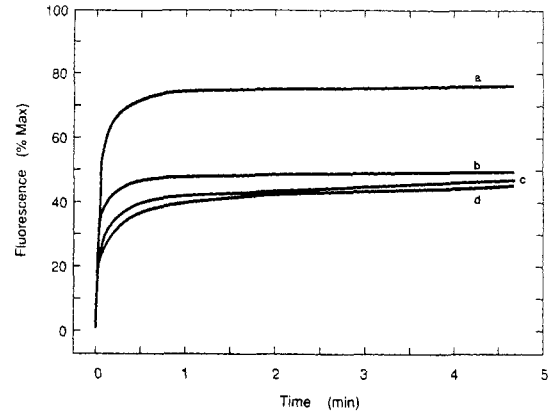


FIGURE 2: Effect of various lipid mixtures on SP 5–18 induced leakage on ANTS/DPX from liposomes. Procedure as in Figure 1. The liposomes (and final leakages) were (a) egg PG LUV (77.3 ± 0.8), (b) canine surfactant lipid LUV (51.0 ± 0.6), (c) synthetic surfactant lipid LUV (47.8 ± 1.1), and (d) egg PG/cholesterol LUV, 85.2/14.8, mol % (46.5 ± 0.9). Values are the mean ± SEM of four experiments and represent the amount of fluorescent probe released after 5 min.

Tes, 1 mM EDTA ± 4 mM $CaCl_2$, pH 7.4, except where the pH was varied) was cleaned by aspiration. After a 10-min equilibration period, LUV (10 μg/mL, final concentration) and then SP 5–18 (1 μg/mL, final concentration) (or vice versa) were injected into the subphase, stirring was started, and surface pressure was recorded.

## RESULTS

### *Measurements of Liposomal Leakage*

*Effects of Lipid Composition.* The curves in Figure 1 show the release of ANTS/DPX from LUV composed of individual phospholipids found in SAM, as a result of the interaction of SP 5–18 with the lipid bilayer. SP 5–18 caused greater release from LUV made of negatively charged phospholipids. The injection of methanol alone (10 μL/mL, final concentration) produced essentially no change over the 5-min period (<5%). The base-line leakage without SP 5–18 over this time was negligible (<2%). In the next series of experiments, the relative proportions of egg PG and DPPC in liposomes were varied. Egg PG, essentially a monoenoic phospholipid, is similar in fatty acid composition to the PG of SAM (King & Clements, 1985). Probe release was extensive (73.5% ± 0.9%) even when the membrane contained a low mole fraction of egg PG (i.e., 10 mol %); however, release from vesicles containing 1 mol % egg PG was considerably lower (20.0% ± 0.8%). These values are the mean ± SEM of four experiments and represent the amount of ANTS/DPX released after 5 min. In addition, the replacement of DPPC by egg PC and of PG by PS did not change the results significantly (data not shown). In all cases, the highest extent of release was obtained with the 80/20 mixture of the zwitterionic lipid with the acidic lipid.

The observation that the release of encapsulated fluorescent markers was sudden and reached a plateau raised the question of whether the addition of more protein would induce the release of the remaining internal contents of the vesicles. Thus, after 4 min of incubation of vesicles with SP 5–18, more protein was introduced. This caused more leakage from egg PG LUV as well as from LUV made from a synthetic surfactant lipid mixture (Syn LUV) and from lipids separated from SP 5–18 during its purification (canine LUV). The lipids used in the Syn LUV and the mole percent of each were as follows: DPPC, egg PC, egg PG, DOPE, and cholesterol, 44.3, 25.8, 13.0, 2.0, and 14.8, respectively. The lipids present in the canine LUV, as determined by TLC and phosphorus analysis (see Experimental Procedures), and the mole percent of each were as follows: lyso-PC, PC, PG, PE, cholesterol, phosphatidylinositol, and sphingomyelin, 4.6, 72.5, 6.7, 0.5, 14.0, 0.5, and 1.2. In each case, the total amount of probe released upon addition of more protein was that which would have been released had this total amount of SP 5–18 been introduced intially (data not shown).

Our results on the relative quenching of ANTS/DPX (or CF) indicate that the addition of SP 5–18 to egg PG LUV caused the release of all the contents of the vesicles with which it interacted (i.e., all-or-none leakage) and that, in the presence of 3 mM calcium, SP 5–18 also promoted lipid mixing of egg PG LUV. The mechanisms are described below. Adding more liposomes with entrapped probe to the protein-interacted egg PG LUV produced no further increase in fluorescence, indicating that no free SP 5–18 in a state capable of lipid interaction was present in the medium and suggesting that all the SP 5–18 had interacted with the vesicles. When the same experiments were repeated in the presence of 3 mM calcium, the same results were obtained: the addition of more protein released more fluorescent probe, and the addition of more liposomes produced no further increase in fluorescence. Adding more Syn LUV or canine LUV with entrapped probe to protein-interacted Syn or canine LUV produced an increase in fluorescence (10–15%), whereas adding egg PG LUV did not (data not shown). The Syn LUV contained enough egg PG (13 mol %) and the canine LUV contained enough PG or other negatively charged phospholipids (6.7 or 7.2 mol % respectively) to cause 65–80% of the fluorescent probe to be released; however, the amount of fluorescent probe released from either of these vesicles was lower than 65%.

As both the Syn LUV and canine LUV contained cholesterol (14.8 mol %), the effect of SP 5–18 was tested on egg PG/cholesterol LUV and DPPG/cholesterol LUV (85.2/14.8, mol %). The effect of SP 5–18 was also tested on vesicles made from a synthetic surfactant lipid mixture without cholesterol (DPPC, egg PC, egg PG, and DOPE, 52.0, 30.3, 15.3, and 2.4 mol %) (Syn No Chol LUV). SP 5–18 caused similar extents of release from Syn LUV, canine LUV, and egg PG/cholesterol LUV (85.2/14.8, mol %) (Figure 2) and from DPPG/cholesterol LUV (85.2/14.8, mol %) (results not shown). Comparable figures in the presence of 3 mM calcium

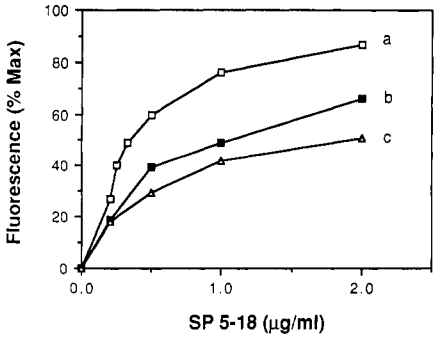


FIGURE 3: Effect of protein concentration on the extent of SP 5–18 induced leakage of ANTS/DPX from liposomes. Procedure as in Figure 1, except that the SP 5–18 concentration was varied. The liposomes were (a) egg PG LUV, (b) canine surfactant lipid LUV, and (c) synthetic surfactant lipid LUV. Values are the mean of four experiments and represent the amount of fluorescent probe released after 5 min. SEM values are all less than 1.

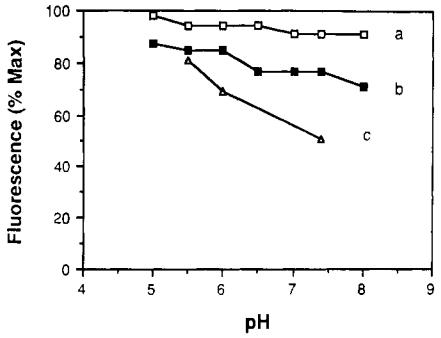


FIGURE 4: Effect of pH on SP 5–18 induced leakage of ANTS/DPX from liposomes. Procedure as in Figure 1. The liposomes were (a) egg PG LUV with 3 mM $Ca^{2+}$, (b) egg PG LUV, and (c) canine surfactant lipid LUV with 3 mM $Ca^{2+}$. Identical curves were obtained for canine surfactant lipid LUV with and without 3 mM $Ca^{2+}$. Values are the mean of four experiments and represent the amount of fluorescent probe released after 5 min. SEM values are all less than 1.

show increases to the following levels: egg PG, 90%; egg PG/cholesterol, 85%; DPPG/cholesterol, 90%. In addition, less probe was released from vesicles containing cholesterol than from their cholesterol-free controls. The Syn No Chol LUV released approximately 20% more fluorescent probe than did the cholesterol-containing Syn LUV. Calcium decreased this cholesterol effect by 10%.

In order to determine how the presence of unsaturated phospholipids affected SP 5–18–lipid interactions, the fatty acid composition of Syn LUV and Syn No Chol LUV was varied. When permeability experiments were performed with the Syn LUV, the amount of fluorescent probe released after 5 min was reduced by 8–10% if either the egg PC was replaced by DPPC or if the egg PG was replaced by DPPG. If both unsaturated phospholipids were replaced by DPPC and DPPG, approximately 25% less probe was released. The same results were obtained in the presence or absence of 3 mM calcium. When permeability experiments were performed with the Syn No Chol LUV in the absence of 3 mM calcium, the amount released was reduced by 16–22% if either the egg PC in this mixture was replaced by DPPC or if the egg PG was replaced by DPPG. If both unsaturated phospholipids were replaced by DPPC and DPPG, approximately 40% less probe was released. When the same experiments were repeated in the presence of 3 mM calcium, the percent fluorescence values were the same for the Syn No Chol LUV and for the vesicles in which the egg PC had been replaced by DPPC. However, if the egg PG was replaced by DPPG or if both unsaturated phospholipids were replaced by DPPG and DPPC, the percent fluorescence values were reduced by approximately 16–18%. In general, unsaturated phospholipids promoted leakage.

*Effect of SP 5–18 Concentration.* The extent of leakage of ANTS/DPX from egg PG LUV increased as the concentration of SP 5–18 was increased at a constant lipid concentration (Figure 3, curve a). Similarly, if the amount of lipid was varied and the amount of SP 5–18 held constant, lower phospholipid/SP 5–18 ratios (w/w) produced more relative leakage (results not shown). SP 5–18 also induced leakage from Syn LUV (Figure 3, curve c) or from canine LUV (Figure 3, curve b). In both cases, when phospholipid/SP 5–18 ratios (w/w) were varied by varying the amount of protein (Figure 3, curves b and c) or varying the amount of lipid (results not shown), lower phospholipid/SP 5–18 ratios (w/w) produced more relative leakage.

*Effect of pH.* The effects of pH on protein-induced leakage are shown in Figure 4. Base-line leakages (without SP5–18) at each pH were all less than 10% over the 5-min test period.

Table I: ANTS/DPX All-or-None versus Graded Dye Release

| PL/SP 5–18 (w/w) ratio | percent release | percent fluorescence remaining in LUV[a]: predicted from graded release[b] | predicted from all-or-none loss[c] | exptl results[d] |
|---|---|---|---|---|
| no SP 5–18 | 0 | 7.3 | 7.3 | 7.3 |
| 20/1 | 52 | 16.0 | 7.3 | 9.8 |
| 10/1 | 68–70 | 26.0–28.0 | 7.3 | 9.7 |
| 9/1 | 72 | 30.5 | 7.3 | 8.6 |
| 8/1 | 75 | 34.5 | 7.3 | 7.3 |
| 6/1 | 84 | 49.5 | 7.3 | 15.9 |
| 20/1 + $Ca^{2+}$ (3 mM)[e] | 88 | 59.5 | 7.3 | 25.2 |
| 10/1 + $Ca^{2+}$ (3 mM)[e] | 92 | 70.0 | 7.3 | 39.0 |

[a] Percent ANTS/DPX fluoresence after addition of detergent ($C_{12}E_8$) to egg PG LUV. [b] From ANTS/DPX quench curve. Assumes all vesicles release the same amount of dye. [c] Assumes vesicles lose all or none of the entrapped ANTS/DPX. [d] Determined after removal of released ANTS/DPX with a Sephadex G-75 column. Values are means of two experiments. [e] Higher percent release may be associated with lipid mixing.

The extent of ANTS/DPX release was higher as the pH of the buffer containing egg PG LUV was lowered from 8 to 5 (Figure 4, curve b). In addition, release was more extensive in the presence of 3 mM calcium throughout the pH range (Figure 4, curve a). When canine LUV were used, more fluorescent probe was released as the pH was lowered (Figure 4, curve c); however, the presence of 3 mM $Ca^{2+}$ did not cause any further increases in permeability.

*All-or-None Release.* Because only a portion of the total encapsulated fluorescent probe was released at each phospholipid/SP 5–18 ratio used, it was of interest to determine whether this was because only a portion of the probe leaked from all the vesicles (partial leakage) or because only a certain population of vesicles had leaked all of their contents (all-or-none leakage). The vesicles used in these experiments were composed of pure egg PG. If all vesicles had leaked only a portion of their contents (partial leakage), then the fluorescence ratio (percent fluorescence) of probe remaining within the liposomes should have increased with increasing release of probe. However, as column 5 of Table I shows, the percent fluorescence within liposomes changed little at each phospholipid/SP 5–18 ratio tested, consistent with the hypothesis that those liposomes which became permeable leaked all of their contents, while the remaining liposomes did not leak at

Table II: Carboxyfluorescein All-or-None versus Graded Dye Release

| PL/SP 5–18 (w/w) ratio | percent release | percent fluorescence remaining in LUV[a]: predicted from graded release[b] | predicted from all-or-none loss[c] | exptl results[d] |
|---|---|---|---|---|
| no SP 5–18 | 0 | 12 | 12 | 12.3 (2) |
| 20/1 | 55–60 | 41–45 | 12 | 11.3 (2) |
| 10/1 | 68–75 | 53–55 | 12 | 10.9 ± 0.3 (4) |
| 6/1 | 88 | 82 | 12 | 13.3 ± 0.4 (4) |
| 20/1 + $Ca^{2+}$ (3 mM)[e] | 88 | 82 | 12 | 13.9 (2) |
| 10/1 + $Ca^{2+}$ (3 mM)[e] | 92 | 90 | 12 | 17.8 (2) |

[a] Carboxyfluorescein fluoresence after addition of detergent ($C_{12}E_8$) to egg PG LUV. [b] From carboxyfluorescein quench curve. Assumes all vesicles release the same amount of dye. [c] Assumes vesicles lose all or none of entrapped carboxyfluorescein. [d] Determined after removal of released carboxyfluorescein with a Sephadex G-75 column. Values are means ± SEM. Number of experiments are in parentheses. [e] Higher percent release may be associated with lipid mixing.

all. The same all-or-none results were obtained with carboxyfluorescein (Table II).

*Measurements of Lipid Mixing*

Because the leakages of fluorescent probes in both of these all-or-none experiments were higher in the presence of 3 mM calcium, we decided to investigate whether this increase could be associated with a lipid mixing/fusion process. Resonance energy transfer between two different fluorescent lipid probes (NBD-PE and RH-PE) was used to monitor lipid mixing/fusion. We found that SP 5–18 induced lipid mixing between two populations of egg PG LUV in the presence of 3 mM calcium. Both SP 5–18 and 3 mM calcium were necessary for this process to occur. The threshold level for fusion of PG LUV by calcium alone is 15 mM (Rosenberg et al., 1983). Therefore, fusion in the presence of 3 mM calcium was due to the SP 5–18 peptides. The extent of lipid mixing was related to the SP 5–18 concentration. Specifically, phospholipid/SP 5–18 (w/w) ratios of 5/1, 10/1, and 20/1 produced 37.0% ± 0.4%, 26.8% ± 1.4%, and 10.0% ± 0.4% lipid mixing. These values are the mean ± SEM of four experiments. It was not possible to perform aqueous contents-mixing assays to monitor membrane fusion (i.e., coalescence of internal aqueous volumes with concomitant mixing of contents) because of the high rates and extents of leakage (at a PL/SP 5–18 ratio of 10/1 w/w), which would have interfered with the accurate interpretation of data. In addition, SP 5–18 did not induce lipid mixing of 100% DPPC LUV in the presence or absence of 3 mM calcium.

*Negative-Staining Electron Microscopy of Liposomes*

Electron micrographs of negatively stained pure egg PG LUV from one preparation showed spherical vesicles with diameters in the range 56–167 nm (mean = 104 ± 5.4 nm) ($n$ = 31) for egg PG LUV (control), 56–178 nm (mean = 124 ± 7.9 nm) ($n$ = 28) for vesicles with $Ca^{2+}$ (control), 50–150 nm (mean = 107 ± 4.3 nm) ($n$ = 50) for vesicles plus methanol (control), 56–156 nm (mean = 102 ± 5.9 nm) ($n$ = 30) for vesicles plus methanol with $Ca^{2+}$ (control), 61–256 nm (mean = 103 ± 5.5 nm) ($n$ = 60) for vesicles plus SP 5–18 (added from methanol), and 83–428 nm (mean = 219 ± 16.0 nm) ($n$ = 32) for vesicles plus SP 5–18 (added from methanol) with $Ca^{2+}$. The addition of SP 5–18 produced some larger vesicles, and the addition of SP 5–18 and $Ca^{2+}$ caused extensive growth of the vesicles (2 times control size), apparently representing various stages of fusion. Values are the mean ± SEM, and $n$ is the number of vesicles measured.

Table III: Surface Chemical Measurements of LUV at 37 °C, pH 7.4[a]

| preparation | time (min) when $\Delta\pi$ = 22 mN/m | $\Delta\pi$ after 1 h (mN/m) |
|---|---|---|
| egg PG LUV | nd | 2 |
| DPPG LUV | nd | 3 |
| DPPC LUV | nd | 3 |
| SP 5–18 → egg PG LUV | 4.3 | 34 |
| SP 5–18 → DPPG LUV | 17.1 | 32 |
| SP 5–18 → DPPC LUV | nd | 2 |
| egg PG LUV → SP 5–18 | 4.3 | 34 |
| DPPG LUV → SP 5–18 | 10.5 | 29 |
| DPPC LUV → SP 5–18 | nd | 1 |
| egg PG LUV + $Ca^{2+}$ + SP 5–18 | 4.2 | 43 |
| canine LUV + SP 5–18 | 24.9 | 24 |
| canine LUV + SP 5–18[b] | 7.3 | 45 |
| canine LUV + $Ca^{2+}$ + SP 5–18[b] | 2.9 | 45 |

[a] All values are means of two experiments. nd = not determined: >1 h. LUV (10 μg/mL, final concentration) and then SP 5–18 (1 μg/mL, final concentration) (or vice versa) were injected into the subphase, stirring was started, and surface pressure was recorded. Subphase buffer: 120 mM NaCl, 2 mM L-histidine, 2 mM Tes, 1 mM EDTA ± 4 mM $CaCl_2$, and pH 7.4. Temperature = 37 °C. [b] pH = 6.

*Measurements of Surface Activity*

The adsorption of lipids from vesicles to the air/water interface was measured by monitoring the change in surface pressure ($\Delta\pi$) with time. The rate of adsorption is given both as the $\Delta\pi$ observed after 1 h and as the time to half-equilibrium surface pressure (Table III). The adsorption of egg PG, DPPG, and DPPC from the subphase was independent of the order in which SP 5–18 and LUV were added, and the velocity of adsorption was egg PG > DPPG ≫ DPPC. Adsorption of these lipids was negligible in the absence of SP 5–18. LUV made from canine surfactant lipid lacking SP 5–18 adsorbed less rapidly when SP 5–18 was added at pH 7.4 than did egg PG; however, the adsorption of canine LUV was greatly enhanced at pH 6.0 and in the presence of $Ca^{2+}$. $Ca^{2+}$ also enhanced the adsorption of egg PG LUV.

## Discussion

The ability of SP 5–18 to interact with LUV and induce leakage in these vesicles provided one way in which to study the interaction of SP 5–18 with various synthetic lipid mixtures. We realize that lipid–protein interactions could occur in a liposomal system without producing leakage. However, if significant rates and extents of leakage are observed, they indicate a lipid–protein interaction which disrupts the permeability barrier of the bilayer, and this result suggests penetration of the protein(s).

The ability of the SP 5–18 group of surfactant apoproteins to increase the permeability of phospholipid vesicles was greatly enhanced by the presence of negatively charged phospholipids. However, the hydrophobic nature of this group of proteins precluded the determination, by binding studies, of the number of SP 5–18 peptides per lipid vesicle. It is interesting that King et al. (1983) have shown that the SP 28–36 group of surfactant apoproteins also promoted leakage of phospholipid vesicles containing PG.

The adsorption of lipids from the subphase to an air/water interface was enhanced by the presence of SP 5–18, negatively charged phospholipids, and 3 mM calcium (Table III). There is thus a similar lipid dependence for the interactions observed in the permeability and adsorption experiments. Both the amount of cholesterol and the phospholipid and fatty acid

composition of the lipid mixtures appear to be important in determining the interaction of SP 5–18 with various lipids and in determining the adsorption of these protein–lipid recombinants to an air/water interface.

Precisely how the SP 5–18 peptides promote surface film formation is still unknown. However, the observation that SP 5–18 destabilized phospholipid bilayers suggests that this type of instability might aid in the spreading of a surface film. Recently, we have also tested the abilities of the SP 5–18 peptides to alter the thermotropic properties of various phospholipid recombinants using differential scanning calorimetry (DSC) (K. Shiffer, unpublished observations). When DSC measurements were performed on DPPC or DPPG multilamellar vesicles, the presence of each of the individually purified SP 5–18 peptides broadened the DSC thermogram and reduced the enthalpy of transition ($\Delta H$) in a concentration-dependent manner. These results suggest that the SP 5–18 peptides are embedded in the hydrocarbon core of the bilayer and modify the lipid environment around them. Together, these data lend support to the hypothesis that surfactant apoproteins are important for the function of pulmonary surfactant. Thus, they are capable of destabilizing lipid bilayers, perhaps those of tubular myelin in the alveolar subphase, of modifying the lipid environment around them, and of promoting the adsorption of lipids to the alveolar air/water interface.

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