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Divalent Cation and Hydrogen Ion Effects on the Structure and Surface Activity of Pulmonary Surfactant†

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ABSTRACT: The structure and surface activity of the extracellular fraction of pulmonary surfactant known as tubular myelin are Ca^{2+} dependent. Previous studies have demonstrated surfactant-specific proteins with monomeric molecular weights of 28 000–36 000 (SP28–36) are associated with this fraction. In reassembled lipoprotein mixtures, SP28–36 promotes the Ca^{2+}-induced aggregation and surface activity of surfactant lipids, but the detailed interactions between Ca^{2+}, SP28–36, and surfactant lipids have not been established. In this study, we investigated the effect of various cations on the aggregation of surfactant lipid liposomes in the presence of SP28–36. SP28–36 reduced the threshold ion concentration for liposome aggregation from >10 to 0.5 mM for Ca^{2+}, Ba^{2+}, and Sr^{2+} but not Mg^{2+} or Mn^{2+}. The liposome aggregation was reversed by ethylenediaminetetraacetic acid and not associated with leakage of carboxyfluorescein. SP28–36 promoted similar liposome aggregation at pH <5 in the absence of divalent cations. Surfactant lipids adsorbed slowly to an air–fluid interface in all ionic conditions unless SP28–36 was present. Both Ca^{2+} and H^+ induced rapid lipid adsorption in the presence of SP28–36. The surface activity of native surfactant had a similar ion dependence. Electron micrographs of native surfactant showed typical tubular myelin structures at pH 7.4 only in the presence of Ca^{2+}. At pH 4.4 in the absence of Ca^{2+}, similar but not identical structures were seen. In the reconstituted system, SP28–36 in the presence of Ca^{2+} induced the formation of larger multilayered structures including parallel bilayers and small areas of squares and triangles with dimensions similar to structures found in the native material. The pH at which the protein-induced changes in lipid aggregation and surface activity occur is similar to the p*I* of SP28–36. Because the marked effects of H^+ and Ca^{2+} on surfactant lipid dispersion and surface activity are similar and are only seen in the presence of SP28–36, we propose that the action of Ca^{2+} at physiological pH is related, at least in part, to neutralization of the negatively charged carboxyl groups on the protein.

Dipalmitoylphosphatidylcholine (DPPC)[1] is widely accepted to be the major component of pulmonary surface-active material (surfactant) that is responsible for the maintenance of low surface tension (Brown, 1964; Notter & Morrow, 1975; Hildebran et al., 1979). However, pure DPPC below its phase transition temperature of 41.5 °C is very slow to adsorb to and spread at an air–water interface (Vilallonga, 1968). Because

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[1] Abbreviations: SP28–36, surfactant-specific proteins with monomeric molecular weights of 28 000–36 000; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; surfactant, surface-active material; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PG, phosphatidylglycerol; CF, carboxyfluorescein; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; LIS, lithium diiodosalicylate.

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rapid surface film formation is thought to be critical for normal lung function (King & Clements, 1972b), other factors which may promote the formation of DPPC films at 37 °C have been sought (King, 1974; Morley et al., 1978; Notter, 1983).

Recent studies suggest that two surfactant-associated protein groups and calcium ions may have an important role in the rapid formation of surfactant films (Suzuki, 1982; King et al., 1983; Benson et al., 1984a; Hawgood et al., 1985a, 1987; Takahashi & Fujiwara, 1986; Yu & Possmayer, 1986). The predominant surfactant proteins (SP28–36) have been characterized in several species as lung-specific glycoproteins with monomeric molecular weights of 28 000–36 000 (Sawada et al., 1977; Sueishi & Benson, 1981; Katyal & Singh, 1981; Phelps et al., 1984; Whitsett et al., 1985a; Benson et al., 1985). The primary sequences of canine and human SP28–36 have recently been published (Benson et al., 1985; White et al., 1985; Floros et al., 1986). Immunohistochemical and precursor labeling studies have shown these proteins are synthesized in type II cells, are stored in lamellar bodies, and are associated with tubular myelin, a latticelike structure, in the alveolar lining layer (Williams & Benson, 1981; Coalson & King, 1984; Whitsett et al., 1985b; Walker et al., 1986). The unusual structure of tubular myelin is reversibly dependent on calcium ions (Gil & Reiss, 1973; Benson et al., 1984b) and is destroyed by trypsin digestion (Benson et al., 1984a). The importance of calcium in enhancing film formation in vitro has been demonstrated for native surfactant (Notter et al., 1983; Benson et al., 1984a) and reassembled complexes of isolated SP28–36 with either synthetic lipid mixtures (King et al., 1983; King, 1984) or surfactant lipids (Hawgood et al., 1985a). In these studies, both liposome aggregation and the rate of surface film formation were calcium dependent.

We have determined the divalent cation specificity for SP28–36-induced changes in surfactant structure and surface activity. The ion sequence suggests direct divalent cation–protein interaction. SP28–36 is rich in negatively charged amino acids and has a p*I* of 4.8–5.2 (Benson et al., 1985; Hawgood et al., 1985b). Because one effect of divalent cations may be to neutralize the surface charge on the protein contributed by carboxyl groups, we have also investigated the effect of changing the net charge of SP28–36 by altering the pH.

Experimental Procedures

Lung Surfactant Preparation and Protein–Lipid Purification. Surfactant was isolated from lung lavage of adult dogs by a series of sedimentation and density gradient centrifugations (King & Clements, 1972a). The buffer used for the lavage was 5 mM Hepes/100 mM NaCl, pH 7.4. The methods to isolate the lipids and the SP28–36 complex have recently been reported in detail (Hawgood et al., 1985a) and were modified only slightly for the work reported here. Briefly, the surfactant preparation suspended in water was injected into an excess of 1-butanol (Sigrist et al., 1977). The surfactant lipids were separated from the majority of the proteins by centrifugation at $10000g_{av}$ for 20 min. The lipids in the butanol phase were further extracted by the method of Folch (Folch et al., 1957) and were stored in chloroform/methanol (2:1 v/v) at −15 °C. The phospholipid concentration was calculated from the phosphorus content (Bartlett, 1959). Phospholipid composition was determined by two-dimensional thin-layer chromatography (Poorthuis et al., 1976).

The delipidated proteins were dried under nitrogen and washed twice in buffered saline containing 20 mM octyl β-D-glucopyranoside to extract serum proteins and other soluble lung proteins. The remaining SP28–36 fraction was solubilized in 100 mM lithium diiodosalicylate in water. The concentration of lithium diiodosalicylate was reduced to less than 10 μM by extensive dialysis against double-distilled water. The protein content was assayed by the method of Lowry (Lowry et al., 1951) using bovine serum albumin in 1% sodium dodecyl sulfate (SDS) as a standard.

Preparation of Liposomes. Unilamellar liposomes of the extracted surfactant lipids and binary mixtures of synthetic phospholipids were prepared in a French pressure cell (3-mL capacity; Aminco, Silver Spring, MD) using methods previously described in detail (Hamilton et al., 1980; Hawgood et al., 1985a). DPPC and egg PG were obtained from Avanti Polar Lipids.

Turbidity Measurements. Turbidity, as a qualitative estimate of liposome and/or protein aggregation, was measured at 400 nm in a Beckman DU spectrophotometer, Model 2400 (Beckman Instruments Inc., Fullerton, CA). Experiments were performed at 37 °C in a 5 mM Hepes or Tris-HCl buffer containing 100 mM NaCl. Liposomes (100 μg) with or without SP28–36 (10 μg) were incubated in 1 mL of the buffer in a glass cuvette. Divalent cations, ethylenediaminetetraacetic acid (EDTA), or NaOH was added to the samples from concentrated stock solutions, and the change in turbidity was recorded.

Carboxyfluorescein Release. Carboxyfluorescein (CF) was obtained from Eastman Kodak and further purified by recrystallization (Blumenthal et al., 1977) and column chromatography to remove hydrophobic contaminants. Small unilamellar liposomes were prepared by sonication and large unilamellar liposomes prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1980). In both cases, the encapsulated aqueous phase contained 50 mM CF. The liposomes were separated from unencapsulated material by gel filtration on Sephadex G-75. Leakage of liposome contents was monitored by following CF fluorescence as it was diluted into the aqueous space from the entrapped self-quenching concentration. The measurements were performed with an SLM-4000 fluorometer using an excitation wavelength of 430 nm. Emission above 520 nm was detected with a 3-68 Corning filter. Maximum CF fluorescence was determined by lysing the liposomes with 0.1% Triton x-100.

Surface Activity. The ability of surfactant, the extracted surfactant lipids, and the lipid–SP28–36 complexes to adsorb to and spread at an air–water interface from a stirred subphase was tested at 37 °C. Samples containing either surfactant (40 μg of phospholipid), the extracted lipid liposomes (40 μg of phospholipid), or the reassembled lipid–protein mixture (40 μg of phospholipids and 20 μg of protein) were incubated at 37 °C for 1 h in 40–100 μL of buffer at various pHs in the presence of 2 mM Ca^{2+} or 5 mM EDTA. Three milliliters of the same buffer was added to a circular teflon trough 2 cm in diameter. For surface pressure measurements, the sample was carefully layered on the bottom of the trough, stirring was started at 240 rpm, and the surface pressure was continuously measured by a platinum dipping plate suspended from a strain gauge (Statham Gold Cell, Hato Ray, Puerto Rico). The signal was amplified and displayed on a chart recorder.

Electron Microscopy. Surfactant was incubated in Hepes buffer either at pH 7.0 in the presence of 5 mM Ca^{2+} or 5 mM EDTA or at pH 4.4 in the presence of 5 mM EDTA for 2 h at 37 °C. Liposomes prepared from the extracted surfactant lipids were incubated in Hepes buffer and 5 mM Ca^{2+} in the presence or absence of SP28–36 for 1 h at 37 °C. The phospholipid to protein ratio was 10:1 (w/w). The suspensions were centrifuged at $100000g_{av}$ at 4 °C for 30 min, and the

pellets were fixed for electron microscopy in 2% glutaraldehyde/1% osmium tetraoxide in either 0.1 M sodium cacodylate (pH 7.0) or 0.1 M sodium acetate buffer (pH 4.4). The preparations were centrifuged in plastic microfuge tubes at 10000*g* for 5 min and were then stored at 4 °C in fixative for 48 h. The pellets were then soaked in 2% aqueous uranyl acetate for 48 h at 4 °C. They were then dehydrated in graded acetone solutions and embedded in Epon. Thin sections were stained sequentially in 5% aqueous uranyl acetate and alkaline lead citrate and were examined in a Zeiss EM-10 electron microscope.

Results

The lipid composition of the liposomes used in this study was similar to the composition of canine pulmonary surfactant previously reported by us (Hawgood et al., 1985a). The major phospholipid was phosphatidylcholine which accounted for 78% of the total. The major anionic species was phosphatidylglycerol (PG) (11% of the total), but small amounts of phosphatidylserine and phosphatidylinositol (3%) were present. The median diameter of the liposomes was 440 Å with a range of 220–1440 Å. The liposomes contained approximately 0.5% protein by weight as assessed by the Lowry method (Lowry et al., 1951) in the presence of 1% SDS. We have recently isolated this protein fraction from chloroform/methanol-extracted canine surfactant lipids (Hawgood et al., 1987). It consists of two polypeptides with monomeric molecular weights of 5000–8000. These proteins, SP5 and SP18, have different amino acid sequences, and neither protein is a fragment of SP28–36. Both proteins are very rich in hydrophobic amino acids and appear to have a role in phospholipid surface film formation (King et al., 1973; Tanaka et al., 1983; Takahashi & Fujiwara, 1986; Yu & Possmayer, 1986; Whitsett, 1986; Hawgood et al., 1987).

The water-soluble canine SP28–36 complex used in this study has been described in detail previously by us. The complex has a major component (M_r 32000) and two minor components (M_r 36000 and 28000) which have the same amino acid sequence but differ in the degree of glycosylation. In addition to the different molecular weight components, there is marked charge heterogeneity with 10 or 11 isoforms ranging from a p*I* of 4.8 to 5.3 (Hawgood et al., 1985b; Benson et al., 1985). The SP28–36 used in this study was solubilized in LIS during the isolation procedure. Although the amount of LIS in the final protein preparation was below the level of detection by the method of Marchesi (Marchesi & Andrews, 1971), the effects of detergent exposure or residual trace detergent on the protein are unknown. We have recently obtained results similar to those described in this study using canine SP28–36 isolated without LIS and human recombinant SP28–36 isolated without exposure of the protein to either organic solvent or detergent (White et al., unpublished results). It is unlikely, therefore, that the protein isolation procedure has significantly affected the results.

Effect of Divalent Cations and pH on the Aggregation of Reassembled Surfactant Lipids and SP28–36. The relation between turbidity, as a quantitative assessment of liposome aggregation, and various divalent cation concentrations in 100 mM NaCl, pH 7.4, can be seen in Figure 1. The ion sequence for the divalent cation induced aggregation of extracted surfactant lipids was $Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} \simeq Ba^{2+}$. The cation concentration required for rapid aggregation of extracted surfactant lipids was 6, 13, 15, 27, and 27 mM, respectively (Figure 1A). Addition of SP28–36 to the liposomes dramatically reduced the threshold concentration of Sr^{2+}, Ba^{2+}, and Ca^{2+} required for rapid aggregation to 0.2, 0.2, and 0.5

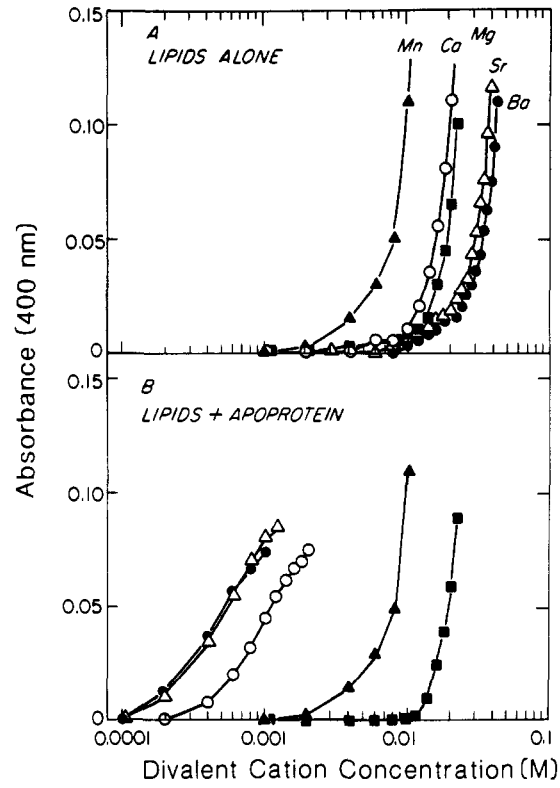

FIGURE 1: Turbidity (absorbance at 400 nm) of surfactant lipids or lipids and SP28–36 as a function of divalent cation concentration. The phospholipid concentration in all samples was 100 μg/mL in a buffer of 5 mM Tris/100 mM NaCl, pH 7.4. The protein concentration was 10 μg/mL. The concentration of the divalent cation was changed every 4 min. The concentration corresponding to the maximal increase in turbidity was taken as the threshold concentration for aggregation. Values are the average of two to five experiments.

mM, respectively, but SP28–36 did not enhance aggregation if Mn^{2+} or Mg^{2+} was the only divalent cation present (Figure 1B).

As can be seen in Figure 2A, the addition of SP28–36 to the surfactant lipids at pH 7.0 did not induce aggregation in the absence of Ca^{2+}. In the presence of SP28–36, the addition of Ca^{2+} to a final concentration of 2 mM induced rapid and extensive liposome aggregation with a stable absorbance reading by 30 ± 5 min. In order to compare the extent of liposome aggregation under varying experimental conditions, the absorbance reading of the lipid–SP28–36 complex at 30 min in the presence of 2 mM Ca^{2+}, pH 7.0, was designated as 100 absorbance units. This pH and calcium ion concentration are close to the reported values for the alveolar liquid in vivo (Nielson et al., 1981; Nielson, 1984). Addition of the protein to the liposomes at low pH (<5.5) induced rapid liposome aggregation in the absence of Ca^{2+} ions. Figure 2A,B shows an inverse relationship between the pH and the extent of liposome aggregation when SP28–36 is included in the reaction mixture. The extent of aggregation for lipoprotein mixtures at pH 7.0 in the presence of 2 mM Ca^{2+} at at pH 4.4 in the absence of Ca^{2+} ions was similar. At a pH below 7.0 but above 4.4, Ca^{2+} ions were required for maximum liposome aggregation. However, at a pH below 4.4, Ca^{2+} had no additional effect on liposome aggregation. The effect of lowering the pH on the aggregation of the liposomes alone was small in the pH range of 7.0–4.0. Further decreasing the pH to <3.0 increased liposome aggregation slightly (Figure 2B). Using these methods, we found no detectable aggregation of SP28–36 alone in the pH range of 7.0–5.0. However, there was some aggregation of the protein at pHs below 4.4 (Figure

Table I: Surface Pressure[a]

	time (min)					
	surfactant			recombinant		
pH	7.5	15	30	7.5	15	30
7.0	7.9 ± 2.4	14.8 ± 3.5	17.0 ± 4.9	13.2 ± 0.9	15.8 ± 1.0	18.3 ± 1.0
7.0 + Ca^{2+}	39.5 ± 2.6	42.9 ± 1.2	44.3 ± 0.4	34.0 ± 6.8	40.9 ± 2.3	44.0 ± 0.0
5.0	20.0 ± 5.4	26.5 ± 3.5	33.9 ± 2.5	15.5 ± 1.1	23.0 ± 0.8	31.0 ± 2.8
4.4	41.5 ± 1.6	42.6 ± 0.3	44.3 ± 0.3	35.5 ± 3.5	41.5 ± 1.5	44.0 ± 0.0

[a] Values (in millinewtons per meter) are mean ± SD of five to eight experiments. Samples containing lung surfactant (40 μg of phospholipids) or lipoprotein–recombinant (40 μg of phospholipids and 20 μg of protein) were incubated for 1 h at 37 °C in Hepes buffer at various pHs in the presence or absence of 2 mM Ca^{2+}. The samples were then placed below a clean surface, and the change in surface pressure was recorded. The subphase phospholipid concentration was 13.3 μg/mL.

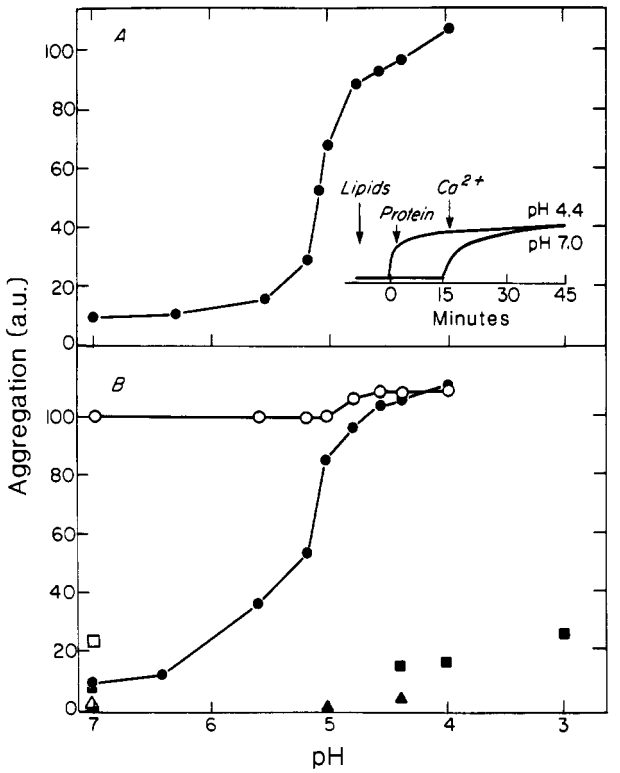

FIGURE 2: H^+- and Ca^{2+}-induced aggregation of surfactant lipids and SP28–36. Phospholipid vesicles (100 μg/mL) were incubated alone for 10 min at 37 °C in two 1-mL glass cuvettes. Protein (10 μg) was then added (0 min), the samples were mixed, and the absorbance at 400 nm was continuously monitored for 15 min. Ca^{2+} was then added to one cuvette from a 100 mM stock solution, and the absorbance was monitored for a further 30 min. An example of the experimental design is shown in the insert in (A). The absorbance reading of the liposomes with SP28–36 in 2 mM Ca^{2+}, pH 7.0 at 30 min, was defined as 100 units of aggregation (a.u.). (A) Aggregation after 15-min incubation of liposomes plus protein. (B) Aggregation after an additional 30-min incubation of the samples ±2 mM Ca^{2+}. Lipids plus protein (●); lipids plus protein plus 2 mM Ca^{2+} (○); lipids alone (■); lipids plus 2 mM Ca^{2+} (□); protein alone (▲); protein plus 2 mM Ca^{2+} (△). Values are the average of five to eight experiments.

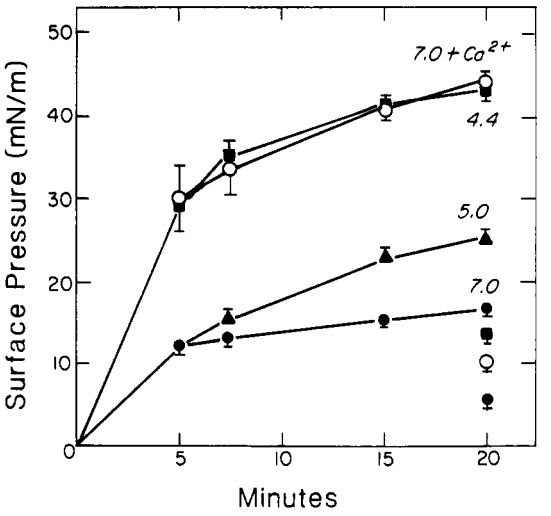

FIGURE 3: pH and Ca^{2+} dependence of surface activity of surfactant lipids and SP28–36. The surface pressure at 37 °C as a function of time for samples containing 40 μg of phospholipids plus 20 μg of SP28–36 in a final volume of 3 mL is shown. The samples were incubated for 1 h at 37 °C in 5 mM Hepes/100 mM NaCl at the indicated pH in the presence or absence of 2 mM Ca^{2+}. Liposomes and protein at pH 7.0 (●), at pH 7.0 + 2 mM Ca^{2+} (○), at pH 5.0 (▲), and at pH 4.4 (■). Liposomes alone at pH 7.0 (●), pH 7.0 + Ca^{2+} (○), and pH 4.4 (■). Values are the mean ± SD of four to seven experiments.

2B). The acid-induced protein aggregation was reversed by increasing the pH above 5.0.

In order to determine if the lipoprotein aggregation was reversible, we either added EDTA to aggregated lipoprotein in 2 mM Ca^{2+} at pH 7.0 or changed the pH from 4.4 to 7.0 by addition of concentrated NaOH. In both cases, aggregation was reversed rapidly, and the turbidity was reduced by 75.1% ± 3.4% (n = 5) and 72.1% ± 6.8% (n = 4), respectively.

The extracted surfactant liposomes contained small amounts of SP5 and SP18 (Hawgood et al., 1987). To determine if these proteins contributed significantly to the divalent cation dependent SP28–36-induced liposome aggregation, similar experiments to those described above were performed with liposomes made DPPC and egg PG (7:3 w/w). Reassembled complexes of these protein-free liposomes and SP28–36 had the same ion sensitivity and specificity for lipoprotein aggregation as the complexes of SP28–36 and extracted surfactant lipids (data not shown).

Carboxyfluorescein Release. Both the large and small liposomes aggregated rapidly when Ca^{2+} was added to a final concentration of 3 mM at pH 7.4 in the presence of SP28–36. Release of CF was minimal despite extensive aggregation and flocculation of the liposomes. The slow leakage of CF at 37 °C appeared to be independent of liposome aggregation and accounted for only 4–6% of the total encapsulated CF after 30 min. The basal rate of CF release was slightly higher with small liposomes at 37 °C, but SP28–36 or Ca^{2+} did not cause an increased rate of release.

Effect of pH and Ca^{2+} Ions on the Rate of Surface Film Formation of Surfactant Lipids and SP28–36. Figure 3 shows the ability of the extracted lipids or the lipid–SP28–36 complex to form a surface film. The rate of adsorption of the reassembled lipoprotein mixtures was slow at pH 7.0 in the absence of Ca^{2+}. The rate of film formation was increased significantly at pH 7.0 by the addition of 2 mM Ca^{2+} or by decreasing the pH without added Ca^{2+}. The rate of surface film formation was the same at pH 7.0 in the presence of Ca^{2+} ions and at pH 4.4 in the absence of Ca^{2+} ions. In both conditions, an equilibrium surface pressure of 44–46 mN/m was attained

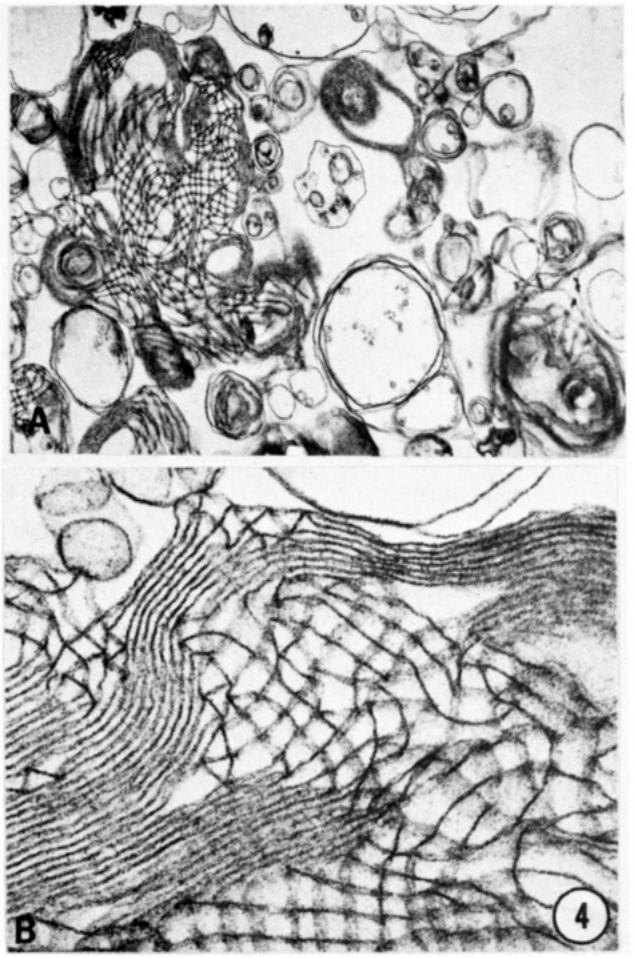

FIGURE 4: Electron micrograph of surfactant incubated at pH 7.0 in the presence of 5 mM Ca^{2+}. Magnification 15000×; insert 60000× magnification.

within 15–20 min with a subphase phospholipid concentration of 13 μg/mL. This represents the maximum surface pressure of an uncompressed surface film of surfactant lipids. As shown in Table I, the rates of film formation of the reassembled lipoprotein and surfactant were similar in all conditions tested, indicating that the reassembled lipoprotein has the same H^+ and Ca^{2+} specificity as the native material.

The surfactant lipids alone formed a surface film very slowly in the presence of either 5 mM EDTA or 2 mM Ca^{2+} in the pH range of 4.0–7.0. The surface pressure of the film generated by the lipids alone at pH 7.0 was only 5.8 ± 2.1 mN/m (n = 8) after 20 min. Lowering the pH to 4.4 increased the rate of surface film formation slightly, and the surface pressure at 20 min was 14.3 ± 1.0 mN/m (n = 5). The liposomes formed from surfactant lipids contained SP5 and SP18, but it is not known whether the organic solvent extractions reduce the amount of these hydrophobic proteins relative to intact surfactant. Recent results indicate that the major effect of SP28–36 on the rate of surfactant lipid surface film formation described here is dependent on the presence of SP18 and/or SP5 (Hawgood et al., 1987). The SP28–36 alone had minimal intrinsic surface activity. The equilibrium surface pressure of the protein alone was 13.8 ± 0.8 mN/m at pH 7.0 in the presence or absence of Ca^{2+} ions, and 13.3 ± 1.3 mN/m at pH 4.4.

Effect of Ca^{2+} and pH on Surfactant Structure. The majority of lipid was organized into unilamellar and multilamellar vesicles of varying sizes, under all conditions. Typical tubular myelin structure was observed in the electron micrographs of

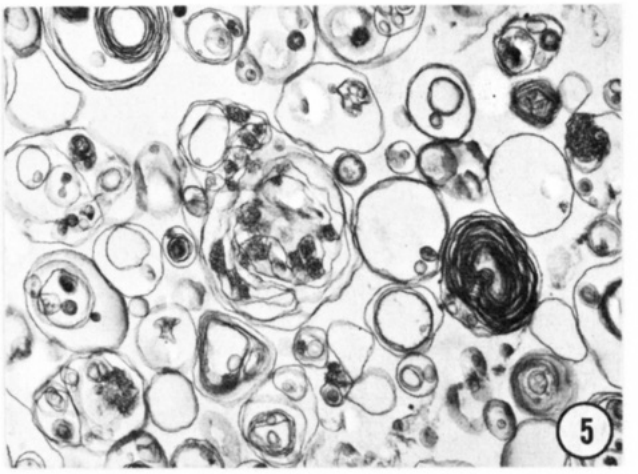

FIGURE 5: Electron micrograph of surfactant incubated at pH 7.0 in the presence of 5 mM EDTA. Magnification of 15000×.

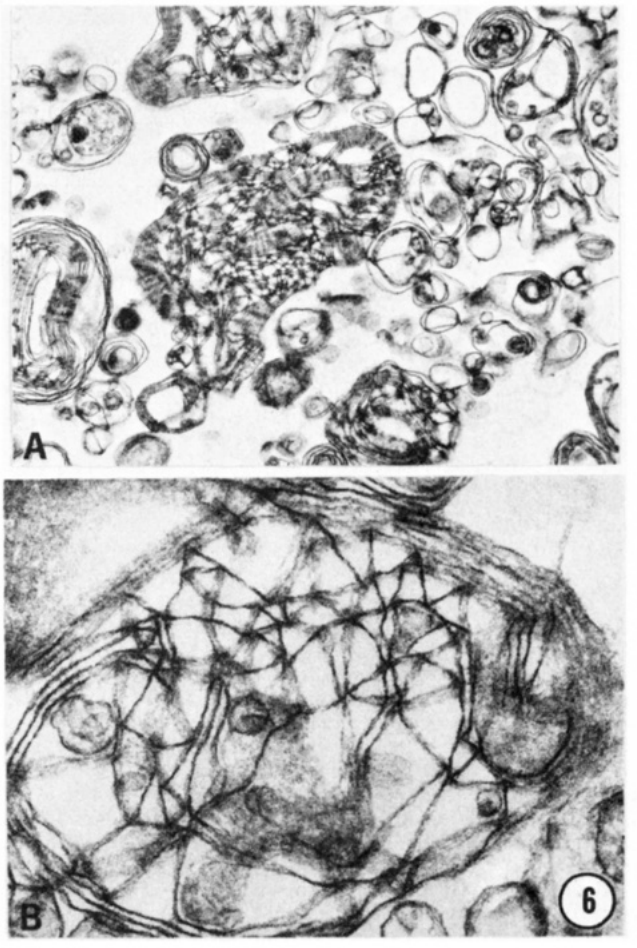

FIGURE 6: Electron micrograph of surfactant incubated at pH 4.4 in the presence of 5 mM EDTA. Magnification 15000×; insert 60000× magnification.

surfactant preparations which had been incubated at pH 7.0 in the presence of calcium (Figure 4). Tubular myelin normally shows mostly a square lattice, but occasional triangular structures were observed. No tubular myelin was observed if EDTA replaced calcium at pH 7.0 (Figure 5). However, at pH 4.4, even in the presence of EDTA, structures similar to but not identical with tubular myelin were observed (Figure 6). At the lower pH, the lattice structure was observed to be triangular more frequently than square.

The extracted surfactant lipid liposomes incubated in the presence of Ca^{2+} were predominantly unilamellar, but some

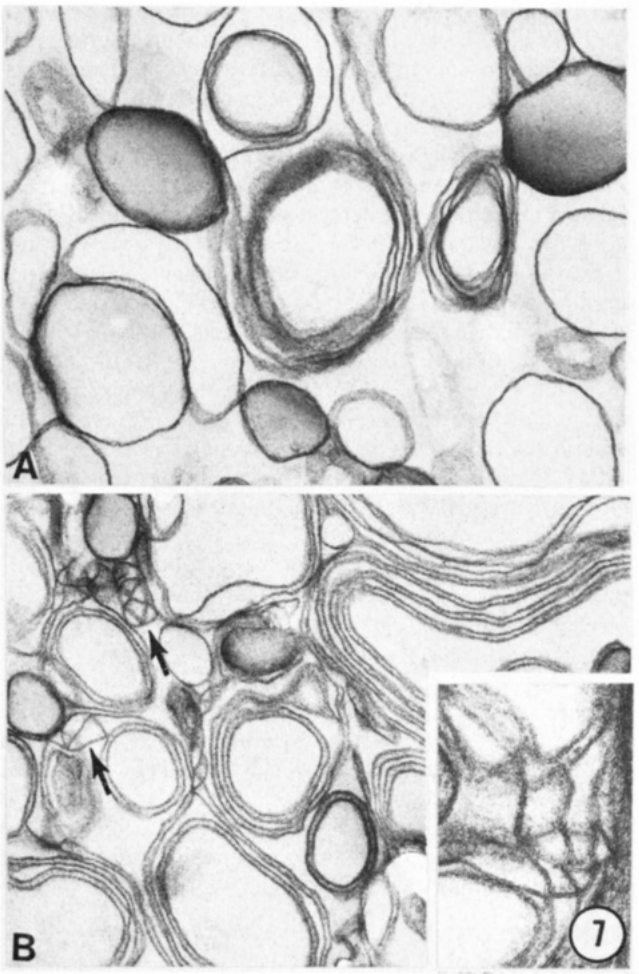

FIGURE 7: Electron micrographs of extracted surfactant lipids (A) and extracted lipids reassembled with SP28–36 (B) in the presence of 5 mM Ca^{2+}. Magnification 48000×; insert in (B) 98000× magnification.

multilamellar forms were seen (Figure 7A). In the presence of SP28–36 (10% by weight) and Ca^{2+}, larger multilayered structures were observed (Figure 7B). Parallel bilayers usually in pairs were common. The paired membranes were separated by 15–25 nm. This space was filled by a fuzzy amorphous coating, presumably protein. Small areas of squares and triangles with a mean length per side of 58 nm (range 44–83 nm) were also present. These structures had similar shapes and dimensions as those seen in the native surfactant.

Discussion

The data presented in this paper are consistent with the hypothesis that both specific proteins and the ionic environment are important in determining both the structure of surfactant particles and their function. In particular, SP28–36 alters the state of phospholipid dispersion in the presence of Ca^{2+} and in a process dependent on hydrophobic proteins enhances the formation of phospholipid surface films (King & MacBeth, 1981; Suzuki, 1982; Notter et al., 1983; Wright et al., 1984; Hawgood et al., 1985a).

The SP28–36 group of surfactant proteins has recently been characterized by several groups (King, 1974; Sueishi & Benson, 1981; Phelps et al., 1984; Whitsett et al., 1985a; Hawgood et al., 1985a; Benson et al., 1985; White et al., 1985; Floros et al., 1986). They are related glycoproteins with monomeric molecular weights of 28 000–36 000. The amino-terminal one-third of the protein has a collagen-like sequence, and the remaining two-thirds of the protein has a globular-like sequence which contains a possible N-linked glycosylation site near the carboxy terminus. The size of the lipid-associated protein in the native structure is unknown, but the delipidated protein in aqueous buffers is an oligomer of at least 300 000 daltons (King et al., 1983). Although the primary structure of this protein has recently been elucidated (Benson et al., 1985; White et al., 1985; Floros et al., 1986), the structure of the lipid binding domain has not been established.

The divalent ion sequence for aggregation of surfactant lipids (Figure 1) correlates well with the sequence of ion binding to PG (Lau et al., 1981), the major acidic phospholipid in pulmonary surfactant (King & MacBeth, 1981). The binding of divalent cations and protons to model lipid mixtures and the resulting bilayer interactions have been extensively studied (Papahadjopoulos et al., 1977; Ohki et al., 1982; Hope et al., 1983; Ellens et al., 1985). The surfactant liposomes without SP28–36 but containing SP5 and SP18 aggregated minimally and formed surface films very slowly at calcium ion concentrations comparable to the alveolar subphase (Nielson, 1984) at any pH tested (see Figure 1A and Figure 3). While lipid–ion and SP5/SP18–ion interactions may have some role in organizing surfactant structure and promoting surface activity (Kobayashi & Robertson, 1983; Notter et al., 1983; Weber & Possmayer, 1984), it is clear that SP28–36 dramatically enhances both these phenomena (Figure 1B and Figure 3). When SP28–36 was included in the reassembled complexes, both the threshold ion concentration and the ion sequence for liposome aggregation were dramatically altered. The protein markedly enhanced the effects of Ca^{2+}, Ba^{2+}, and Sr^{2+} but not Mg^{2+} or Mn^{2+}. This ion specificity and sensitivity suggest that specific divalent cation–protein interactions may occur. Both Ba^{2+} and Sr^{2+} have similar size:charge ratios to Ca^{2+} and have the necessary coordination flexibility for specific protein–ion interactions (Levine & Dalgarno, 1983).

The amount of SP28–36 bound to the liposomes was not directly assessed in this study, but we have subsequently determined by sedimentation of the lipoprotein complex or sucrose gradient centrifugation that in the presence of Ca^{2+} greater than 90% of the SP28–36 is associated with the lipids (H. Efrati et al., unpublished results). The results of King et al. (1983) indicate that divalent cations are not required for the phospholipid–SP28–36 interaction. It is unlikely, therefore, that the divalent cation specificity reported here for the protein-dependent liposome aggregation or surface activity reflects variable divalent cation dependent binding of SP28–36 to the liposomes.

The similarity of the effects of SP28–36 at pH 7.0 in the presence of Ca^{2+} and at pH <5.0 in the absence of Ca^{2+} on the aggregation and adsorption of the reassembled lipoprotein complexes was striking. At pH <5.0, there was very little change in the properties of the surfactant lipids if SP28–36 was not present. At pH <5.0, the charge contributed by the carboxyl groups of the acidic residues in SP28–36 would be largely neutralized. This may promote protein–protein interactions which could contribute to the observed changes in the state of lipid dispersions. At a physiological pH, Ca^{2+} may also modify the net protein charge.

It has been shown in other systems that certain proteins can promote the Ca^{2+}-induced interaction of lipid bilayers. In some cases, the threshold concentration of Ca^{2+} required for aggregation and/or fusion of the model lipid systems was lowered to physiological levels by the presence of a Ca^{2+} binding protein (Creutz et al., 1979; Zimmerberg et al., 1980; Hong et al., 1981). Other proteins promote close bilayer contact when the

pH is reduced even in the absence of divalent cations (Schenkman et al., 1981; Blumenthal et al., 1983; White et al., 1983; Eidelman et al., 1984). In both cases, it is thought that the ions cause a conformational change in the protein with exposure of extra hydrophobic domains. These conformational changes trigger protein–protein or protein–lipid interactions (Creutz et al., 1979; LaPorte et al., 1980; White et al., 1983; Doms et al., 1985). The results of intrinsic fluorescence studies suggest a small conformational change in SP28–36 after the addition of Ca^{2+} (King et al., 1983). As all the aromatic amino acids are found at the carboxy-terminal end of SP28–36 (Benson et al., 1985; White et al., 1985), larger conformational changes may be induced by Ca^{2+} at sites other than the extreme carboxy terminus.

The aggregation of the surfactant proteoliposomes was not followed by extensive bilayer fusion or lysis within the time frame of these experiments. The aggregation was rapidly reversed by chelation of Ca^{2+} with EDTA and was associated with only minimal leakage of liposome contents. These data are seemingly in conflict with those of King and co-workers, who reported that Ca^{2+} caused 70% of the entrapped dye to leak from liposomes containing 51% PG in 30 min even in the absence of surfactant protein (King et al., 1983). These differences may be due to differences in the lipid composition, the presence of very hydrophobic proteins in the surfactant lipoproteins, or the purity of the CF used (Hong et al., 1981). Reversible changes in the structure of tubular myelin with the chelation of Ca^{2+} ions are seen in native surfactant (Benson et al., 1984a), and we have shown here similar, but not identical, changes in the tubular myelin structure with changes in the pH. The bilayer interactions in the native tubular myelin structure appear to represent at least partial membrane fusions at discrete sites, but reversible aggregation may also play a role in the formation of this structure. Despite the retention of liposome internal content as assessed by CF release and the rapid reversal of Ca^{2+}-induced lipoprotein aggregation, SP28–36 induced major changes in liposome structure. Negative staining of similarly reconstituted mixtures of SP28–36 and extracted surfactant lipids (Hawgood et al., 1985a) or mixtures of DPPC and DPPG (King et al., 1983) revealed extensive, Ca^{2+}-dependent aggregation of the liposomes and formation of heterogeneous multilayered structures. It is unlikely that small amounts of tubular myelin-like structure could be detected in negatively stained preparations. In the present study using transmission electron microscopy, structures resembling those found in fetal lung surfactant prepared by fixation in situ (Williams, 1977) and in lavage surfactant (Manabe, 1979) were detected in the reconstituted SP28–36–lipid mixtures. These structures include parallel membranes separated by amorphous material and the squares and triangles characteristic of native tubular myelin. The dimension and overall configuration of both these structures in the reconstituted system are similar to those reported in native surfactant by many investigators. Similar structures were recently reported in reconstituted mixtures of surfactant lipids and proteins extracted with *N*-acetylcysteine from the lavage of patients with alveolar proteinosis or adult rabbit surfactant (Hook et al., 1986). These investigators did not observe tubular myelin-like structures with purified protein preparations, but important protein subunit interactions may have been disrupted during purification.

There was a strong correlation in all our studies between particle aggregation and the ability of the sample to form a surface film. A similar dependence between the rate of surface film formation and the state of lipid dispersion has been reported in other lipid systems (Notter & Morrow, 1975; Goerke, 1981). The effects of SP28–36 on phospholipid aggregation were not dependent on the presence of the hydrophobic proteins SP5 and SP18. In our studies, the ion-dependent surface properties of native surfactant could only be duplicated when SP28–36 was included in the reassembled complex, but, as previously shown, the effects of SP28–36 on surface activity, unlike aggregation, are dependent on the presence of SP5/SP18 (Hawgood et al., 1987). The rate of surface adsorption of native surfactant showed the same pH and divalent cation dependence as the reassembled complex. The speed at which surface films are formed in vitro suggests that bulk transfer of lipids into the interface is taking place (Goerke & Clements, 1986) but the molecular events underlying surface film formation or the advantages of the unique tubular myelin structure in this process are not yet understood. The ability of Ca^{2+} to cause reversible conformational changes in proteins with rapid association and dissociation rates (Levine & Dalgarno, 1983) makes the Ca^{2+} ion a very favorable participant in the dynamic phenomenon of tubular myelin formation and the generation of surface films.

Although our data suggest that Ca^{2+} may bind to SP28–36, the evidence is indirect, and a specific Ca^{2+} binding site or significant effects of Ca^{2+} on protein conformation have not yet been identified. We cannot rule out the possibility that some of these ion effects are mediated or augmented by the small amounts of other proteins which are still associated with the extracted surfactant lipids used in this study. It is possible that as well as binding Ca^{2+} directly, SP28–36 enhances the interaction between Ca^{2+} and the anionic lipids or other proteins of pulmonary surfactant.

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Registry No. Ca, 7440-70-2; Ba, 7440-39-3; Sr, 7440-24-6; Mg, 7439-95-4; Mn, 7439-96-5; H^+, 12408-02-5.

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