INTERACTION OF C-REACTIVE PROTEIN WITH PULMONARY SURFACTANT

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by

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Table of Contents

Materials and Methods

Results

List of Figures

List of Tables

Abstract

The influence of the acute inflammatory phase protein human C-reactive protein (CRP) on the adsorption of porcine pulmonary surfactant from a subphase into the air-water interface with and without dynamic surface compression has been investigated. CRP was shown to detract from the ability of surfactant to rapidly adsorb to the air-water interface at a molar ratio of 0.03 : 1, protein to phospholipid (weight ratio, 0.5 : 1). On a weight basis, CRP was found to be more effective than fibrinogen or globulin at reducing the adsorption rate of surfactant. The effect of CRP required the presence of calcium, and was reversed by the addition of phosphocholine, in a concentration dependent manner. The inhibition of surfactant adsorption by CRP was effectively eliminated by the addition of phosphocholine at a molar ratio of 300 : 1, phosphocholine : CRP, but it was not diminished by the addition of identical molar ratios of o-phosphoethanolamine or DL-α-glycerophosphate at the same molar ratios. These data suggest that the potent inhibition of surfactant adsorption by CRP is primarily a result of a specific interaction between CRP and the phosphocholine headgroup of surfactant lipids in the subphase and that it can be reversed by the water-soluble CRP ligand, phosphocholine. Experiments were performed to determine the effect of the addition of the complement protein C1q on the adsorption of porcine lipid extract pulmonary surfactant from the subphase to the air-water interface with and without dynamic compression. C1q, at a weight ratio of 10% (C1q : PL), when added to lipid extract surfactant, which is void of SP-A, increased the adsorption rate of the lipid extract surfactant to approach the rate at which whole surfactant, which contains 5-7% SP-A, by weight. C1q at this weight ratio did not detract from the ability of lipid extract surfactant from attaining a minimum surface tension under dynamic

compression. Albumin, when added to lipid extract surfactant at the identical weight ratio at which C1q was a added, detracted from the ability of lipid extract surfactant to adsorb to the air-water interface. Albumin, also detracted from the ability of this mixture in attaining a minimum surface tension under dynamic compression. This suggests that C1q, which shares quaternary structural homology with SP-A, increases the adsorption of lipid extract surfactant by a similar molecular interaction as that of SP-A. Serum CRP levels, expressed μg/ml and as percentage of total protein, were elevated in patients with ARDS and those patients at risk of ARDS compared to normals. A weak correlation exists between the serum CRP in ICU patients, expressed as percentage of total protein and the APACHE II score. This suggests that CRP levels may be useful in the clinical evaluation of patients in the ICU.

Introduction

Surfactant Introduction

Pulmonary surfactant, a biologically complex mixture of lipid and proteins, is essential for normal lung function. The terminal air-filled sacs are coated with this substance which reduces the surface tension of the alveolar air-water interface to very low values. The function of pulmonary surfactant, which results its physical characteristics, is to reduce the potentially large energy requirement needed to expand the alveolar surface and enable the alveoli to resist the collapsing forces which exist at physiological transpulmonary pressures. In addition, surfactant may have other roles in the lung such as preventing pulmonary edema formation (Pattle, 1965; Clements, 1961) and aiding the pulmonary defence system to resist and combat infection (e.g. LaForce et al., 1973). Deficiency of pulmonary surfactant in prematurely born infants can contribute to respiratory distress syndrome. In the adult, the inhibition of pulmonary surfactant function can lead to adult respiratory distress syndrome (ARDS). Both of these syndromes can lead to similar complications with respect to respiratory pathophysiology, and they are associated with significant morbidity and mortality.

Alveolar Structure and Pulmonary Surfactant Composition

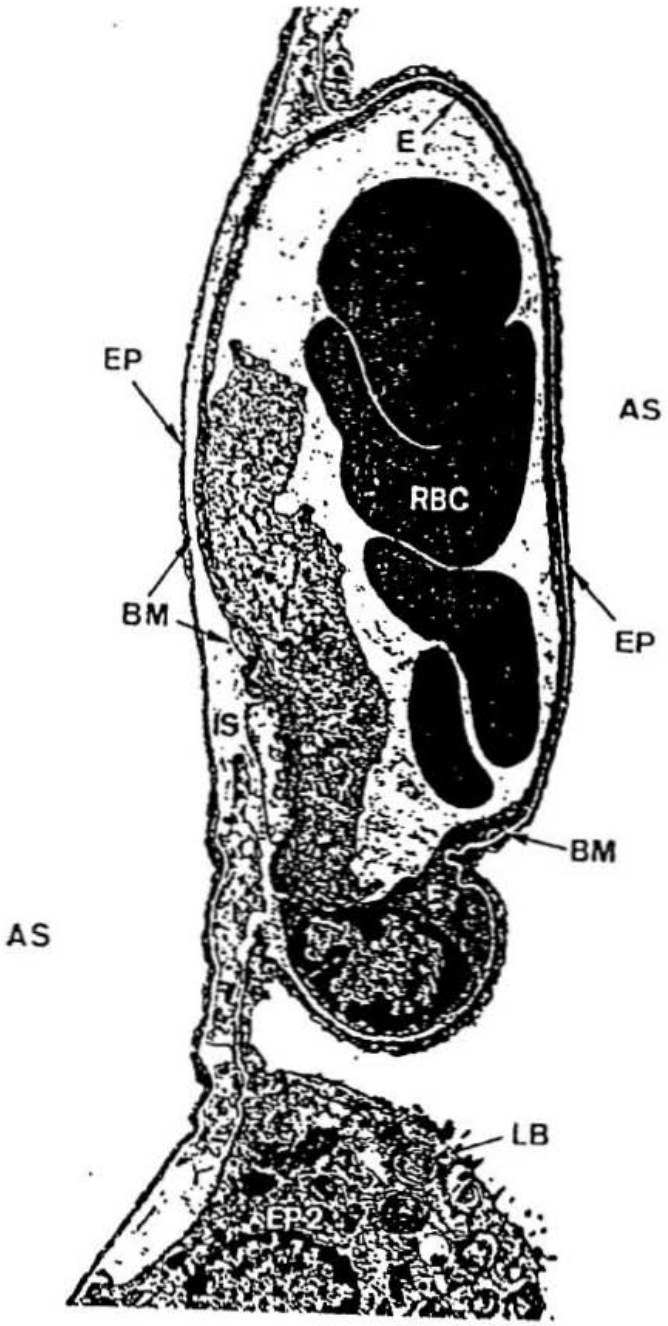
Alveoli are the terminal air-spaces through which respiration occurs. The adult lung contains ~ 300 million alveoli which have an average diameter of ~ 25 μm. The alveoli are not closed sacs in an anatomical sense, rather, they are interconnected by fluid-filled pores called alveolar pores of Kohn (Bastacky, 1994). Histological examination using electron microscopic

techniques has revealed that the alveolar-capillary membrane consists of four components: contiguous tissue elements in the intercalated interstitial space, capillary endothelium and its basement membrane, alveolar epithelium and its basement membrane, and a surfactant lining (Murray, 1986), Figure 1.

The interstitial space which separates the alveolar epithelium and capillary endothelium basement membranes is anatomically divided into two regions. The thin portion of the septum is considered to be the air-blood exchange region and is the area in which the two basement membranes appear to be fused. The thick portion is located where the basement membranes are separated by an interstitial space containing elastic fibres, collagen fibres and sparse fibroblasts. Pulmonary capillaries weave throughout the interstitial space. The capillary endothelium is primarily composed of cytoplasmic extensions of endothelial cells which form thin vascular tubes. These cells allow gas and liquid exchange and carry-out important non-respiratory functions of the lung. The alveolar epithelium consists of a continuous layer of tissue made up of, principally, two cell types. Type I cells, or squamous pneumocytes, have broad thin extensions and cover ~ 93% of the alveolar surface. They possess few cytoplasmic organelles, are highly differentiated and do not divide. Alveolar type II cells, or granular pneumocytes, are more numerous than type I cells, but because of their cuboidal shape they occupy ~ 7% of the alveolar surface. These cells are the primary surfactant producing and surfactant storage cells of the lung and also play a key role in recycling of pulmonary surfactant. They are easily identified by their microvilli and osmiophilic lamellated inclusion bodies which contain pulmonary surfactant.

The composition of pulmonary surfactant has been determined by analysis of material which has been obtained from bronchoalveolar lavage (BAL) or lung homogenates after

Figure 1. Reproduction of an electron micrograph of a cross-section of an alveolar membrane. From Murray, 1986, with permission.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purification by centrifugation in sucrose or sodium bromide. Any review of the studies of the compositional analysis of lung surfactant quickly reveals that the exact content of each component varies, however, this variability is small and is partially dependent on which method of extraction is employed. The lavage method of surfactant recovery is most common, and Tanaka and Takei (1982) have suggested that surfactant made from the lung lavage method should contain fewer contaminants from blood and tissue than surfactant material made by other methods. There has also been criticism of the BAL method with respect to how accurately the recovered surfactant represents the actual surfactant of the lung. Harwood et al. (1976) suggested that lung surfactant obtained by the lavage method may be contaminated with material from the upper airways or it may represent a partial recovery of the total extracellular surfactant pool. Also, the lung lavage process may promote the release of intracellular surfactant; therefore, the BAL may contain surfactant from both the intracellular and extracellular pools (Shelley et al., 1982). In spite of this, most biochemical analyses of lung surfactant yield similar results (e.g. King & Clements, 1972; Harwood et al., 1975; Shelley et al., 1982).

Pulmonary surfactant is composed of lipids and specifically associated proteins in a weight ratio of ~ 9 : 1, lipid to protein. Phospholipids, comprise nearly 90% of the total lipid content. One distinguishing characteristic of pulmonary surfactant is the high content of phosphatidylcholine, comprising ~ 80% of the phosphoglyceride content, of which nearly 40% is dipalmitoylphosphatidylcholine (DPPC). Surfactant contains the acidic phospholipids phosphatidylglycerol, ~ 8%, and phosphatidylinositol, ~ 1%, along with sphingomyelin, ~ 2%, lysophosphatidylcholine, ~ 2%, and a small amount of free fatty acids (Shelley et al., 1982). Surfactant also contains cholesterol, in amounts up to 7-8% by weight, which on a molar basis

constitutes a significant proportion, (up to 15 mol%) (King & Clements, 1972).

Macklin (1954) was first to suggest that alveolar type II cells synthesize, store and secrete surfactant. Since then, evidence which suggests that alveolar type II pneumocytes are the primary sites of synthesis of surfactant and storage of lipids has accumulated. For example, biochemical and pulse-chase studies of lung phosphatidylcholine have shown that lamellar bodies of alveolar type II cells are the main storage sites of surfactant in the lung (Chevalier & Collett, 1972; Baritussio et al., 1981). The phospholipid content of lavage fluid was found to be nearly identical to that which was found in lamellar bodies (Robertson et al., 1984). Dobbs et al. (1982) supported this conclusion by studying the physical and chemical properties of the lipid and protein mixture of lamellar bodies and determined them to be very similar to that of extracellular surfactant. They have also incubated alveolar type II cells with [C^{14}]-acetate, and found that the distribution of secreted material was very similar to the phospholipid content of surfactant isolated from lung lavage.

Almost 10% of the total weight of pulmonary surfactant is composed of the specifically associated proteins. These proteins are named surfactant protein-A (SP-A), surfactant protein-B (SP-B), surfactant protein-C (SP-C), and surfactant protein-D (SP-D) according to Possmayer (1988). In the 1980's a large body of research was focused on elucidating the genomic and structural organization of these proteins, as well as, on determining their biological functions. To this end, the genomic organization of these proteins has been identified and their amino acid sequences have been determined from genomic and cDNA libraries; however the precise role of these proteins in the complete biological function of pulmonary surfactant remains to be delineated.

The largest and most abundant surfactant protein by mass is the hydrophillic surfactant protein-A. SP-A has a monomeric molecular weight of 28 000-36 000, depending on its extent of glycosylation and species of origin. Each monomer contains a collagen-like N-terminal, which is rich in the gly-X-pro(OH-pro) sequence. The C-terminal regions of SP-A form triple helices through non-covalent protein-protein interactions, as in collagen (Floros et al., 1985). The monomers are linked by disulfide bridges near the N-terminal which stabilizes the molecule by cross-linking adjacent polypeptide chains within and between the triple helices (Voss et al., 1988). Six trimers associate to form the native octadecameric structure which has a molecular weight of ~ 700,000. SP-A contains a large globular glycosylated non-collagenous carboxy-terminal region which is essential for the correct quaternary structure of the molecule (Spissinger et al., 1991). The flower bouquet-like structure of SP-A resembles that of the quaternary structure of the complement protein C1q (Voss et al., 1988 and ref. cited within).

Surfactant is ideally suited to play a role in the host defence system of the lung because it covers the entire lung surface, which is potentially exposed to many pathogens. SP-A has been implicated in the host defence system of the lung (e.g. Tenner et al., 1989; Van Iwaarden et al., 1990; Weber at al., 1990; Manz-Keinke et al., 1992; Wright and Youmans, 1993). The ability of SP-A to bind to carbohydrates and lipids enables it to attach to the surface of several pathogens, including the herpes simplex type I virus (Van Iwaarden et al., 1991), the opportunistic pathogen *Pneumocystis carinii* (Zimmerman et al., 1992) and several types of bacteria (e.g. McNeely & Coonrod, 1993). It has also been shown that SP-A can stimulate chemotaxis of macrophages (Hoffman et al., 1987) and stimulate the phagocytic activity of monocytes and macrophages to sheep erythrocytes which have previously been opsonized with IgG or IgM (Tenner et al., 1989).

SP-A can stimulate the production of oxygen free radicals by macrophages (Van Iwaarden et al., 1990) and it can bind to C1q receptors of U937 cells and upregulate C1q receptor production by these cells (Malhotra et al., 1992). Therefore, SP-A is homologous to the complement protein C1q with respect to these functions, however, SP-A can not substitute for C1q in the formation of hemolytically active C1 (Tenner et al., 1989).

Immunocytochemistry experiments have revealed that SP-A is present in alveolar type II cells, alveolar macrophages and in a suopopulation of bronchiolar epithelial cells (Clara cells) (Walker et al., 1986). *In situ* hybridization experiments have located the presence of SP-A mRNA in alveolar type II cells and in Clara cells but not in alveolar macrophages (Phelps & Floros, 1988). Ultrastructurally, in both alveolar type II cells and Clara cells, SP-A has been detected in the endoplasmic reticulum, the Golgi bodies, some multivesicular bodies and on the cell membrane (e.g. Williams & Benson, 1981; Walker et al., 1986; deMallo et al., 1993). Evidence suggests that SP-A secretion from these cells occurs via two different routes, one in which it is secreted along with surfactant in lamellar bodies, and via a route which is independent of these organelles. It was shown by Froh et al. (1993) that the kinetics of secretion of SP-A are different from those of lamellar bodies and the concentration of SP-A in tubular myelin is higher than that in lamellar bodies. In alveolar macrophages, SP-A is located in lysosomes and other organelles associated with catabolism (Williams & Benson, 1981; Walker et al., 1986).

SP-A shares some sequence homology with another hydrophillic surfactant protein, which was initially identified by Ng et al. (1983) and referred to as a class D surfactant protein (Phelps & Taeusch, 1985). The two proteins are functionally dissimilar. SP-D is a collagenous 43 kDa glycoprotein with fewer irregularities in the collagen-like region than SP-A. Unlike SP-A, SP-D

contains no cysteine residues in the collagenous domain thus preventing interhelical disulfide cross-linking. SP-D possesses intrahelical glycosylation in the N-terminus whereas in SP-A it is located in the carboxyterminus. SP-D has been located in nonlamellar secretory compartments of alveolar type II cells and in the apical electron dense secretory granules of the Clara cells (Crouch et al., 1992) and it has been shown to be synthesized by alveolar type II cells (Persson et al., 1988).

Pulmonary surfactant also contains two hydrophobic proteins both of which are extracted from surfactant along with lipids by organic solvents and are referred to as proteolipids. The larger of the two is SP-B. It has 79 amino acids with a monomeric molecular weight of ~8 000 and exists as a homodimer (Curstedt et al., 1990). SP-B is relatively high in cysteine residues and it was suggested that disulfide bridges may be important in stabilizing the conformation of SP-B (Hawgood, 1989). The location of the disulfide-bridges have been determined and they were shown to result in the mature SP-B molecule appearing as three loops, a central loop surrounded by two smaller loops (Johansson et al., 1991).

The other hydrophobic surfactant protein is SP-C. It has 35 amino acids with a molecular weight of ~ 5 000. Mature SP-C is an α-helical membrane-spanning molecule (Waring et al., 1990) and it also belongs to that class of proteins known as proteolipids which are soluble in organic solvents. SP-C is a hydrophobic molecule with two cysteine residues near the N-terminal which are covalently linked to palmitic acids by thioester bonds (Curstedt et al., 1987).

SP-B has been localized, immunohistochemically, and its mRNA has been detected in alveolar type II cells and in Clara cells (Phelps & Floros, 1988; Stahlmon et al., 1992). SP-C and its mRNA has been observed exclusively in the alveolar type II cells (Phelps & Floros, 1991).

The surfactant system of the lung is in a state of constant flux. The calculations of Wright and Clements (1987) suggested that there is not a large intracellular or extracellular pool of surfactant. They have estimated that the type II cells must secrete approximately 11 to 47% of the lamellar body pool per hour and in a steady state, an equal amount of surfactant must be removed per hour. Although several assumptions were required for their estimation of surfactant pool turnover time, surfactant production, secretion, and catabolism appears to be tightly and accurately controlled by several mechanisms so that the amount of surfactant at the alveolar air-water interface remains constant under varying conditions, such as exercise. There are several elegant reviews on this subject (e.g. Wright & Clements, 1987; Wright & Dobbs, 1991).

Pulmonary Surfactant Life-Cycle

It is thought that the fusion of the lamellar body limiting membrane with the plasma membrane is the primary step in surfactant secretion. Electron microscopic studies have demonstrated the presence of lamellar bodies in alveolar type II cells which appeared to be in the process of exocytosis (Ryan et al., 1975; Williams & Benson, 1981). Lamellar bodies transform into a complex lattice-like structure known as tubular myelin when they enter the extracellular space (Williams, 1977). The factors which are responsible for this conversion are not known, but it has been shown to require the presence of calcium (Sanders et al., 1980; Notter et al., 1986; Suzuki et al., 1989).

The clearance of pulmonary surfactant can occur via three mechanisms. Studies (e.g. Hallman et al., 1982) showed that alveolar type II cells can degrade surfactant lipids, after being internalized, probably by endocytosis (Williams, 1977). Surfactant lipids can be recycled, without

intracellular degradation through reincorporation into lamellar bodies and resecretion (Jacobs, 1983). Degradation of phospholipids, such as phosphatidylcholine, occurs in alveolar macrophages (Stern et al., 1986) and in alveolar type II cells (Chander et al., 1987). When surfactant is degraded by these cells, it appears to be associated with catabolic organelles, such as lysosomes, where the action of phospholipases takes place. It appears that little catabolism of surfactant occurs in the alveolar subphase in the healthy state (Ozarzun & Clements, 1977). Alternatively, instead of being recycled, surfactant can be cleared from the lung by movement up the muco-ciliary escalator to the oesophagus where it could be swallowed or expectorated. It has also been suggested than a very small amount of surfactant could be transferred across the epithelial/endothelial barrier into the blood or lymph and subsequently transported to the kidney or liver and used to synthesize new lipids or excreted (Wright & Dobbs, 1991 and ref. cited within).

Physical Properties of Pulmonary Surfactant

Pulmonary physiologists have been aware of the role of surface forces in lung mechanics and the importance of the lung air-water interfacial film since the seminal work of von Neergaard in 1929 (von Neergaard, 1929). Von Neergaard attributed the difference in the recoil forces between the fluid and air-filled lung to the action of surface tension at the alveolar air-water interface. He (von Neergaard, 1929) suggested that a special material which lowered surface tension existed in the lung. Pattle (1955, 1958) provided evidence for this material in the bubbles of foam which was expelled from lungs. The subsequent work of many investigators has shaped our current and incomplete understanding of the physical properties of pulmonary surfactant (e.g.

Clements, 1977; Bangham et al., 1979).

Central to the study of the physical properties of lung surfactant is the contention that the physical characteristics of the alveolar air-water interface strongly influence the mechanical properties of the lung (Radford, 1954, 1957). Therefore, the physical characteristics of pulmonary surfactant, as they relate to physiological function, must be relevant to lung mechanical properties.

Morphometric studies of rabbit lungs have show that the surface area of the lungs varies with lung volume (Gil et al., 1979). Spontaneously-formed surfactant films are able to lower the surface tension of an aqueous subphase to ~25 $mN \cdot m^{-1}$. This equilibrium spreading pressure occurs when the components of pulmonary surfactant are in equilibrium with components in the subphase. The minimum surface tension of the lung at functional residual capacity was determined to be less than 9 $mN \cdot m^{-1}$ (Schürch et al., 1976). Compression of surfactant films in leak-free balances produced extremely low surface tensions which approached 0 $mN \cdot m^{-1}$ (e.g. Klaus et al., 1961). This value of minimum surface tension is in good agreement with *in vivo* measured values obtained from cat lungs by Schürch (1982). Further, Schürch (1982) has shown that the cat lung, held at 40% of total capacity maintained a surface tension of less than 1 $mN \cdot m^{-1}$ for more than one hour. Experiments such as this revealed that films of pulmonary surfactant must not only be able to reduce surface tension to near zero values but they should be able to maintain this value for an extended period of time at low lung or alveolar volumes. The unusual composition of pulmonary surfactant enables it to possess several special physical characteristics which in turn enable surfactant to perform its physiological role in lung mechanics (King & Clements, 1972).

Studies by Clements (1962), Brown (1964), and Clements (1967) were substantiated and expanded on by others who have indicated that DPPC is the component of pulmonary surfactant which enables it to attain sustained low surface tension upon compression. Films of DPPC must be below the phase transition temperature (Tc) to attain a low surface tension. The phase transition temperature is the temperature at which phospholipids undergo a change from an ordered gel phase to a disordered liquid-crystalline phase, which for hydrated DPPC is 41-42°C (Ladbrooke et al., 1968). At temperatures above the Tc, the acyl chains of phospholipid possess too much intramolecular motion for it to be able to be compressed into a highly ordered rigid state. Under most situations, this does not pose a problem since core body temperature rarely exceeds 37 °C, and surfactant is high in DPPC content. Monolayers of DPPC, at temperatures below the Tc, are able to sustain high surface pressures (e.g. Hawco et al., 1981). During compression, a monolayer of DPPC undergoes a change from a fluid state, a liquid expanded phase, to a more ordered state, a liquid condensed phase, via the main phase transition. At extremely high surface pressures, a less compressible solid phase of DPPC is formed (Mohwald, 1990). Monolayers of unsaturated lipids, such as DOPC, at temperatures below the Tc, do not withstand very high surface pressures, and collapse at a lower surface pressure than DPPC (Tchoreloff, 1991; Nag & Keough, 1993). Surface pressure isotherms of monolayers of mixtures of DPPC and DOPC show plateau regions at low to intermediate surface pressures where liquid expanded-liquid condensed transitions occur in pure DPPC monolayers (Nag & Keough, 1991). Using electron microscopy (Tchoreloff, 1991) and fluoresence microscopy (Nag & Keough, 1993) it has been visualized that domains of condensed lipids coexist with domains of partially ordered fluid lipids and it has been suggested that the condensed domains of mixed lipid monolayers of

DPPC and POPC are enriched in DPPC (Nag & Keough, 1993). Therefore, it might be speculated that upon compression of mixed lipid monolayers, reorganization of the lipid species in the monolayer may occur such that DPPC rich and DPPC poor regions are formed.

At higher surface pressures a process of selective exclusion or squeeze-out is believed to occur whereby non-DPPC lipid and protein components of surfactant are displaced from the interface to produce a monolayer which is enriched in DPPC. The squeeze-out of non-DPPC lipids from mixed lipid monolayers has been investigated by several groups (e.g. Watkins, 1969; Bangham, 1979; Hawco et al., 1981a,b; Keough, 1985; Boonman et al., 1987; Egberts et al., 1989; Mendelsolm, 1993). Another possible mechanism for the production of DPPC-rich films of pulmonary surfactant is that during dynamic compression and expansion, selective insertion of DPPC into surfactant films may occur. However, there is little direct evidence for this. Schürch et al. (1989), using the captive bubble technique, have found that initial compression isotherms of lipid extract surfactant films, adsorbed from the subphase, resembled those of solvent-spread mixed films of saturated and unsaturated phospholipids. Repeated cycling of these adsorbed films produced isotherms which mimicked the behavior of pure DPPC more closely than the initial isotherm. Schürch et al. (1989) have interpreted this DPPC-like behavior of repeatedly cycled surfactant films as occurring because of the squeeze-out of non-DPPC lipids and hydrophobic surfactant proteins.

Non-DPPC components of surfactant may not be squeezed-out of the monolayer independently. The unique composition of pulmonary surfactant may lead to a more effective selective exclusion of non-DPPC components than that observed with simple mixed lipid models. This may not only be promoted by its special lipid composition, but it may involve the presence

of surfactant proteins (Curstedt et al., 1987). Yu and Possmayer (1991) have shown that SP-B enhances the surface refinement characteristics of PG and that SP-A may facilitate this property of SP-B. The results of Perez-Gil et al. (1991) supported the involvement of SP-B in the selective removal of PG from surfactant monolayers and have shown that SP-C may also be involved in this process. Therefore, further experiments with complex lipid and protein mixtures, which more closely resemble natural surfactant, are necessary to acquire a comprehensive understanding of possible synergistic effects with respect to selective exclusion of non-DPPC components form surfactant monolayers.

The process of formation of a monolayer at the air-water interface from lipids and proteins in the bulk phase will be referred to as adsorption. Adsorption of surfactant to the air-water interface has been shown to occur very rapidly (e.g. Kobayashi & Robertson, 1983; Holm et al., 1985 and ref. cited within; Keough et al., 1989). This is necessary to maintain a adequate level of surfactant at the air-water interface since there is not a large surfactant pool in the alveolar interstitial space (Dobbs & Wright, 1991). The calculations of Goerke and Gonzales (1981) and Keough (1985) suggested that since dispersions of surfactant lipids in the subphase can form a monolayer in seconds, surfactant must adsorb as assemblages of lipids and not as individual surfactant components.

Phospholipids with rigid acyl chains adsorb more rapidly to the air-water interface at temperatures above the transition temperatures than below them (e.g. Gershfeld & Tajima, 1979). Therefore, the properties which make DPPC ideal for reducing surface tension to ~ 0 $mN \cdot m^{-1}$, namely a high gel-to-liquid transition temperature and its ability to pack tightly in a gel state, decrease its ability to adsorb with facility. Addition of the major non-DPPC lipids, unsaturated

PC (Tinker & Low, 1982; Egberts et al., 1989) and unsaturated PG (Fleming & Keough, 1988; Egberts et al., 1989), in amounts which are found in surfactant, broadens and lowers the transition temperature from of that of DPPC. It has been shown that the addition of unsaturated phosphatidylglycerol (Meban, 1981; Notter et al., 1982; Notter et al., 1983; Yu et al., 1984), unsaturated phosphatidylinositol (Meban, 1981), phosphatidylethanolamine (Yu et al., 1984; Notter et al., 1983) and cholesterol (Meban, 1981) to aqueous dispersions of DPPC enhanced the adsorption of the lipid mixtures to the air-water interface. Therefore, one of the biophysical functions of the non-DPPC lipid components of surfactant may be to fluidize the rigid acyl chains of DPPC at body temperature which increases adsorption of surfactant lipids to the air-water interface. The fluidizing behavior of non-DPPC lipids of surfactant has another effect on the behavior of DPPC; that is to increase the respreading of the DPPC rich film after surfactant film collapse. There is evidence that the addition of unsaturated PC and cholesterol increases the respreading of a mixture of DPPG and POPG; however, unsaturated PC and cholesterol were shown to increase the minimum surface tension compared to the more simple lipid mixtures (Fleming & Keough, 1988). The increase in minimum surface tension which is produced by these lipids in monolayers of more simple lipid mixtures may not have as deleterious an effect in monolayers of whole surfactant (see above discussion).

Freeze-fracture studies of foam from calf lung surfactant have demonstrated vesicles and funnel-like structures in contact with the interface, and tubular myelin-like structures at and very near the air-water interface (Sen et al., 1988). Tubular myelin is an unusual structure consisting of arrays of long tubes of square cross-sections with bilayer sides (e.g. Williams, 1978) and a body of circumstantial evidence suggests that it is the precursor of surfactant films. The presence

of tubular myelin appears to be coincident with the rapid adsorption of surfactant (Magoon et al., 1983; Benson et al., 1984; Efrati et al., 1987). Gil and Reiss (1973) have found tubular myelin to be rich in protein compared to other forms of surfactant, such as lamellar bodies which are the precursor of tubular myelin (Osterlaken-Dijksterhuis et al., 1991). Others have shown that tubular myelin is rich in SP-A (Wright et al., 1984), and presumably SP-B and SP-C as well, since these proteins are present in lamellar bodies. The formation of tubular myelin requires DPPC and PG, calcium and at least SP-A and SP-B (although tubular myelin is formed when SP-A, SP-B, and SP-C are present with the surfactant lipids and calcium) (Williams et al., 1991).

Therefore, non-DPPC surfactant components facilitate the adsorption, initial spreading, and respreading after surfactant film collapse while not impeding the surface tension lowering ability of surfactant DPPC upon compression. This orchestra of biophysical behaviours of individual components of surfactant is necessary for the proper function of pulmonary surfactant. When the surfactant system of the lung is impeded from functioning properly lung dysfunction can occur and lead to life-threatening illness such as ARDS.

Adult Respiratory Distress Syndrome

In 1967, Ashbaugh et al. (1967) described the development of acute respiratory failure in twelve patients with tachypnea, hypoxemia, and a loss of lung compliance. Post-mortem examination of the lungs of several of the patients revealed areas of atelectasis, haemorrhage, oedema and hyaline membrane formation. They have postulated that lung surfactant function was abnormal and that it contributed to the pathophysiology of the condition. Shortly thereafter, this condition was referred to as adult respiratory distress syndrome (ARDS) (Petty & Ashbough,

1971). ARDS is estimated to affect ~ 150 000 people per year in the United States and has a mortality rate of 50-70% despite advances in the supportive therapy (Villar & Slutsky, 1989).

ARDS is a well-known cause of acute respiratory failure which can effect previously healthy adults and children. ARDS can occur after a variety of pulmonary or systemic insults. These include pulmonary and non-pulmonary sepsis, shock, inhalation of smoke or toxic gases, oxidant injury, liquid or gastric aspiration, and thoracic and nonthoracic trauma such as fractures and burns (e.g. Royall & Levin, 1988).

Sepsis is the most common clinical condition associated with ARDS with 20-40% of patients with sepsis developing ARDS (Wiener-Kronish et al., 1990). At post-mortem examination, the source of infection was usually in the abdomen in patients with clinical evidence of infection and positive blood cultures. In contrast, in patients with negative blood cultures, the origin of infection was more likely to be in the lung (Bell et al., 1983).

ARDS has three phases. During the first 5 days, the early phase, the patient typically has severe alveolar oedema, with a large number of inflammatory cells accumulating in the lung, primarily neutrophils in the lung interstitium (Wiener-Kronish et al., 1990). The pathology of the second phase occurs at approximately 5-10 days after initial onset and primarily involves the interstitium of the lung. Some patients develop an accelerated fibrosing alveolitis and ultrastructural studies have shown proliferation of alveolar type II cells (Matthey, 1989). The highest risk of superimposed infection appears to be within 6-10 days after initiation of ventilation (Langer et al., 1989). At this stage, lung damage can result from impairment of blood and lymphatic drainage and the presence of plasma in the airways. This, along with impairment of muco-ciliary transport is believed to contribute to the development of nosicomial pneumonia which

is common at this stage of ARDS (Wiener-Kronish et al., 1990). The final stage of ARDS, the chronic stage, lasts 10-14 days after the initial onset of the syndrome and is characterized by varying degrees of lung dysfunction, emphysema, pulmonary vascular obliteration and areas of pulmonary fibrosis (Wiener-Kronish et al., 1990).

The treatment of ARDS can be considered to be aggressively supportive. Continuous positive airway pressure (CPAP) and positive-end expiratory pressure (PEEP) are believed to decrease progressive alveolar collapse, reduce interstitial oedema, and increase functional residual capacity (Weigelt, 1987), thus limiting the severity of hypoxia. Further, PEEP may prevent surfactant aggregation (Wysazogrodski et al., 1975) and reduce oxygen toxicity by reducing inspired oxygen requirements (Petty & Ashbough, 1971). The difference in oncotic pressure between the pulmonary capillaries and interstitial space is correlated with the amount of pulmonary oedema formed (Wiener-Kronish et al., 1990). Therefore, fluid management is important to maintain low capillary pressures, thereby reducing the hydrostatic transcapillary pressure gradient. Corticosteroids have been the pharmacologic agents most widely used to treat ARDS because of their anti-inflammatory properties (Weigelt et al., 1985); however, a large scale study Luce et al. (1988) have failed to demonstrate their beneficial effects in terms of prevention or improving the outcome of ARDS, indeed showing increased morbidity in the treated group.

The current treatment and prevention of ARDS has not been particularly successful. Effective therapeutic strategies will be facilitated by a more comprehensive understanding of the underlying factors which govern the pathologies of ARDS.

The early pathology of ARDS typically shows severe pulmonary edema. This results from pulmonary microvasculature injury which can vary from an increase in permeability of the

pulmonary capillary bed to total disruption of portions of the lung microvasculature. Many of the mediators of ARDS are primarily responsible for the destruction of microvascular integrity.

Unlike the pathologic and cardiorespiratory changes of ARDS, the underlying mediators which are believed to be responsible for ARDS have not been fully delineated. ARDS can result from an alteration of several homeostatic mechanisms which occur simultaneously, and often synergistically, to produce the clinical conditions of ARDS. Leukocytes, platelets, red blood cells, macrophages, 0_2-free radicals, proteolytic enzymes, lysosomes, complement, fibrin and fibrin degradation products, histamine, and endotoxin are included in this list of mediators (Royall & Levin, 1988).

There is considerable evidence to implicate leukocytes as playing a key role in the damage to the pulmonary capillaries seen in ARDS. Tate and Repin (1983) have shown that peripheral leukopenia is associated with ARDS and complement-activated neutrophils accumulate in the lung (Hammerschmidt et al., 1980). The complement components C3a and C5a which are neutrophil chemotactic stimulants are elevated in the BAL of patients with ARDS (Robbins et al., 1978) and the degree of complement activation, which can result in leukocyte aggregation has been reported to be a predictive factor of the development of ARDS (Duchateau et al., 1984).

For the survivors of ARDS the prognosis of full recovery of pulmonary function is good (e.g. Lakshminarayan & Hudson, 1978; Alberts et al., 1983); however, the literature is not comprehensive with respect to long term recovery of ARDS survivors. Most patients are asymptomatic or have minor dyspnea upon exertion. Lung volumes tend to improve during recovery and become normal within 6 to 12 months after initial recovery although ~ 30% of patients demonstrate hypoxemia at rest and have abnormalities in the diffusing capacity of CO.

Pulmonary Surfactant and ARDS

In the initial description of ARDS, Ashbough et al. (1967) suggested that surfactant abnormalities may be partially responsible for the pathological conditions of this syndrome in their patients. They reported an increase in the minimum surface tension of surfactant recovered from minced lungs of ARDS patients at autopsy. Contrary to this finding, Petty et al. (1977, 1979) reported that the surfactant obtained from lungs of patients with ARDS produced normal minimum surface tension upon compression but the compressibility of the film was 5-10 times higher than normal. They suggested that the loss of film elasticity may contribute to the abnormal pressure-volume characteristics of the lungs of these patients.

Several groups have provided additional evidence that surfactant function is abnormal in ARDS. In a number of studies using a flexible bronchoscopy, BAL samples were obtained during different stages of the syndrome. Gregory et al. (1991) and Hallman et al. (1982) noted a 2-4 fold increase in minimum surface tension of patients with ARDS compared to normals. On the other hand, Pison et al. (1989), in agreement with the data of Petty et al. (1977, 1979) did not observe abnormal minimum surface tension in such patients. They (Pison et al., 1989) did observe decreased hysteresis of the surface tension-surface area relationship in ARDS patients compared to normals.

Measurement of surfactant function has also been correlated with the degree of respiratory failure of patients with ARDS. In a study by Pison et al. (1989), serial BAL samples were collected prospectively from patients who subsequently developed ARDS. They showed that decrease in hysteresis area was markedly more pronounced in groups of patients with high overall ARDS scores than in patients with mild pulmonary dysfunction. Gregory et al. (1991) found a

2-fold increase in the minimum surface tension of patients who were at risk of developing ARDS compared to normals. Others (e.g. Seeger et al., 1990) have noted a significant correlation between the level of surfactant function and severity of lung dysfunction in ARDS patients. It is clear that surfactant abnormalities exist in patients with ARDS and that the degree of impairment of surfactant function is correlated with severity of lung dysfunction.

Accurate quantitation of alveolar surfactant pool size in patients with ARDS is not feasible because samples consist of relatively small amounts of BAL and the techniques of standardizing the recovery of BAL have not yet been established. Also, variabilities in the method of surfactant isolation such as different centrifugation forces may alter measurement of surfactant pool size. Given this, it is not surprising that there are inconsistencies in reports of surfactant pool size and surfactant composition in patients with ARDS and in animal models of ARDS.

Hallman et al. (1982) and Pison et al. (1989) have reported no change in the total phospholipid pool size recovered from patients with ARDS compared to a control group. Three groups, Gregory et al. (1991), Seeger et al. (1990), and Pison et al. (1990), however, have reported decreased phospholipid content in such samples. In animal models when ARDS was induced by intravenous injection of oleic acid (Casals et al., 1989) and bilateral vagotomy (Berry et al., 1986) no change in surfactant pool size was reported. A decrease in surfactant pool size was reported when the lung was exposed to 100% oxygen (Holm et al., 1985). In another study, ARDS was induced by exposure to 85% oxygen and an increase in surfactant pool size was reported (Low et al., 1988). These differences may result from variation in the level of damage to alveolar type II cells with severe damage resulting in decreased surfactant pool size while more mild forms of damage may produce a hyperplastic response and a concomitant increase in

surfactant pool size (Royall & Levin, 1988).

Altered phospholipid composition has consistently been reported in human studies and animal models of ARDS. Typically, decreased quantities of saturated phosphatidylcholine and phosphatidylglycerol are associated with increased amounts of phosphatidylinositol, phosphatidylethanolamine, sphingomyelin, and lysophosphatidylcholine (Hallman et al., 1982; Gregory et al., 1989; Pison et al., 1989). Additionally, Gregory et al. (1991) reported that, when compared to normals, the levels of SP-A and SP-B are decreased in BAL of patients at risk of ARDS and in those with ARDS. These surfactant composition abnormalities became more pronounced as the severity of lung injury increased. Save for the abnormal levels of phosphatidylglycerol, the relative concentrations of the surfactant constituents returned to normal as the lung recovered from injury. Modification of the amount of phosphatidylglycerol and phosphatidylinositol in rabbit surfactant by dietary means seemed not to interfere with the function of surfactant (Beppu et al., 1983; Hallman et al., 1985; Liau et al., 1985). Although the decrease in the amount of DPPC and phosphatidylglycerol is associated with ARDS, an abnormal amount of phosphatidylglycerol and phosphatidylinositol in the lung may not negatively affect surfactant function *in vitro*, especially if the level of phosphatidylserine, another acidic lipid with a polyhydroxy head-group, increases as phosphatidylglycerol decreases.

Gregory et al. (1991) have stated that the decreased levels of SP-A and SP-B in the surfactant of patients with ARDS may be due to altered synthesis or damage or both of surfactant in the alveolar interstitium. Also, the changes in surfactant phospholipid composition may be related to altered surfactant uptake and synthesis by alveolar type II cells. It seems especially likely that the decrease in phosphatidylglycerol and increase in phosphatidylinositol is due to

abnormal metabolism of these lipids by alveolar type II cells (Hallman & Gluck, 1976). Additionally, these changes may be due to contamination from membrane phospholipids which results from damaged alveolar type II cells, lung tissue or inflammatory cells (Gregory et al., 1991). Compared to normals, the activity of phospholipase A was not increased in the BAL or the plasma of rabbits (Hallman et al., 1982) or patients (Casals et al., 1989), both of which had respiratory failure. Plasma contains a higher concentration of lysophosphatidylcholine than that which is found in the lung (Holm et al., 1991). Therefore, increased lysophosphatidylcholine in the BAL of patients with ARDS may not result from an increased breakdown of phosphatidylcholine but probably results from the extensive pulmonary edema which is the hallmark of ARDS. Leakage of protein-rich edema into the alveolar space and subsequent alteration of lung surface tension caused by detrimental surfactant-plasma protein interactions has been suggested to be important in the pathophysiology of RDS (e.g. Taylor & Abrams, 1966) and ARDS (e.g. Ashbaugh et al., 1967).

Measurement of the protein content of BAL samples from ARDS patients has shown significantly increased levels compared to those of normal controls (Pison et al., 1989). They, (Pison et al., 1989) instilled 5 consecutive 20 ml volumes of 0.15 M NaCl and withdrew it under negative pressure with an overall recovery of 60-70%. In a multicentre study, Gregory et al. (1991) determined that BAL samples from patients with ARDS had significantly lower phospholipid to protein ratios than samples obtained from healthy volunteers. Further, the phospholipid to protein ratio of ARDS patients was significantly lower than in patients who were determined to be at risk of developing ARDS. Therefore, leakage of plasma proteins into the lung, an early event in the pathogenic sequence of ARDS, appears to be related to the severity of

lung dysfunction which is associated with this syndrome.

Many investigators have determined the effects of blood some of and its protein components on the function of pulmonary surfactant *in vitro*. To investigate the effect of inhibitors on the adsorption facility of surfactant, investigators have used the surface adsorption apparatus (King & Clements, 1972) and the pulsating bubble surfactometer (Enhorning, 1977). Table 1 is a brief list of protein inhibitors of pulmonary surfactant adsorption.

Authors	Inhibitor(s) of Surfactant Adsorption		
Tierney and Johnson (1965)	blood	serum	
Holm et al. (1985)	albumin		
Fuchimukai et al. (1987)	albumin	fibrinogen	serum
Holm and Notter (1987)	haemoglobin		
Holm et al. (1988)	albumin	fibrinogen	haemoglobin
Keough et al. (1989)	albumin	fibrinogen	globulin
Kobayashi et al. (1991)	pulmonary edema fluid		
Amirkanian and Taeusch (1993)	CRP	fibrinogen	
Seeger et al. (1993)	albumin	fibrinogen	haemoglobin
Seeger et al. (1993)	fibrinogen		
Cockshutt et al. (1993)	albumin	fibrinogen	globulin

Table 1.

Evaluation of Illness

There is a recognized need for a system which standardizes information on patients admitted and treated in intensive care units (ICUs) (Griner, 1972). The inability to classify groups of patients based on severity of illness has limited both the evaluation of intensive care and assessment of new therapies (Knaus et al., 1981, and ref. cited within). A widely accepted index for determining the severity of illness is a scale which assesses the probability of mortality (Kricher, 1976). Several systems have been designed to serve this purpose (e.g. Cullen et al., 1977). The first widely accepted scoring system to classify groups of acutely ill patients based on severity of illness was the APACHE I (acute physiology and chronic health evaluation) system (Knaus et al., 1981). The APACHE disease classification system is based on the hypothesis that the severity of acute disease can be measured by quantifying the degree of abnormality of multiple physiological variables. The APACHE I system is composed of two parts: a physiology score representing the degree of acute illness and a preadmission health evaluation indicating health status before acute illness. The APACHE I index was used to prognostically stratify acutely-ill patients and assist investigators in comparing the success of new and different forms of therapy. This systems can be used to classify all ICU patients, save those with myocardial infarction or burns for whom another classification scheme exists. The physiological portion of the APACHE I scoring system was designed to measure objectively the degree of acute illness by surveying 34 physiological measurements, the sum of which yields an acute physiology score. These physiological measurements are performed within the first 24 hours of patient admission to ICU.

Knaus et al. (1981) suggested that the degree of physiological abnormality alone does not

accurately reflect severity of illness. For example, the same degree of hypercapnia in a patient with chronic obstructive pulmonary disease is less alarming, clinically, than in a previously healthy patient. Therefore, the chronic health evaluation serves to modify the acute physiology score by considering patient history.

Refinement of the APACHE I scoring system with the intent of developing a more simplified and more clinically useful yet statistically accurate and valid, patient classification system, has lead to the development of the APACHE II scoring system (Knaus et al., 1985). The APACHE II scoring system has been revised in an attempt to improve the risk prediction by reevaluating the selection and weighting of physiological variables which examine how differences in patient selection for and timing of admission to ICU relates to outcome across several hospitals (Knaus et al., 1991). The use of clinical judgment and documented physiological relationships to choose variables and assign weights serves as a basis of the APACHE II as it did for the APACHE I. Age and severe chronic health problems have been incorporated into the APACHE II. The number of physiological measurements taken in the first 24 hours of ICU admission, however, has been reduced from 34 to 12. This was accomplished by ommitting physiological variables which were measured infrequently, had little explanatory power, or were redundent. Also, unlike the APACHE I system, the APACHE II scoring index can provide the clinician with a systematic evaluation of how an individual patient's severity of disease influences outcome (Knaus et al., 1985).

Acute Phase Response

A number of the body's homeostatic mechanisms are altered following various types of

tissue injury and infection. The body has developed many ways to counteract this imbalance and return the body to a normal homeostatic state. One such mechanism is known as the acute phase response. The term "acute phase" was introduced in 1941 by Avery and colleagues (Albernaethy & Avery, 1941; MacLeod & Avery, 1941) to refer to patients who were acutely ill with infection. Also included in this original definition was a finding of the presence, in the patients' sera, of C-reactive protein (CRP), a protein which had been previously discovered in the sera of acutely ill individuals by Tillett and Francis (1930). They, Avery and colleagues, found that CRP existed only in acute phase sera. As new techniques of detection and quantitation were developed it became clear that the acute phase response involves adjustment in the serum concentrations of several proteins, and that CRP exists in the sera of healthy individuals, albeit, at much lower concentrations (e.g. Pepys & Baltz, 1983).

Today the acute phase response is defined as a characteristic pattern of alteration in the plasma concentration of a number of proteins, which include protease inhibitors, coagulation proteins, lipoproteins and proteins with other functions (Pepys & Baltz, 1983). The concentrations of most of these proteins increase by varying amounts during the acute phase response while some decrease. These are referred to as positive and negative acute phase proteins, respectively.

There are many types of stimuli which are known to elicit the acute phase response in man, such as surgery and other types of physical trauma; for example, bone fractures, burn injuries, and tissue infarctions. This response can also be initiated by chemical trauma, ischemic necrosis, malignant neoplasia and inflammatory stimuli such as bacterial, viral, fungal or parasitic infection (Kushner, 1982). Experimentally, in animal models such as the rabbit, the acute phase response

can be induced by the injection of turpentine, a local inflammatory substance, or alternatively, by the injection of a small amount of bacterial lipopolysaccharide which can provoke a major acute phase response without causing other clinical evidence of toxicity (Kushner, 1982).

Production of C-reactive Protein

Plasma CRP is synthesized exclusively by hepatocytes (Hurlimann et al., 1966). It is also synthesized by a subset of peripheral blood lymphocytes, but this CRP remains bound to the surface of these cells (Ikuta et al., 1986; Kuta & Baum, 1986). Recently, Egenhofer et al. (1993) have shown that CRP is synthesized by monocytes and macrophages, and that these cells, as well as natural killer cells, do not secrete CRP into general circulation. CRP is known to exist in two antigenically distinct conformations, either as a native pentamer in general circulation or as a neoantigenic determinant which is bound to certain cells, neoCRP (Plempa et al., 1983).

Kushner and Feldmann (1978), Baltz et al. (1980), Benson and Kleiner (1980), and Courtory et al. (1981) have shown that increased circulating concentrations of acute phase proteins of hepatic origin result from an increase in the number of hepatocytes being recruited for synthesis. These authors have shown that initial synthesis is located in the periportal distribution, but as acute phase stimuli increase, most hepatocytes become recruited to produce acute phase proteins. The experiments of Courtary et al. (1981) have shown that most hepatocytes are capable of synthesizing acute phase proteins and are able to simultaneously increase the levels of several acute phase proteins.

The cascade of events which lead to the production of acute phase proteins begin with the activation of several cells types such as macrophages, epithelial cells, keratinocytes and mast cells

in response to the broad range of stimuli which elicit the acute phase response. It is the activation of macrophages and concomitant cytokine release which is primarily responsible for the increased production of acute phase proteins from hepatocytes during this response (Akira et al., 1990).

It was originally suggested that the cytokines Il-1 and TNFα were primarily responsible for altering the synthesis of acute phase proteins which causes the majority of the physiological and biochemical features of the acute phase response (Dinarello, 1984; Beulter et al., 1986; Oppemheim, 1986). Indeed, Perlmutter et al. (1986) have shown that these cytokines are able to regulate the expression of acute phase proteins in human hepatoma cells. Others, however, suggested that an additional factor (hepatocyte stimulating factor) was necessary for complete control of hepatic acute phase protein production (e.g. Richie & Fuller, 1983). Then in 1987, Gauldie et al. (1987) identified the additional potent hepatocyte stimulating factor as the cytokine IL-6.

The myriad of events which occur during the acute phase response are primarily a result of the change in plasma concentrations of the acute phase proteins (Kushner & Pepys, 1983). This is an attempt to bolster a homeostatic response to infection or trauma due to the broad spectrum of biological activities of the acute phase proteins.

Biochemical Properties of CRP

C-reactive protein belongs to the pentraxin family of proteins and as the name implies, consists of five identical subunits with an molecular weight of ~23 000 (Gotschlich et al., 1965). Each subunit is synthesized as a 214 amino acid precursor of which an 18 amino acid stretch is a signal peptide (Tucci et al., 1983). CRP subunits are non-glycosylated, contain one disulfide

bridge (Olivera et al., 1977) and associate non-covalently to form the pentameric ring molecule (Gotschlick & Edleman, 1965; Osmand et al., 1977). Ken et al. (1990) noted that the functional state of circulating CRP may be related to its conformation, whether it is aggregated, cleaved, or in its native pentameric form. Pepys and Balts (1983) stated that CRP would probably be aggregated or cleaved during a local response to inflammation. In general, however, circulating CRP is most likely to be in the native pentameric form in both healthy individuals and those experiencing the acute phase response.

CRP was initially characterized and purified from sera by its ability to bind to and precipitate with pneumococcal C-polysaccharide in the presence of calcium (Tillett & Francis, 1930). The work of Gotschlick and Edelman (1967) have shown that CRP binds calcium with a stoichiometry of 1-2 moles per subunit, this was later supported and expanded upon by studies, using circular dichroism, which have shown that calcium binding caused a conformational change in CRP (Young & Williams, 1978) and by the use of monoclonal antibodies (Kilpatrick et al., 1982). The site at which CRP binds calcium consists of a highly-conserved amino acid stretch, residues 133-147, and it is thought to bind calcium via four acidic residues (Liu et al., 1987).

The first to explore the ligand binding properties of CRP, beyond precipitation with C-polysaccharide from different sources, were Gotschlich and Edelman (1965). Their work was initiated by earlier findings by Hornung and Berenson (1963) who found that uridine monophosphate could inhibit the binding of CRP to C-polysaccharide. Gotschlich & Edelman, (1965) tested a number of potential inhibitors in order to determine which groups were required to inhibit the binding of CRP to C-polysaccharide. Their conclusion, that phosphate monoesters were necessary to block the binding to C-polysaccharide, preceded the work of Volanakis and

Kilpatrick (1971) who determined the relative inhibitory powers of several phosphate monoesters to perform this function. Volanakis and Kilpatrick, (1971) have shown that phosphocholine was especially inhibitory and that DL-α-glycerophosphate and O-phosphoethanolamine possessed less inhibitory power with respect to binding of CRP to C-polysaccharide and they have shown that the association constant of CRP for phosphocholine was 2×10^5 M^{-1} at 5°C.

Liu et al. (1987) suggested that phosphocholine binding occurs at the highly conserved residues 51-66 with residues Lys-57-Arg-58 binding to the PO_4^{2-} moiety and the cationic residues Asp-60-Glu-62 binding to the choline moiety. This hypothesis was supported by the work of Swanson and Mortensen (1990) who found that a synthetic peptide corresponding to amino acid residues 47-63 of CRP bound phosphocholine and reacted with a monoclonal antibody which was specific for the phosphocholine binding region of CRP. The phosphocholine binding region is thought to be located nearly perpendicular to the plane of the CRP molecule i.e., facing away from the edge (Roux et al., 1987).

The binding of CRP to C-type and type 27 polysaccharides is explained by a binding to phosphocholine moieties in these polysaccharides. CRP has also been shown to bind to depyruvylated type 4 capsules which do not contain phosphocholine (Higginbotham et al., 1970). This was subsequently explained by the binding of CRP to polymers of galactose (Pepys et al., 1977). Also, in the absence of calcium, CRP binds to a number of polycations, such as poly-L-serine and poly-L-lysine (DeCamelli et al., 1980). The binding of CRP to phosphocholine and to galactosyl polymers explains the large number of endogenous and exogenous ligands for CRP (e.g. Kolb-Backofen, 1991).

The biological roles which CRP are believed to play in the host defence system is in part

reflected by its ligand specificity. Once CRP is attached to most of its ligands, it is also able to activate the classical complement pathway via an interaction with the first component of this pathway, C1q (Kaplan & Volanakis, 1974). In the absence of additional signals, however, CRP unlike immunoglobulins, is unable to lyse cells through the formation of the membrane attack complex via an interaction with the components C5 through C9 (Berman et al., 1986). The site at which C1q binds CRP was proposed in 1991 by Jiang and Gewurz (1991) and has since been delineated to involve two cationic domains on the collagen-like region of C1q (Jiang et al., 1992).

Interaction of C-reactive Protein with Lipids

Various groups have investigated the binding of CRP to suspensions of phospholipids and phospholipids plus cholesterol. Volanakis and Wirtz (1979) have shown that with liposomes formed from phosphatidylcholine alone or with phosphatidylcholine plus cholesterol (15 mol%) no binding occurred between CRP and these structures. They have also shown that the incorporation of stearylamine (10 mol%) into the phosphatidylcholine-containing liposomes did not cause CRP binding. Subsequently, in 1981, Mold et al. (1981) have shown that the incorporation of increasing amounts of cholesterol (up to 33 mol%) to liposomes composed of dimyristoylphosphatidylcholine (44 mol%), stearylamine (15 mol%) and galactosylceramide (8 mol%) did not increase CRP-binding. They have demonstrate that the incorporation of stearylamine and galactosylceramide into liposomes of phosphatidylcholine plus cholesterol resulted in binding of CRP which was calcium independent and was not inhibited by phosphocholine nor was it affected by the substitution of dimyristoylphosphatidylethanolamine for dimyristoylphosphatidylcholine. The ligand to which CRP was binding in the Mold et al. (1981)

study was the positively charged stearylamine (Tsujimoto et al., 1981).

The incorporation of lysophosphatidylcholine into phosphatidylcholine-containing liposomes has been shown to be a very effective method of inducing the binding of CRP to these vesicles (Volanakis & Wirtz, 1979; Volanakis & Narkates, 1981; Anderson et al., 1982). This binding is calcium-dependent and is inhibited by free phosphocholine. The incorporation of lysophosphatidylcholine into phosphatidylcholine-containing liposomes is thought to disrupt the molecular organization of the bilayer by causing membrane irregularities (Weltzien, 1979 and ref. cited within) and this may consequently increase exposure of phosphatidylcholine to CRP (Volanakis & Wirtz, 1979). Kushner and Kaplan (1961) reported that CRP is in close association with membrane structures of altered and necrotic cells but not with normal cells. Indeed, Narkates and Volanakis (1982) have shown that treatment of erythrocytes with phospholipase A_2, which cleaves phosphatidylcholine to produce lysophosphatidylcholine or lysis of the cellular membranes by osmosis, causes the binding of CRP to the erythrocytes. It has been suggested that this may be relevant to the biological function of CRP such that damaged cells or foreign pathogens which have been opsonized with CRP may be more attractive to phagocytic cells (Kaplan & Volanakis, 1974).

Therefore, despite the potentially large number of endogenous ligands to which CRP can bind it appears to do so under special circumstances which are facilitated by a disruption of cell membranes. This requirement of CRP for an exposed ligand appears to protect most membranes of the body from the destructive implications of CRP binding.

Clinical Usefulness of Measuring Serum CRP Levels

Since the discovery of CRP in the serum of patients with pneumonia in 1930 (Tillett & Francis, 1930) interest in the acute phase response and whether detecting changes in the concentrations of acute phase proteins could provide clinically useful information has increased.

The first of several researchers to investigate the possibility that CRP may be useful in the clinical management of disease were Kroop and Shackman (1954). They detected elevated serum CRP levels in patients with myocardial infarction. Shelter et al. (1955) in a study of 113 cases, including normal cases and patients with a variety of diseases, found that CRP was elevated in a majority of instances of tuberculosis, Hodgkin's lymphoma, Ewing's sarcoma, multiple myeloma and other malignancies. The usefulness of determining the levels of serum CRP compared to other measurements made during the acute phase response was noted as early as 1957 (Yocum & Doerner, 1957). They noted that an elevated CRP level was a good indication of the presence of inflammation or necrotic processes. Subsequently, Hedlund (1961) found that serum CRP increased in cases where elevated temperature was associated with inflammation. Two years later, Belfrage (1963) found that in over 900 cases, serum CRP level was a better indicator of infection than fibrinogen, haptoglobin, α-globulin and erythrocyte sedimentation rate. As more work was completed it became clear that major elevations in serum CRP concentrations occur in most severe infections (e.g. Kenny et al., 1981; Pepys, 1981; Gewurz et al., 1982). It has been shown that the degree of elevation of CRP corresponds reasonably well with the severity of infection (e.g. Sabel & Hanson, 1974; Kushner & Feldman 1978; Sabel & Wadsworth, 1979; Macintry et al., 1982) and severity of tissue damage (Kushner et al., 1978; de Beers et al., 1982).

Elevation of CRP levels in the serum of individuals afflicted with one or more of the stimuli which often lead to an acute phase response also occurs in those who suffer from ARDS.

Previously, Kew et al. (1990) have investigated the possible correlation between elevated CRP levels in the serum of individuals in an intensive care unit and the stages of pulmonary dysfunction which lead to ARDS. They determined that sera of patients at high-risk of developing ARDS and those with ARDS contained significantly elevated levels of CRP compared to that of normal subjects. They defined patients at high-risk of ARDS as those who failed to meet the criteria of ARDS, but possessed one or more of the following: sepsis syndrome, requiring pulmonary aspiration, nonthoracic trauma or hypotension. Sepsis syndrome required the presence of two of the following criteria: temperature $>39°C$ or $<36°C$; peripherial white blood cell count <3000 or $>12\,000$ cells $\cdot$ mm^{-3}, positive blood culture for a commonly recognized pathogen or a strongly suspected source for systemic infection from which a known pathogen had been identified. The following were also necessarily present: a deleterious systemic response to infection such as metabolic acidosis, systemic arterial hypotension with systolic blood pressure of <80 mm Hg for more than two hours, or systemic vascular resistance of <800 dynes $\cdot$ $sec^{-1} \cdot cm^{-2}$. Pulmonary aspiration required a witnessed event with or without the suction of gastric contents from the trachea. Trauma and hypotension required acute nonthoracic trauma, including surgical operations, associated with blood loss and systemic systolic blood pressure of <80 mm Hg for more than two hours or the requirement of vassopressor agents fro longer than two hours. They (Kew et al., 1990) defined patients with ARDS as those having roentgenographic evidence of bilateral alveolar infiltrates, possessing a pulmonary capillary wedge pressure of <15 mm Hg, and a total static pulmonary compliance of <50 ml $\cdot$ cm^{-1} H_20 and a PaO_2/FiO_2 ratio of <200 while the patient was receiving positive end expiratory pressure from the mechanical ventilator. The levels of CRP in the BAL obtained from patients with ARDS were found to be elevated

compared to those of normals (Li et al., 1989). Li et al. (1989) identified patients with ARDS as those who required intubation and mechanical ventilation with positive end expiratory pressure of >5 cm H_2O, had diffuse radiographic infiltrates and severe hypoxemia injury, and a FIO_2 of >0.40 in order to maintain a $PaO_2 > 50$. They also had reduced total respiratory system compliance (<50 cc/cm H_2O) and a pulmonary capillary wedge pressure of <16 mm Hg.

Complement Protein C1q

The complement system is composed of a group of proteins, found in body fluids or on cell membranes, which when activated, lead to specific sequential interactions which produce many physiological effects. Complement plays a major role in the mediation of inflammation, in the regulation of phagocytic activity, and in the metabolism of immune complexes (Loos, 1983). Complement activation may be initiated by chemical activators (e.g. lipopolysaccharide), by interactions with certain classes and subclasses of immunoglobulins or by an interaction with CRP. Therefore, complement serves as an effector system for antibodies and CRP when they are bound to substrates.

C1q is the first component of the classical complement pathway and possesses a " flower bouquet-like" quaternary structure which is similar to that of SP-A. IgM and IgG subsets 1, 2, and 3 have receptors for C1q in the constant region of their heavy chains (Shulman et al., 1987) which become exposed once the immunoglobulin has attached to a specific antigen. Activation of C1q is dependent upon cross-linking of at least two of the six binding sites with immunoglobulin receptors. At least two molecules of IgG or one molecule of the pentameric IgM are necessary for this to occur. In addition, upon binding to a substrate, the pentamer, CRP, can

initiate C1q activation. Once activated, C1q activates $C1r_2$ which in turn cleaves $C1s_2$ by an intramolecular, autocatalytic mechanism (Weisset et al., 1986). The resulting molecule C1 esterase, may continue the pathway by catalysing the assembly of C3 convertase, or it may be inactivated by the binding of C1 esterase inhibitor to C1r and C1s, causing them to loose their attachment to C1q, thereby stopping the pathway.

C1q is synthesized primarily by macrophages (Loos, 1983), which not only secrete C1q but also have C1q associated with their surface (Loos et al., 1980). The molecular weight of C1q is ~410 000 (Ziccardi & Cooper, 1977). The normal mean value for C1q in sera was shown to be ~127 $\mu l \cdot ml^{-1}$ (Delamarche et al., 1988) and serum C1q has been shown to range between 70 to 276 $\mu l \cdot ml^{-1}$ (Ziccardi & Cooper, 1977). C1q, unlike several other complement proteins, such as C1s, is not an acute phase protein. Therefore, its serum concentration is not expected to be altered by inflammation, injury or stress.

Statement of the Problem

The relevant literature with respect to surfactant dysfunction by circulating protein inhibitors has been reviewed. ARDS, affect thousands of people a year and has a mortality rate of 50-70%. The early stages of this syndrome are associated with severe pulmonary edema and it is thought that the inhibition of surfactant function by plasma proteins contributes to the development of ARDS. Many of the etiologies which commonly cause ARDS also result in the acute phase response. CRP, an acute phase protein, can increase in serum concentration up to 1000-fold during this response. CRP has been shown to be elevated in the lung of patients with ARDS compared to normals and CRP binds phosphocholine. CRP also binds phosphoethanolamine and glycerophosphate, although with less avidity than phosphocholine.

These water soluble CRP ligands constitute the headgroups of three of the phospholipids of pulmonary surfactant, with phosphocholine being the most abundant. The complement protein C1q is homologous to the hydrophilic surfactant protein SP-A in it's quaternary structure. SP-A has been shown to have beneficial effects on the physical behavior of pulmonary surfactant. Therefore, this thesis is put forth to seek answers to the following questions which have not been fully addressed in the literature.

Does CRP affect surfactant adsorption?

Is the effect calcium-dependent?

Is the effect of CRP specific for certain surfactant component(s)?

Can the effect of CRP be diminished and is the effect reversible?

Does the level of CRP in the serum and sputum of acutely ill patients correlate with the severity of pulmonary dysfunction which is associated with ARDS and does it correlate with the APACHE II scores of these individuals?

Can some the beneficial effects of SP-A on surfactant adsorption be substituted for by the addition of the complement protein C1q to surfactant which is void of SP-A?

Does C1q affect the minimum surface tension of lipid extract surfactant?

Research Plan

The ability of surfactant to adsorb rapidly to the air-water interface was tested in the presence and absence of CRP, calcium, and in combinations of CRP and three CRP ligands, phosphocholine (Cl^-), O-phosphoethanolamine, and DL-α-glycerophosphate using a surface adsorption apparatus (Keough et al., 1987). The effect of CRP was compared to that of other

known serum protein inhibitors of surfactant function. Adsorption experiments were performed with C1q and lipid extract surfactant which was void of SP-A, using the surface adsorption apparatus. Adsorption along with the ability of this mixture to obtain a minimum surface tension was tested using a pulsating bubble apparatus.

Sera and sputum samples were collected serially from patients in an ICU, and sera was also be collected from healthy volunteers. These samples were assayed to determine the CRP and total protein levels. Values are expressed as CRP as a percent of total protein and were reviewed in relation to severity of lung dysfunction associated with ARDS and the APACHE II score of patients in the ICU.

Materials and Methods

Pulmonary Surfactant Isolation

Surfactant which was used in these experiments was isolated from porcine lungs which were obtained from the abattoir of Newfoundland Farm Products, St. John's. The lavage procedure was employed as a method to obtain initial material for surfactant isolation.

Only lungs which did not appear bloody, were without areas of extensive haemorrhage and did not contain large lacerations, were lavaged. The lungs from freshly slaughtered pigs were packed in ice at the abattoir so that the tracheas were extended above the ice to minimize contamination of the inner surface of the lung by blood or melting ice. In the laboratory, the lungs were removed from the ice and the external surfaces were washed with cold water. Any accompanying tissue or debris around the tracheas or lungs was removed to facilitate handling of the lungs and lavage extraction.

The lavage procedure consisted of first inserting a Tygon tube into the trachea so that one end of the tube was near the bronchial junction. This was secured by a nylon cable tie which was placed around the trachea. The lungs were distended with 0.15 M NaCl at 4°C until the surface became firm, and the lungs were gently massaged. This process was intended to facilitate suspension of surfactant in the saline solution in the lung. Any minor cuts on the surface of the lungs were pinched between the fingers. The lavage was extracted through the Tygon tube which was connected to a reservoir and a water aspirator via a three way valve. Lavage was collected in the reservoir which was surrounded with ice. Lavage from each pair of lungs was handled separately to minimize the chance of contamination of lavage fluid from one lung with potentially inhibitory material of another. Also, lavage fluid which appeared red or "bloody" in colour, was

discarded.

The lavage fluid was processed by a modification of the method of King and Clements (1972) (appendix 1). This procedure consisted of four centrifugations, the third being in sodium bromide. It was noted that with each centrifugation, the surfactant material became whiter in colour and it was assumed that contaminating material such as blood proteins and cells or components thereof were removed from the surfactant rich material.

The phospholipid content of the surfactant was calculated from the phosphorus content which was obtained by a modification of the method of Bartlett (1959), as described by Keough and Kariel (1987) (appendix 2). Surfactant protein content was determined by a modification of the method of Lowry et al. (1951) (appendix 3).

Preparation of Surfactant for Interfacial Property Measurement

An aliquot of surfactant was removed from storage at -70°C and was equilibrated at room temperature. It was briefly vortexed (5 seconds duration), sonicated and vortexed again. Sonication consisted of two sets of 10 short bursts (0.5-1.0 seconds duration) at a power of 30 Watts using a Branson sonifier which was equipped with a tapered microtip. The suspension was cooled on ice for two minutes between the two sets of sonication bursts. The surfactant was diluted in 0.15 M NaCl, in the presence or absence of 0.005 M $CaCl_2$, briefly vortexed and incubated overnight at 4°C.

Preliminary experiments were conducted to determine which method used for final stages of surfactant preparation prior to its use in experiments was most conducive to obtaining consistent results. After the overnight incubation, the surfactant was vortexed thoroughly (20-30

seconds) and the surface behaviour was tested using the surface adsorption apparatus and the pulsating bubble surfactometer. Consistent results could not be obtained with identical surfactant preparations when they were finally mixed only by vortexing, nor did water bath sonication (Bransonic 220) for 2 minutes yield consistent surface behaviour. It was assumed that the surfactant suspensions were not homogeneous with respect to the distribution of surfactant or certain components thereof and that this produced inconsistent results. Therefore, it was necessary to probe sonicate the surfactant in the manner described above prior to the first experimental test of the day and before every third experimental test thereafter.

Lipid extracts of surfactant were prepared by the method of Bligh and Dyer (1959). An aliquot of surfactant was removed from storage at -70°C, equilibrated to room temperature, vortexed, probe sonicated and vortexed in the manner described above. A volume of surfactant was chosen for lipid extraction such that the amount of lipid extract surfactant which was produced was sufficient for approximately 30 experimental tests. This volume was designated as 0.8 volume. To this, 1.0 volume of chloroform and 2.0 volumes of methanol were added and the phases were vigorously mixed. Another 1.0 volume of chloroform and 1.0 volume of water were added, the phases were mixed and allowed to separate. The top aqueous layer was removed and discarded and the remaining organic layer, which contained the hydrophobic surfactant lipids and hydrophobic proteins was evaporated to dryness under a stream of nitrogen. This surfactant was suspended by vortexing in a small volume of 0.15 M NaCl so that the concentration of the lipid extract surfactant was similar to that of the natural surfactant. It was immediately stored at -70°C with an aliquot being assayed for phosphorus content by (Keough & Kariel, 1987) (appendix 2) and protein concentration by a modification of the method of Lowry et al. (1951) (appendix 3).

Preparation of Plasma Proteins and CRP Ligands

Human CRP, from ascites fluid, was obtained in a solution composed of the following (M): NaCl, 0.15; $CaCl_2$, 0.002; tris, 0.020; pH=7.5, in 0.1% NaN_3. According to the supplier (Calbiochem, La Jolla, CA), the CRP was 95% pure by SDS-PAGE. This purity was confirmed by SDS-PAGE. The concentrations of the CRP solutions which were mixed with surfactant were determined by an ELISA (appendix 4).

When necessary, calcium was removed from the CRP solution using Centricon 3 microconcentrators (W.R. Grace & Co., Beverly, MA). This procedure consisted of filling the top of a concentrator chamber with CRP solution, to which EGTA had been added for a final EGTA concentration of 0.002 M. The top and bottom chambers of the concentrator were separated by a membrane with a 3000 Da exclusion limit. This apparatus, containing the CRP solution was centrifuged for thirty minutes at 3020 x g_{av}. The top chamber was refilled with the same solution, save the CRP, and centrifuged and this procedure was repeated. EGTA was subsequently removed by three thirty-minute centrifugations, as described above, in the presence of 0.15 M NaCl and 0.005 M tris-HCl, pH=7.4. The concentration of the resulting CRP solution was determined by an ELISA (appendix 4).

To prepare stock solutions of human fibrinogen and C1q, $CaCl_2$ was added to lyophilized fibrinogen (type I, fraction III, ~70% clottable protein, 12% Na citrate, 18% NaCl) and to lyophilized human complement component C1q (0.5 M NaCl, 0.05 M tris, pH=7.3) so that the calcium was present at a final concentration of 0.005 M. Solutions of human albumin (essentially fatty acid free, fraction V) and globulin (Cohn Fraction IV) were prepared by the addition of NaCl

and $CaCl_2$ in doubly distilled water so that the final concentration of these ions was 0.15 M and 0.005 M, respectively. These proteins were solubilized by gentle shaking and were stored in aliquots at -20° C. An aliquot of each solution was used to assay the protein concentration by a modification of the method of Lowry et al. (1951) (appendix 3).

The water soluble CRP ligands were dissolved in similar solutions as that of the plasma proteins and surfactant mixtures. The concentration of ligand was determined from the weight of ligand which was added.

Surfactant Adsorption in a Stirred Subphase

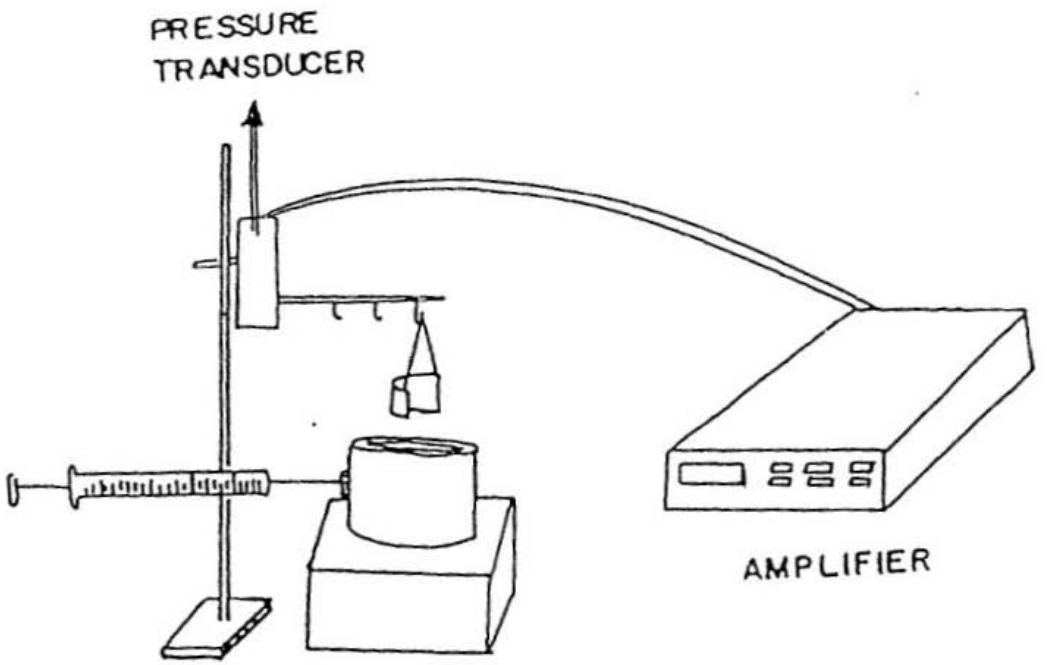
Dispersions of whole and lipid extract surfactant were mixed with solutions of protein or protein + ligand so that the concentration of surfactant in each experiment remained constant. In control dispersions, identical concentrations of surfactant alone or mixtures of surfactant + protein were mixed with buffer solution to achieve final concentrations which were identical to their respective test mixtures of surfactant + protein, or surfactant + protein + ligand. Solutions of CRP, fibrinogen or globulin were added to the surfactant suspension immediately subsequent to sonication. Solutions of C1q or albumin were added to the lipid extract surfactant in a similar fashion. Surfactant suspensions, and mixtures of surfactant + protein, and surfactant + protein + ligand were briefly vortexed (5 seconds) and incubated at room temperature for two minutes prior to measurement of their interfacial properties. Surfactant was diluted such that when it was mixed with volumes of solutions which may or may not have contained plasma proteins or plasma protein + ligand, consistent concentrations of each of the components were obtained at similar volumes. Surfactant concentrations are reported as phospholipid concentrations by multiplying

the phosphorus concentration by 25.

Surface tension versus time adsorption experiments were performed using an adsorption apparatus which was similar to that of King and Clements (1972) as previously described by Perez-Gil et al. (1990), (figure 2). Briefly, ~120 μl of surfactant or an equal volume of mixtures of surfactant + protein, or surfactant + protein + ligand were injected slowly (requiring a time period of ~5 seconds) into 5 ml of stirred subphase which was contained in a Teflon beaker. The subphase, which contained 0.15 M NaCl with or without 0.005 M $CaCl_2$, was stirred continuously. Subphase temperature was maintained at 37°C ± 2°C. Surface tension, measured with a platinum dipping plate that had been roughened by scratching with emery paper, was monitored as a function of time. With the platinum dipping plate suspended above the subphase, the surface adsorption apparatus was zeroed according to the operating instructions of the TSAR-1 computerized transducer readout (Torrance, CA). The flag was submerged into the subphase and raised so the that only the bottom 1/4 was submerged. This process was repeated before every experimental test.

The amount of surfactant used in these experiments was chosen as the amount of surfactant which, in the presence of 0.005 M $CaCl_2$, adsorbed to the air-water interface rapidly enough so that it reduced the interfacial surface tension to 25 ± 3 $mM \cdot m^{-1}$ within 5 minutes. In the absence of calcium, the amount of surfactant used was that which reduced the surface tension to the same value within 20 minutes. Surfactant adsorption in the presence of calcium is poor. Therefore, the amount of surfactant required to meet these conditions was greater than that used for the adsorption of surfactant in the presence of calcium. If the criterion for an equilibrium spreading pressure of ~25 $mN \cdot m^{-1}$ in 5 minutes were to be achieved in the absence of calcium, a very large

Figure 2. Diagram of a surface adsorption apparatus.


PRESSURE
TRANSDUCER
AMPLIFIER

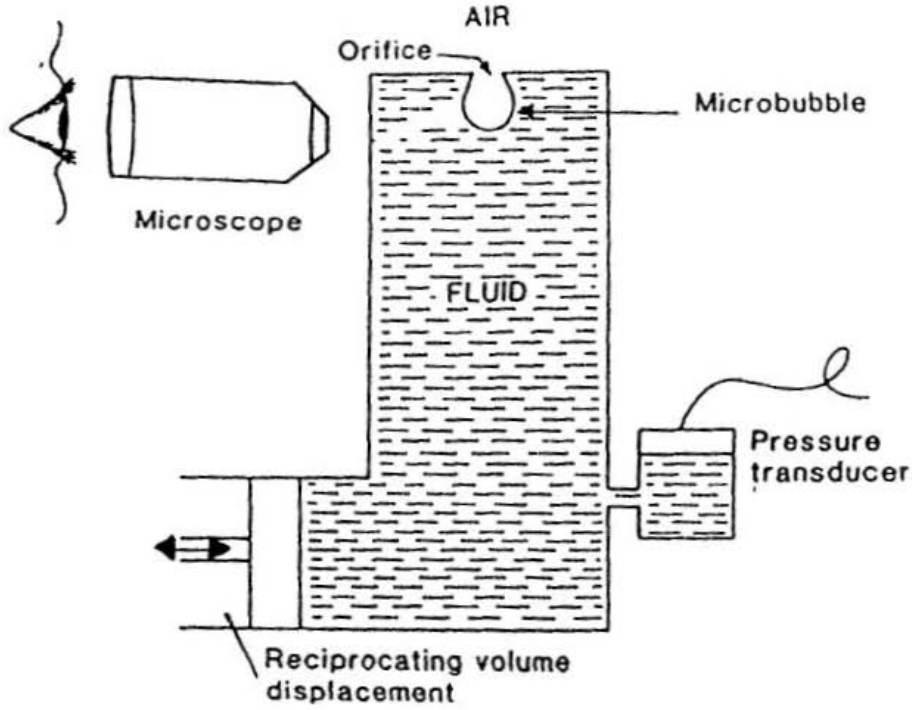
amount of surfactant and a unreasonably costly amount of CRP would be required for the experiments. Results are expressed as surface tension versus time and symbols represent mean ± 1 SD.

Surfactant Properties in a Pulsating Bubble Surfactometer

Dispersions of surfactant were prepared in an identical manner for use with the pulsating bubble surfactometer (Enhorning, 1977) as were those which were tested using the surface adsorption apparatus. Lipid extract surfactant was mixed with the C1q or albumin solutions such that the concentration of surfactant remained constant and C1q was present at either 5% or 10% and albumin was present at 10% of the phospholipid content, by weight. Control mixtures were prepared from whole or lipid extract surfactant were mixed with solutions of saline and calcium to achieve the same surfactant concentration as that of the test mixtures and final concentrations of 0.15 M NaCl and 0.005 M $CaCl_2$.

Two phenomena can be studied using the pulsating bubble surfactometer (figure 3). The pulsating bubble surfactometer can be operated in a pulsating mode. The principle of the pulsating bubble surfactometer, in this mode, is to record the pressure which is required to form a bubble which is pulsated between maximum (0.55 mm) and minimum bubble radii (0.40 mm). This pressure is equal to the pressure difference ($\triangle P$) across the bubble interface. The pressure difference is related to the surface tension by the LaPlace Law, $\triangle P = 2\gamma/r$ (r is the bubble radius), so that, in the absence of surfactant, where the surface tension is constant, the highest pressure difference occurs when the bubble is at its smallest size, $\triangle P_{min}$. The presence of well-functioning

Figure 3. Diagram of a pulsating bubble surfactometer , after Enhorning, 1977.


AIR
Orifice
Microbubble
Microscope
FLUID
Pressure
transducer
Reciprocating volume
displacement

pulmonary surfactant causes the surface tension to be very low on compression to a small surface area (small bubble radius) and, thus "inverts" the behavior of ΔP so that ΔP is lowest at minimum bubble size. The pulsating bubble surfactometer provides a mechanical model of an alveolus wherein the bubble, like an alveolus, undergoes cyclic compression and expansion. Therefore, the effect of inhibitors of surfactant adsorption can be investigated simultaneously with their effect on surface refining of surfactant at the air-water interface. Surface adsorption alone can be monitored when a bubble is formed and it is not pulsated. In this respect, the pulsating bubble surfactometer is similar to the surface adsorption apparatus, however, since surface tension (related to ΔP) is not measured with a dipping plate the possibility of contact-angle artifact is avoided (Hills, 1984). The complication of such studies is that the subphase is not stirred and there may be diffusion gradients influencing the properties of surfactant adsorption. Pressure difference across the bubble interface was used to express the data instead of surface tension because is was not assumed that the bubbles always had a spherical shape, especially when they have very low surface tensions.

Whole surfactant, lipid extract surfactant and mixtures of lipid extract surfactant + 5% and 10% C1q and 10% albumin, by weight, were assayed for surface activity according to the procedures outlined for use of the pulsating bubble surfactometer, (appendix 5). The samples were assayed for 180 pulses or until the pressure difference across the bubble interface at minimum bubble radius became approximately zero. Results are expressed as pressure difference across the bubble interface at maximum and minimum bubble radii versus pulse number, and symbols represent means $\pm$ 1 SD.

Collection and Preparation of Serum and Sputum

Blood and sputum samples were obtained as discards from routine management of patients in the Intensive Care Unit of the General Hospital, St. John's, Newfoundland under the supervision of Dr. S. Peters. An application to the Human Investigations Committee, Memorial University of Newfoundland-Faculty of Medicine was submitted on December 14, 1989, application # 798 titled "Protein Inhibition of Pulmonary Surfactant in Airway Secretion in Sera of Patients Requiring Mechanical Ventilation." Full approval was granted on January 11, 1991 from the viewpoint of ethics as defined in terms of reference of the faculty committee. Blood and sputum samples were obtained as discards during routine procedures and personal identification was not used to identify the samples, therefore, informed consent for the samples was deemed not necessary by the committee.

Blood samples were collected from patients via an arterial catheter. The procedure is outlined in The General Hospital Nursing Unit Departmental Manual Procedure for Arterial Catheter-Blood Sampling, Number III-20, revised 1991-02. Sputum samples were obtained according to The General Hospital Nursing Unit Departmental Manual Procedure for the Collection of Sputum by Tracheal Aspiration in a Patient with a Tracheostomy, Number IV-h-20, revised 1990-02. Blood was also collected from the brachial artery of healthy volunteers by a qualified technician.

Blood, sputum and samples were labelled with a patient number, a sample number, the date on which the samples were collected, and the sample type. Sample labels enabled the correlation of the level of lung dysfunction and the APACHE II index of a patient with CRP expressed as a percentage of total protein for each sample. Before and during the time at which

the samples were assayed no details of the physiological status of the patient were discussed. All samples were refrigerated immediately after collection, and blood was allowed to clot at 4°C. Blood and sputum were centrifuged in a bench top centrifuge at maximum velocity for 5 minutes at room temperature. Serum and the fluid portion of the sputum (which was able to be displaced by a 200 μl autopippette) were divided into small aliquots, labelled and frozen at -20°C.

Blood and sputum were collected from the time that mechanical ventilation was required until it was no longer necessary or the patient expired. Blood samples were also collected from the index finger of normal healthy volunteers using the Glucolet 2 automatic lancing device (Elkhart, IN) and Fingerstix lancets (Elkhart, IN). No distinction was made between blood obtained by this method or via the brachial artery. Sera which was obtained from capillary blood was handled in an identical manner as that from patients in the ICU. The CRP concentration of these samples was determined by an ELISA (appendix 4) and total protein concentration was determined by a modification of the method of Lowry et al. (1951) (appendix 3).

CRP-depleted serum from a patient in the ICU was produce by phosphocholine-sepharose (PC-sepharose) affinity column chromatography. The PC-sepharose was obtained from Sigma Chemical (St. Louis, MO). Prior to use, the column, which contained ~20 ml of PC-sepharose was washed by eluting 500 ml of buffer solution, 0.15 M NaCl; 0.01 M tris-base; 0.01 M tris-HCl; 0.002 M $CaCl_2$; pH=7.5 which contained 0.002 M phosphocholine (Cl^-). This ensured that any residual CRP or other contaminants which may have been retained on the column were removed. The optical density of the eluted buffer solution at 280 nm at the beginning and at the end of the PC-wash was 0.00. To remove non-solid phase phosphocholine, the column was washed with 500 ml of the same buffer solution as above which did not contain phosphocholine.

Again the optical density at 280 nm before and after the second wash was 0.00. Approximately 2.0 ml of serum was loaded on the column and eluted with buffer solution which did not contain phosphocholine. The dilution of this serum as a result of the column chromatography was not determined, however, an effort was made to minimize the dilution by using only those fractions which appeared straw-coloured, presumably containing the eluted serum. To an aliquot of this serum, a minimum volume of concentrated diluent was added so that the serum would contain similar amounts of diluent components as test sera. Also, a known concentration of CRP in a minimal volume of concentrated diluent was added to another aliquot. A maximum dilution of 15 has been calculated for this serum. The actual final dilution was most likely lower than this. A known concentration of CRP was added to serum from another patient in the ICU which had not been exposed to phosphocholine-affinity column chromatography and assayed by the ELISA (appendix 4). Human serum with a known amount of CRP (Lot No. 191232) was purchased form Behring Diagnostics (Marburg, Germany). The CRP content of these sera were assayed by ELISA (appendix 4). The CRP concentration of the standard which was used in the ELISA and that which was added to two sera samples was determined by the method described Lowry et al. (1951) (appendix 2).

Statistical Analysis of CRP Concentrations Versus Illness

Sera samples obtained from 19 patients in the ICU were analyzed to determine if a correlation existed between CRP expressed as % total protein in the sera and APACHE II scores. A Pearson correlation coefficient was calculated for these data. The Pearson correlation coefficient is a measure of the degree to which two variables are correlated.

ICU patients were also classified into two groups; those at risk of developing ARDS; and those who developed ARDS. Patients were defined as being at risk of developing ARDS or having ARDS if they required mechanical ventilation for more than 48 h and had conditions associated with an increased risk of ARDS or indicative of this syndrome, such as those stated in the introduction. These data are expressed as serum %CRP/total protein and as serum CRP (μg/ml). Wilcoxon rank sum tests was used to analyze these data. This method of statistical analysis is the non-parametric analogue of the two sample *t*-test which is suitable for use with small sample sizes and employs random sampling of two independent populations.

Many of the sputum samples contained solid material which was likely composed of clotted blood and tissue. Often, upon centrifugation, this material did not separate from the less viscous portion of the sputum. Therefore, too few sputum samples were assayed to be appropriate for statistical analysis.

Results

The Effect of C-reactive Protein on the Adsorption and Surface Tension Lowering Ability of Pulmonary Surfactant

Before the effect of plasma proteins on porcine surfactant function were investigated, using either the pulsating bubble surfactometer or the surface adsorption apparatus, control dispersions of surfactant alone were tested to determine the surface behaviour of each surfactant preparation. This allowed the selection of surfactant preparations which possessed the physical properties which are considered to be associated with normal functioning pulmonary surfactant, i.e. rapid adsorption and the ability to lower surface tension to near zero upon compression. Therefore, by selecting surfactant preparations which behaved in this manner, it is likely that they will more closely resemble the normal *in vivo* surfactant system of the lung. Employing isolated surfactant preparations which functioned well also enabled the use of lower surfactant concentrations, thereby sparing the surfactant preparations and proteins and ligands which were added to the surfactant and therefore minimizing time and expense.

The concentration of surfactant used with the surface adsorption apparatus was deliberately chosen so as to obtain a measurable rate of change in surface tension with time which was not " saturating" or " instantaneous" as is sometimes used in such adsorption experiments. Similarly, the concentration of surfactant used in experiments with the pulsating bubble surfactometer was also chosen in the same manner as not to be "saturating" with respect to the rate of change in surface tension with time during dynamic compression and expansion of the surfactant film. This allowed any positive or negative influences of proteins or other additives on the rate of adsorption

alone on the rate of adsorption during dynamic compression and expansion to be easily observable.

Figure 4 shows that when porcine surfactant at 0.781 $\mu g \cdot ml^{-1}$ of phospholipid was injected in the subphase which contained 0.15 M NaCl and 0.005 M $CaCl_2$, even 25 minutes after injection, the surface tension of the subphase was reduced by only 2 $mN \cdot m^{-1}$. A higher concentration of surfactant (16.4 $\mu g \cdot ml^{-1}$) reduced the subphase surface tension to 33 $mN \cdot m^{-1}$ in 25 minutes. These amounts of surfactant were inadequate to allow a sufficiently rapid rate of adsorption. An aliquot of surfactant at 36.7 $\mu g \cdot ml^{-1}$ reduced the subphase surface tension to 30 $mN \cdot m^{-1}$ in less than 1 minute. Within 5 minutes after injection, that concentration of surfactant lowered the surface tension of the saline and calcium subphase to near 25 $mN \cdot m^{-1}$. This limit is in keeping with the equilibrium spreading pressure of phospholipids ie. $\pi \sim$ 45-48 $mN \cdot m^{-1}$. Surfactant at this concentration, while having a sufficiently rapid adsorption rate, was "saturating" with respect to the subphase concentration because a lower concentration of surfactant (27.5 $\mu g \cdot ml^{-1}$) had an initial adsorption rate which was almost a rapid and also approached equilibrium spreading pressure in 5 minutes. Therefore, a surfactant phospholipid concentration of 27.5 $\mu g \cdot ml^{-1}$ was used in studies with the surface adsorption apparatus.

Effect of C-reactive Protein on Pulmonary Surfactant Adsorption

The effect of CRP on the rate of adsorption of porcine pulmonary surfactant was investigated (figure 5). Within 5 minutes after injection, surfactant alone lowered the surface tension of the saline and calcium subphase to 25 $mN \cdot m^{-1}$. When the surfactant was mixed with CRP and injected into the subphase, however, the adsorption rate was substantially decreased, so

Figure 4. Change in surface tension with time after injection of four different concentrations of porcine surfactant into a stirred subphase. ●, Porcine surfactant (0.781 $\mu g \cdot ml^{-1}$ PL, n=3); ▽, (16.4 $\mu g \cdot ml^{-1}$ PL, n=3); ▾, (27.5 $\mu g \cdot ml^{-1}$ PL, n=14); ○, (36.7 $\mu g \cdot ml^{-1}$ PL, n=3). Subphase and incubation mixtures contained 0.15 M NaCl and 0.005 M $CaCl_2$. In this and subsequent figures which express data which was obtained using the surface adsorption apparatus or the pulsating bubble surfactometer, concentrations are expressed as final subphase concentrations. Values are expressed as mean ± 1 SD; standard deviations are shown for every second measurement are not shown when they fall within the range denoted by the symbols.

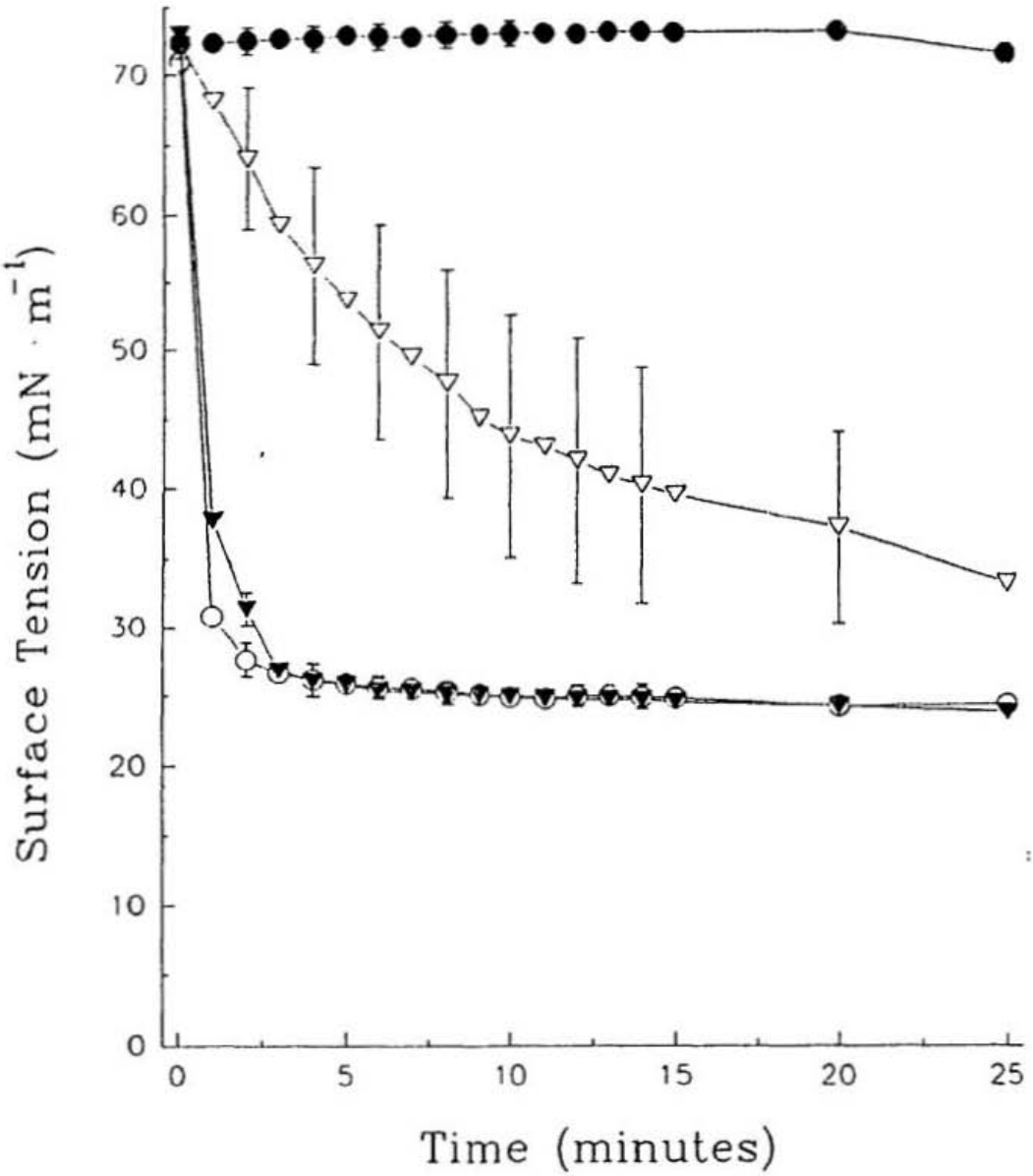
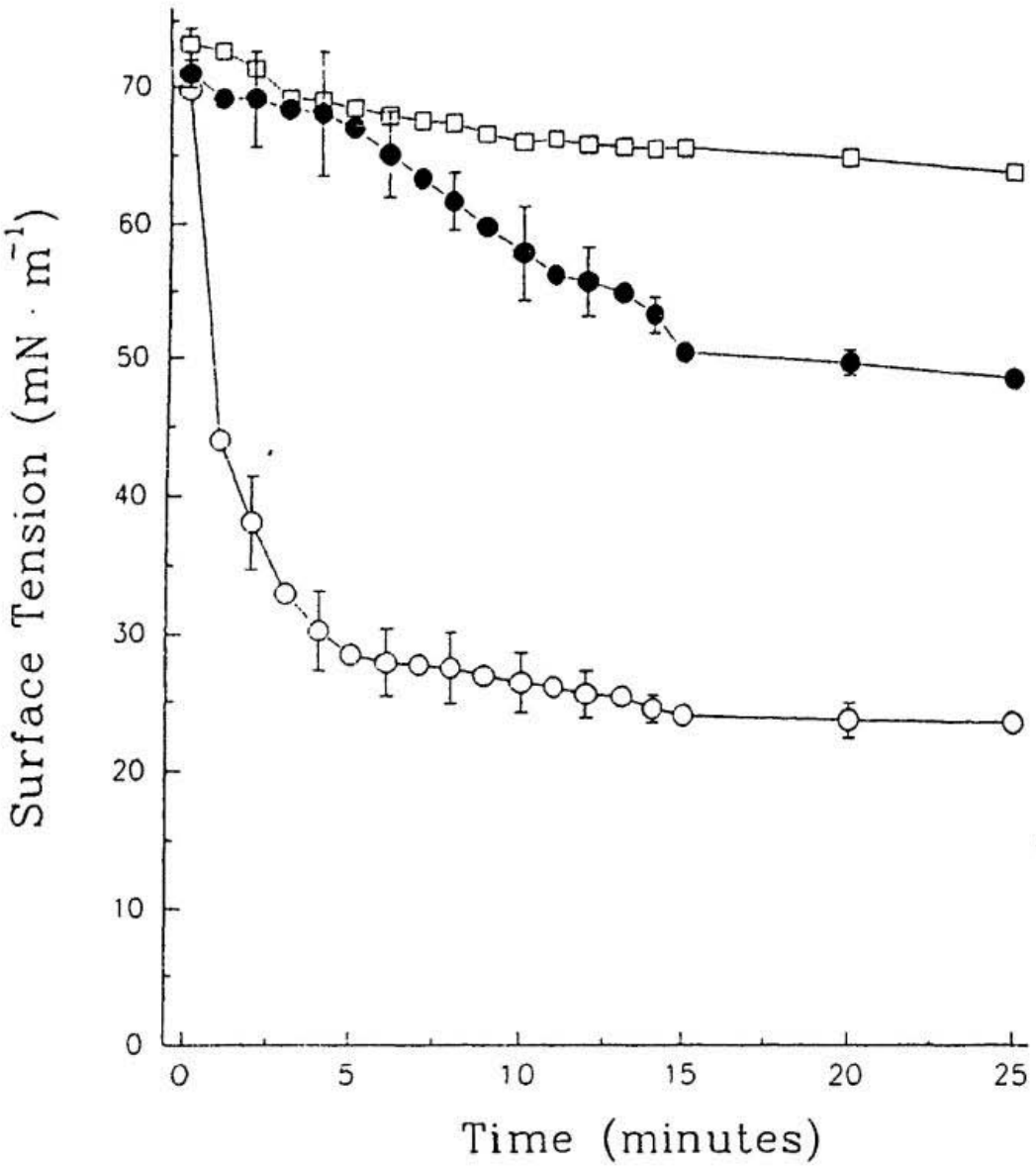

Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

Figure 5. Change in surface tension with time after injection of porcine surfactant, CRP and a mixture of CRP + porcine surfactant into a stirred subphase. ○, Porcine surfactant (39.0 $\mu g \cdot ml^{-1}$ PL, n=22); □, CRP (19.5 $\mu g \cdot ml^{-1}$, n=3); ●, CRP + porcine surfactant (19.5 $\mu g \cdot ml^{-1}$ CRP : 39.0 $\mu g \cdot ml^{-1}$ PL, n=3). Subphase, surfactant and CRP solutions contained of 0.15 M NaCl and 0.005 M $CaCl_2$. Since the variability in the rate of adsorption of surfactant alone between different sets of experiments was small the mean and standard deviations for control surfactant mixtures were calculated for entire data set obtained from the controls in the series of experiments shown in figures 5, 6, 7, 10, 12, and 13.


Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

that at 25 minutes after injection, the surface tension was reduced to only 50 mN · m^{-1}. The adsorption of an amount of CRP alone, which was equivalent to that used in the test conditions, was slow under these conditions since it did not contribute substantially to the lowering of interfacial surface tension in the interface. The variability in the rate of adsorption of surfactant alone between different sets of experiments was small. The mean and SD for the adsorption of control surfactant mixtures which were used in each experiment were calculated for the entire data set obtained from the control for a number of experiments and the averaged values are reported in figures 5, 6, 7, 10, 12, 13.

The inhibitory effect of CRP on the adsorption rate of porcine surfactant, in the presence of calcium, was compared to that of two concentrations of human fibrinogen (figure 6). It is evident that CRP inhibited the adsorption of surfactant more extensively than an identical weight of fibrinogen. In fact, for fibrinogen to approach the inhibitory capacity of CRP, it was necessary for fibrinogen to be present at six times the concentration at which CRP was present. The molecular weight of CRP is ~110 000 and that of fibrinogen is ~340 000. Therefore, on a molar basis it required at least twice as much fibrinogen to approach the inhibitory capacity of CRP with respect to surfactant adsorption. Subphase injections of fibrinogen alone at final concentrations of 19.5 and 117.0 $\mu g \cdot ml^{-1}$ decreased the surface tension of the subphase, 25 minutes after injection, by ~5 mN · m^{-1} and ~12 mN · m^{-1}, respectively.

The inhibitory effect of CRP on porcine surfactant adsorption was compared to that of globulin (figure 7). Globulin, when present with surfactant at twice the weight at which CRP was present, produced a substantial inhibitory effect on surfactant adsorption to the air-water interface. This effect of globulin, however, was not as potent as that of CRP. Figure 7 also shows that in

Figure 6. Effect of CRP and the effect of fibrinogen on the rate of adsorption of porcine surfactant. —, Porcine surfactant (39.0 $\mu g \cdot ml^{-1}$ PL, n=22); ■, fibrinogen (19.5 $\mu g \cdot ml^{-1}$, n=3); ▵, fibrinogen (117.0 $\mu g \cdot ml^{-1}$, n=3); ●, CRP + porcine surfactant (19.5 $\mu g \cdot ml^{-1}$ CRP : 39.0 $\mu g \cdot ml^{-1}$ PL, n=3); ▾, fibrinogen + porcine surfactant (19.5 $\mu g \cdot ml^{-1}$ fib. : 39.0 $\mu g \cdot ml^{-1}$ PL, n=3); ▿, fibrinogen + surfactant (177.0 $\mu g \cdot ml^{-1}$ fib. : 39.0 $\mu g \cdot ml^{-1}$ PL, n=3). Subphase and incubation suspensions of surfactant and surfactant + inhibitors used for injection into the subphase contained 0.15 M NaCl and 0.005 M $CaCl_2$.

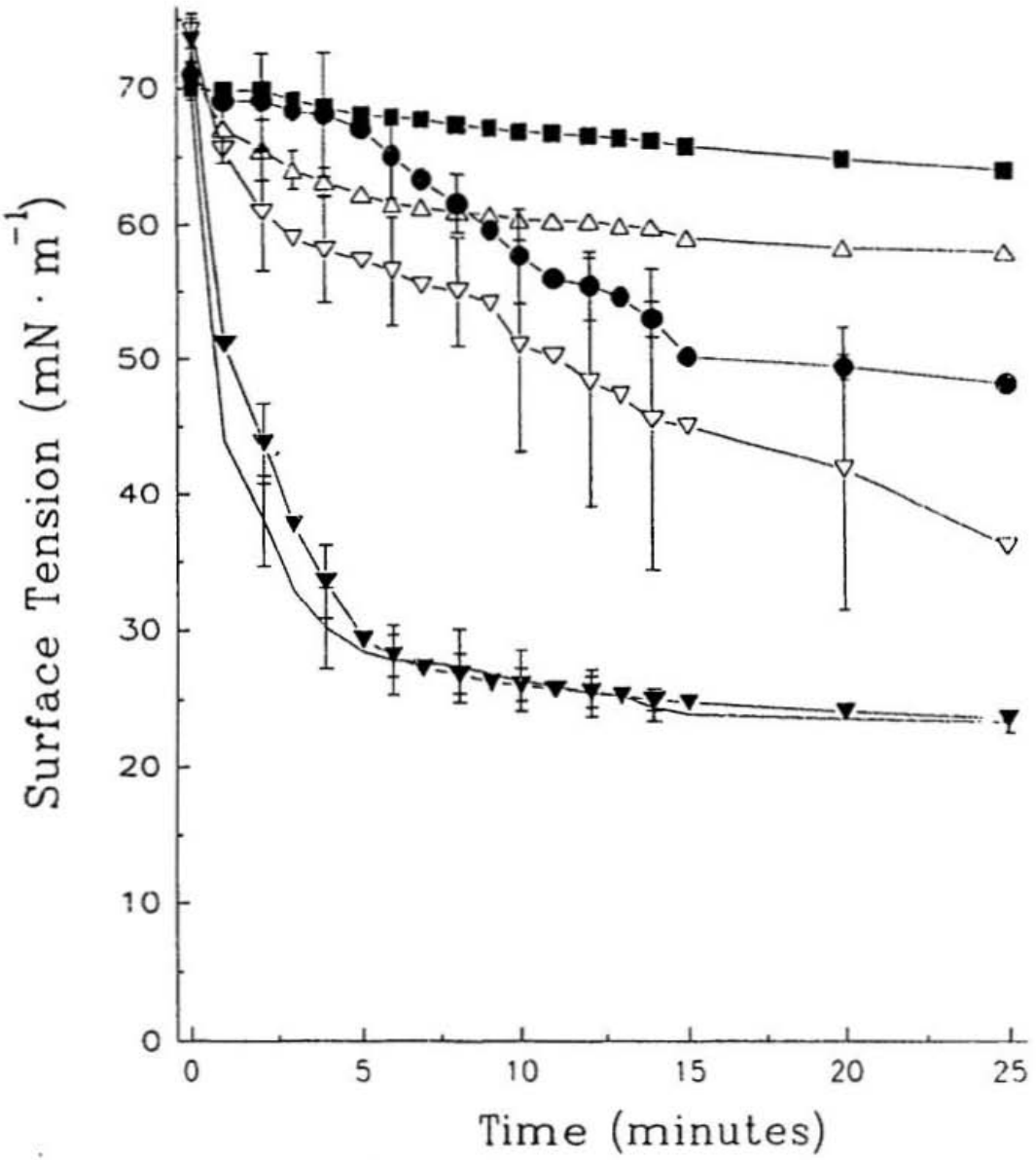
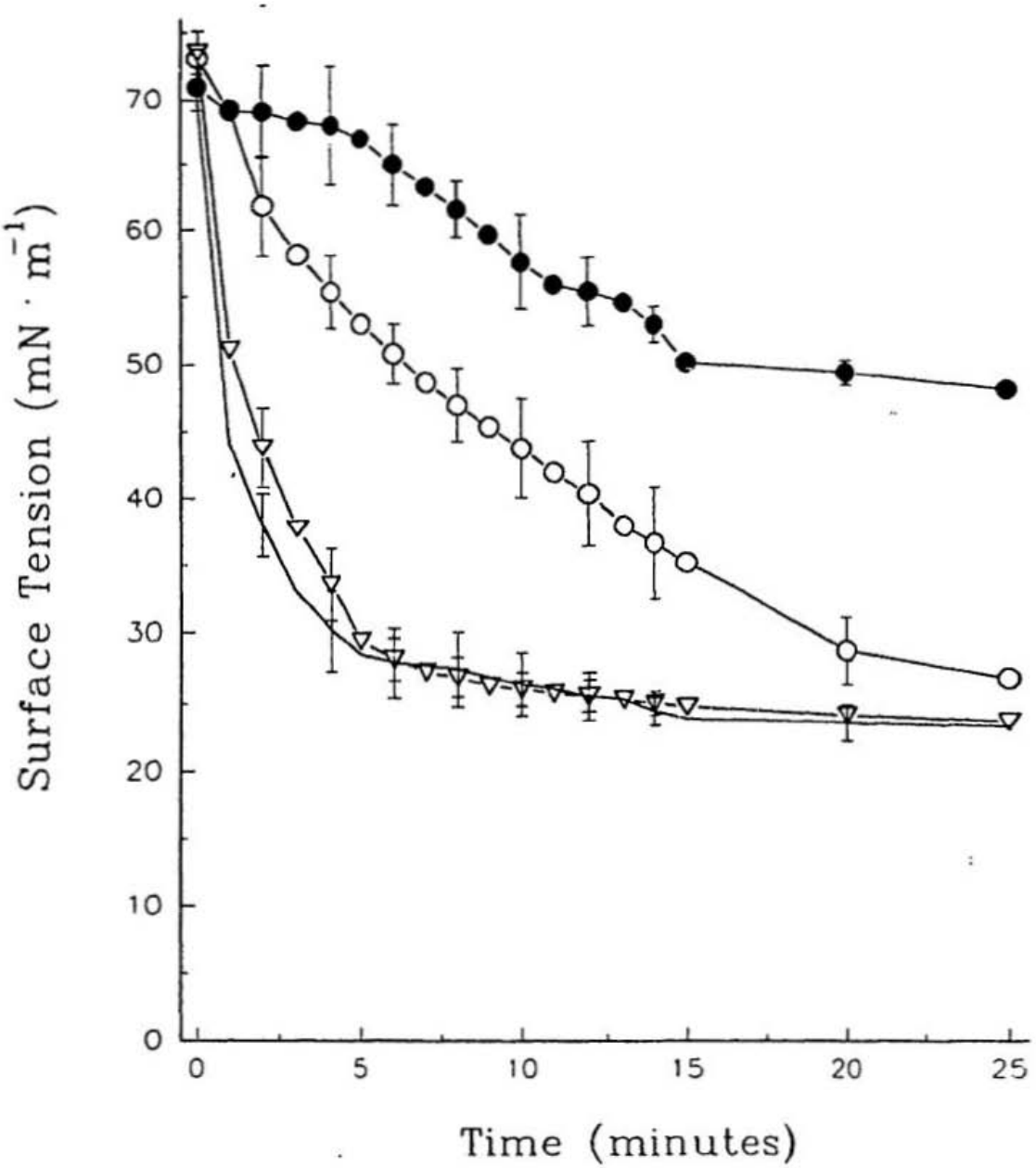

Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

Figure 7. Effect of CRP and globulin on the adsorption rate of porcine surfactant. -, Porcine surfactant (39.0 $\mu g \cdot ml^{-1}$ PL, n=22); ●, CRP + porcine surfactant (19.5 $\mu g \cdot ml^{-1}$ CRP : 39.0 $\mu g \cdot ml^{-1}$ PL, n=3); ▽, globulin + porcine surfactant (39.0 $\mu g \cdot ml^{-1}$ globulin : 39.0 $\mu g \cdot ml^{-1}$ PL, n=3); ○, globulin (390 $\mu g \cdot ml^{-1}$, n=3). Subphase and incubation mixtures contained 0.15 M NaCl and 0.005 M $CaCl_2$.


Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

the absence of surfactant, subphase injections of globulin (39.0 $\mu g \cdot ml^{-1}$) considerably reduced surface tension by 23 $mN \cdot m^{-1}$ over a 25 minute time period.

Effect of Calcium on the Inhibition of Pulmonary Surfactant Adsorption by C-reactive Protein

The effect of calcium on the inhibition of surfactant adsorption by CRP was investigated and the results are shown in Figure 8. In the absence of added calcium, the rate of adsorption of surfactant was lower than in its presence. CRP had only a small effect on the rate of surfactant adsorption (compare figures 5 and 8). Therefore, calcium was necessary for the inhibitory effect of CRP on surfactant adsorption.

Effect of Calcium on the Inhibition of Pulmonary Surfactant Adsorption by Fibrinogen

It has previously been shown that the proteolytic cleavage of fibrinogen into fibrin monomers is calcium dependent and Seeger et al. (1993) have shown that polymerizing desAABB-fibrin, induced by thrombin, associates with surfactant phospholipids. Therefore, calcium was omitted from the subphase and from suspensions of porcine surfactant, porcine surfactant + fibrinogen, and fibrinogen solution (figure 9). This ensured that cleavage of fibrinogen into fibrin monomers did not occur in the presence of residual thrombin which may have been retained during the isolation of fibrinogen. In the absence of added calcium, fibrinogen, when present with surfactant at an identical weight at which CRP was present in Figure 8, did not inhibit the adsorption of surfactant. This was a much lower concentration of fibrinogen than that which inhibited surfactant adsorption in the above fibrinogen experiment in which calcium was present.

Figure 8. Adsorption of porcine surfactant and a mixture of porcine surfactant + CRP in the absence of calcium. ○, Porcine surfactant (78.1 $\mu g \cdot ml^{-1}$ PL, n=5); ●, CRP + porcine surfactant (39.0 $\mu g \cdot ml^{-1}$ CRP : 78.1 $\mu g \cdot ml^{-1}$ PL, n=3). Subphase and incubation suspensions and solutions contained 0.15 M NaCl.

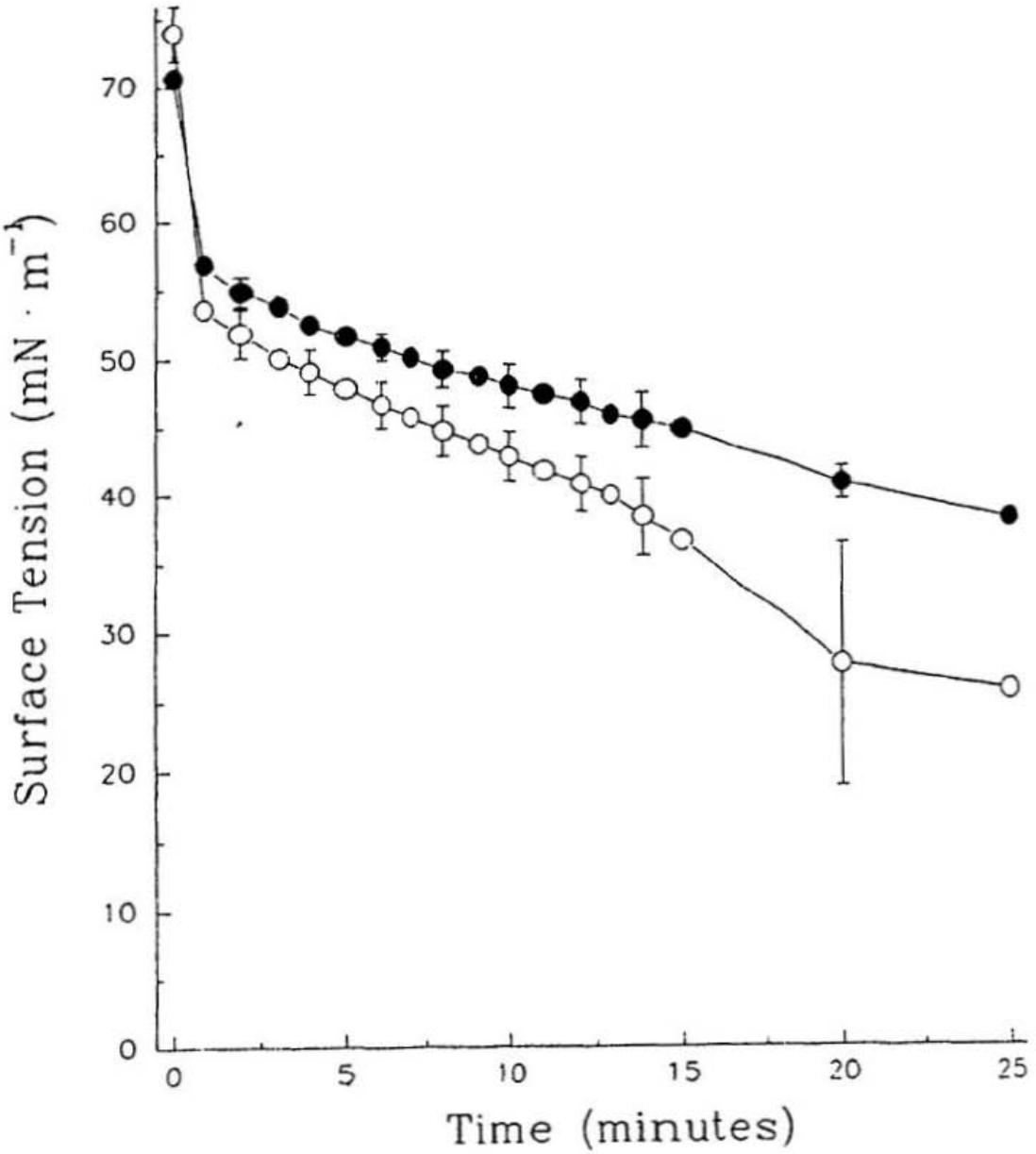
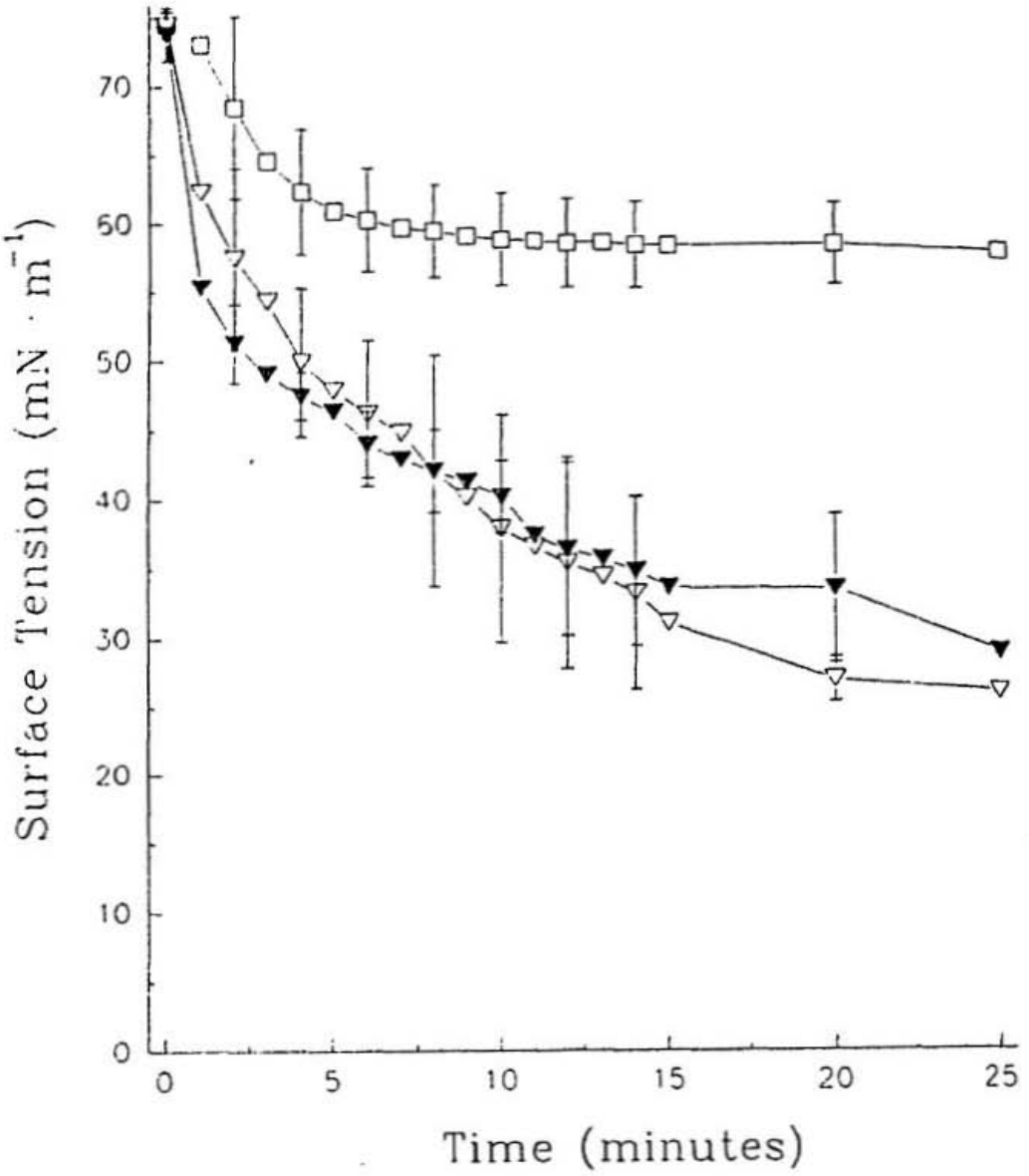

Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

Figure 9. Adsorption of porcine surfactant, fibrinogen and a mixture of fibrinogen + porcine surfactant in the absence of calcium. •, Porcine surfactant (58.6 $\mu g \cdot ml^{-1}$ PL., n=5); □, fibrinogen (29.3 $\mu g \cdot ml^{-1}$, n=5); ▽, fibrinogen + porcine surfactant (29.3 $\mu g \cdot ml^{-1}$ fib. : 58.6 $\mu g \cdot ml^{-1}$ PL, n=5). Subphase and incubation mixtures contained 0.15 M NaCl.


Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

This figure also shows that, in the absence of added calcium, fibrinogen alone reduced the subphase surface tension by 15 $mN \cdot m^{-1}$, 25 minutes after injection.

Effect of Three C-reactive Protein Ligands, Phosphocholine, DL-∝-Glycerophosphate, and o-Phosphoethanolamine on the Inhibition of Pulmonary Surfactant Adsorption by C-reactive Protein

To better understand the nature of the inhibition of surfactant adsorption by CRP, surfactant was mixed with CRP at the same weight ratios as those in Figure 5. Three different concentrations of the water-soluble CRP ligand phosphocholine, which is the headgroup of the most abundant surfactant lipid, phosphatidylcholine, were added to the CRP + surfactant mixtures (figure 10). The addition of phosphocholine to the CRP + surfactant mixture at a phosphocholine : CRP molar ratio of 300 : 1, almost completely eliminated the inhibitory effect of CRP on surfactant adsorption. Addition of phosphocholine at the lower molar ratios of phosphocholine : CRP of 225 : 1, and 150 : 1, did not decreased the inhibitory capacity of CRP on surfactant adsorption as effectively as phosphocholine at a 300 : 1 ratio. A comparison of the effect of adding three different concentrations of phosphocholine to CRP + surfactant mixtures reveals that phosphocholine reduced the inhibitory effect of CRP on surfactant adsorption in a concentration dependent manner.

Pulmonary surfactant also contains phosphatidylglycerol and phosphatidylethanolamine which contain headgroups to which CRP is known to bind although with less avidity than with phosphocholine. To further probe the mechanism by which CRP decreases the rate of adsorption of pulmonary surfactant to the air-water interface, two water-soluble CRP ligands, DL-∝-

Figure 10. Effect of phosphocholine on the adsorption of mixtures of CRP + porcine surfactant. —, Porcine surfactant (39.0 $\mu g \cdot ml^{-1}$, n=22); □, phosphocholine (5.86 x 10^{-5} M, n=3); CRP + porcine surfactant + phosphocholine (19.5 $\mu g \cdot ml^{-1}$ CRP : 39.0 $\mu g \cdot ml^{-1}$ + phosphocholine at molar ratios of phosphocholine : CRP; ○, 300 ligand : 1 CRP; ●, 225 ligand : 1 CRP; ▽ 150 ligand : 1 CRP). n=3 for each of the CRP + porcine surfactant + phosphocholine. Subphase and incubation mixtures contained 0.15 M NaCl and 0.005 M $CaCl_2$.

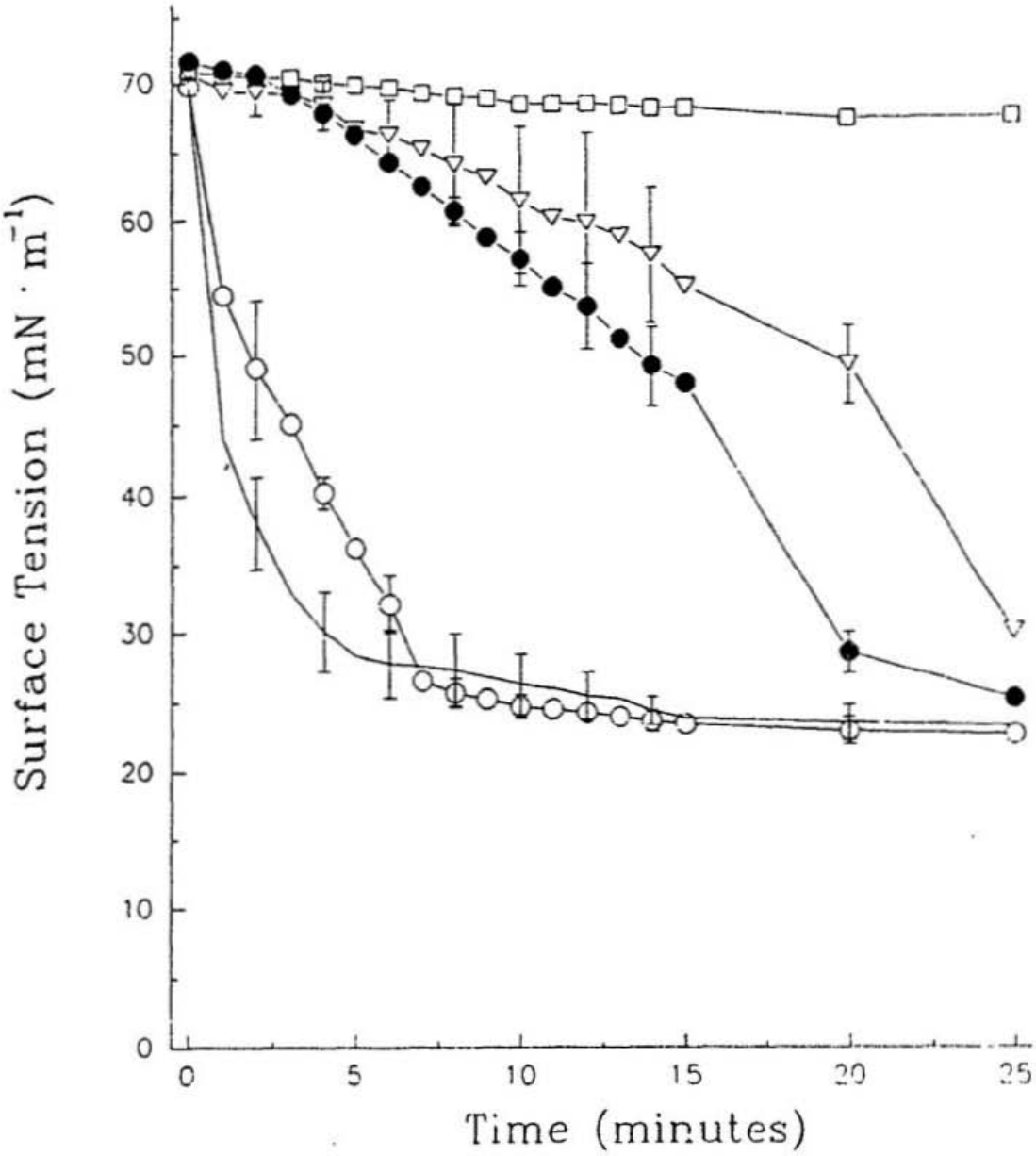
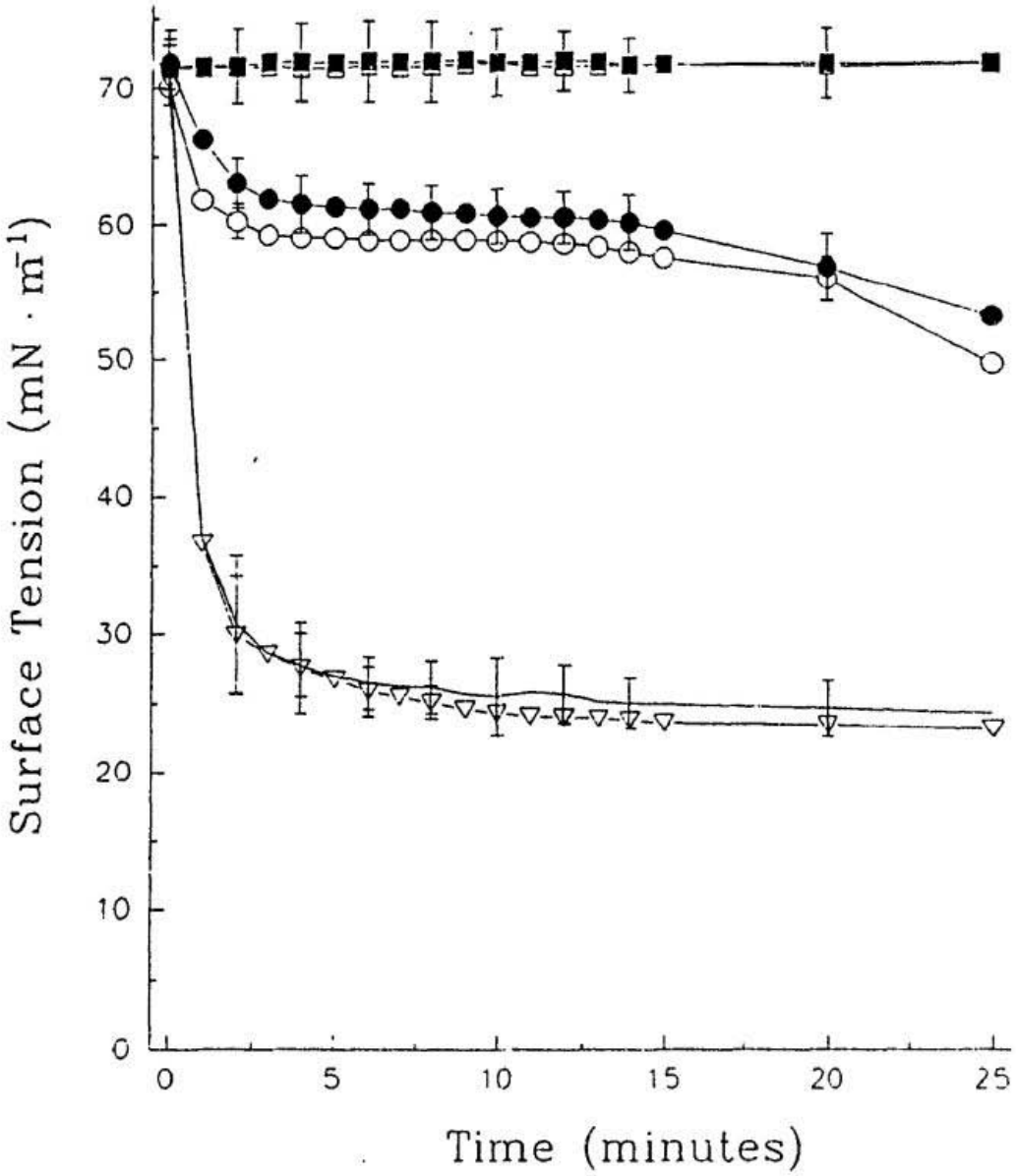

Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

Figure 11. Effect of three CRP ligands on the adsorption of mixtures of CRP + porcine surfactant. —, Porcine surfactant (27.0 $\mu g \cdot ml^{-1}$ PL, n=14); ■, o-phosphoethanolamine (5.86×10^{-5} M); □, DL-α-glycerophosphate (5.86×10^{-5} M); CRP + porcine surfactant + ligand (13.7 $\mu g \cdot ml^{-1}$ CRP : 27.0 $\mu g \cdot ml^{-1}$ PL + ligands; ▽, phosphocholine; ●, o-phosphoethanolamine; ○, DL-α-glycerophosphate). CRP ligands were present with CRP and surfactant at molar ratios of 300 ligand : 1 CRP and n=3 for each of the CRP + surfactant + ligand mixtures. Subphase and incubation mixtures contained 0.15 M NaCl and 0.005 M $CaCl_2$.


Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

glycerophosphate and o-phosphoethanolamine were added to CRP + surfactant mixtures. The effect of these CRP ligands on the capacity of CRP to inhibit porcine pulmonary surfactant adsorption were compared to that of phosphocholine (figure 11). While phosphocholine at a molar ratio of 300 : 1 phosphocholine : CRP almost completely eliminated the inhibitory effect of CRP on surfactant adsorption, identical molar ratios of the other two CRP ligands, o-phosphoethanolamine and DL-α-glycerophosphate did not noticeably decrease the capacity of CRP to inhibit surfactant adsorption (compare figures 5 and 11). In the absence of surfactant, these CRP ligands alone, like phosphocholine alone, did not substantially reduce the subphase surface tension over the 25 minute time period.

To determine if the inhibitory effect of CRP on porcine surfactant adsorption is reversible by the addition of phosphocholine, surfactant incubated with CRP was injected into the subphase in an identical manner as that of that in Figure 5 (figure 12). At 8.5 minutes after injection of the CRP + surfactant mixture, an aliquot of phosphocholine, at a final subphase concentration of 5.86 x 10^{-5} M (molar ratio of phosphocholine : CRP of 300 : 1) was injected. This concentration of phosphocholine reversed the CRP effect on surfactant adsorption, enabling the CRP + surfactant + phosphocholine mixture to attain a surface tension 25 $mN \cdot m^{-1}$ within 25 minutes while the negative control CRP + surfactant mixture required approximately 75 minutes to achieve this value for surface tension.

The effect of the addition of phosphocholine on the adsorption of porcine pulmonary surfactant, in the absence of CRP, was investigated (figure 13). This figure shows that the addition of phosphocholine at a concentration of 5.86 x 10^{-5} M did not affect the adsorption of surfactant to the air-water interface. This is the same concentration of phosphocholine which,

Figure 12. Change in surface tension with time for porcine surfactant, a mixture of CRP + porcine surfactant alone and a mixture of CRP + porcine surfactant to which an aliquot of phosphocholine was added at 8.5 minutes after injection of the CRP + porcine surfactant mixture. ○, Porcine surfactant (39.0 $\mu g \cdot ml^{-1}$ PL, n=22); ●, CRP + porcine surfactant (19.5 $\mu g \cdot ml^{-1}$ CRP : 39.0 $\mu g \cdot ml^{-1}$ PL, n=3); ▽, CRP + porcine surfactant (19.5 $\mu g \cdot ml^{-1}$ CRP : 39.0 $\mu g \cdot ml^{-1}$ PL, to which phosphocholine (5.86×10^{-5} M, final subphase concentration, n=3) was injected at 8.5 minutes. Incubation mixtures, phosphocholine solution and subphase contained 0.15 M NaCl and 0.005 M $CaCl_2$.

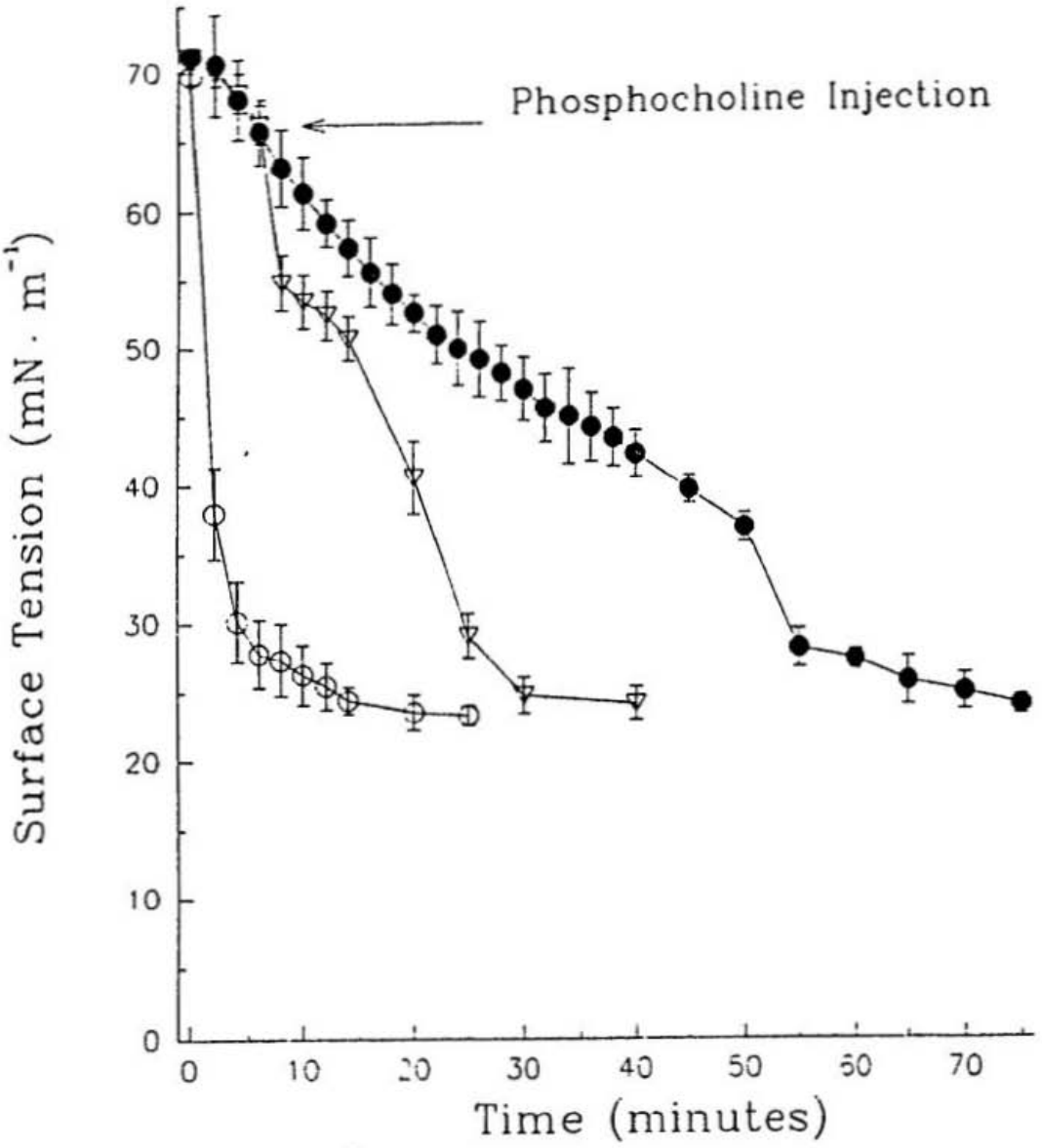
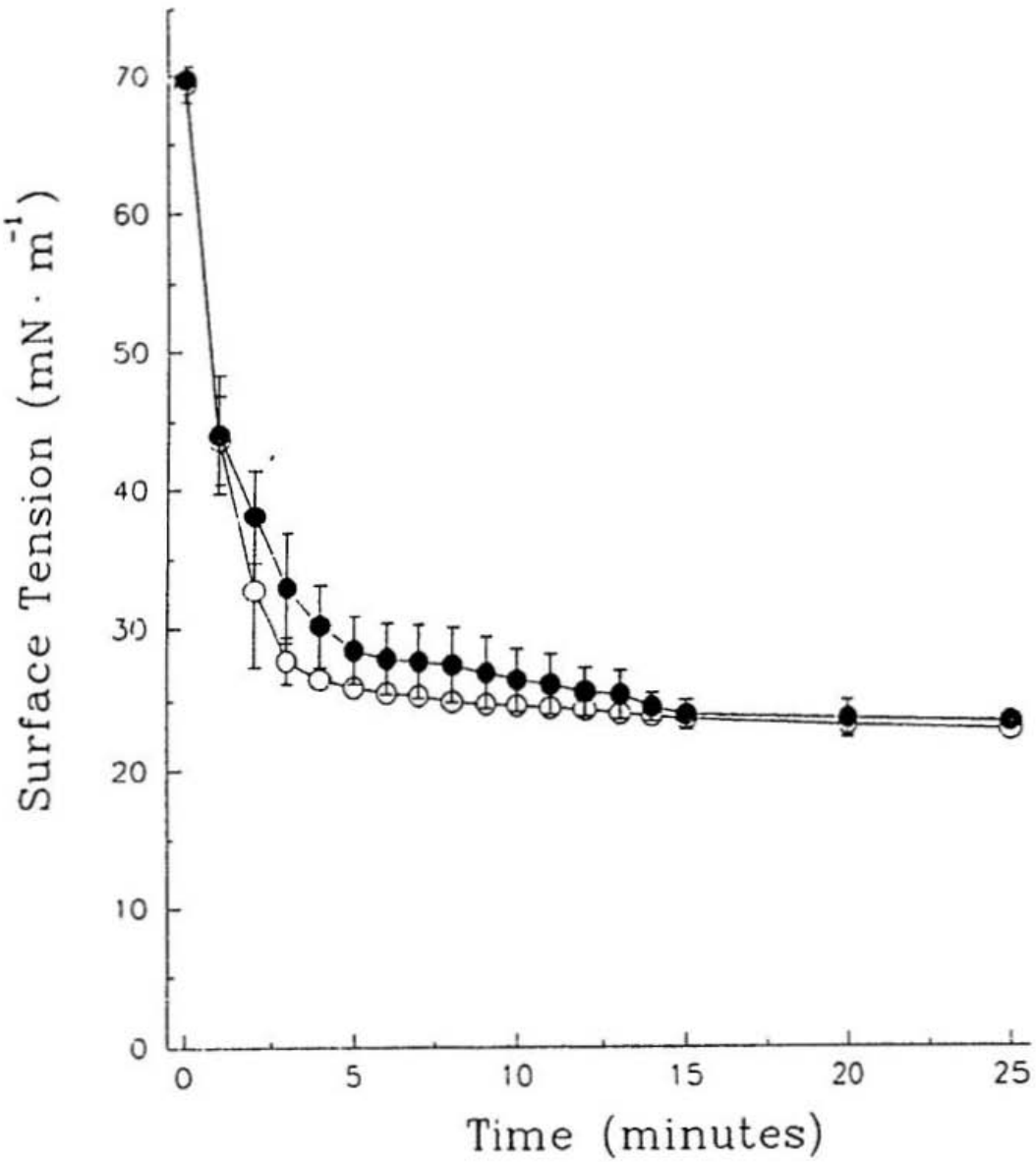

Phosphocholine Injection
Surface Tension (mN · m⁻¹)
Time (minutes)
0
10
20
30
40
50
60
70

Figure 13. Effect of phosphocholine on the adsorption of porcine pulmonary surfactant, in the absence of CRP. ○, Porcine surfactant (39.0 $\mu g \cdot ml^{-1}$, n=22); ●, porcine surfactant (39.0 $\mu g \cdot ml^{-1}$) + phosphocholine (5.86×10^{5} M, n=3). Subphase, surfactant suspensions and surfactant + phosphocholine mixtures contained 0.15 M NaCl and 0.005 M $CaCl_2$.


Surface Tension (mN · m^{-1})
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (minutes)

when mixed with CRP + surfactant prior to injection into the subphase, almost completely eliminated the inhibitory effect of CRP on surfactant adsorption. Also, this concentration of phosphocholine, when injected after the adsorption of CRP + surfactant had begun, partially reversed the inhibitory effect of CRP on surfactant adsorption.

Effect of C-reactive Protein on the Adsorption of Pulmonary Surfactant During Dynamic Surface Compression

The pulsating bubble surfactometer provides a mechanical model of the alveolus wherein the bubble, like the alveolus, undergoes cyclic compression and expansion. Therefore, the effect of inhibitors of surfactant adsorption can be investigated simultaneously with the effect of surface refining of surfactant and inhibitory components at the air-water interface. Surface adsorption alone can be monitored when a bubble is formed but not pulsated. During experiments using the pulsating bubble surfactometer, the surface behaviour of surfactant, protein, and surfactant + protein mixtures was monitored using the pulsating bubble technique.

Whole surfactant was initially tested at several concentrations to determine which concentration would reduce the value of ΔP_{min} to approximately zero at an appropriate rate, which was not instantaneous (see above). Figure 14 shows that porcine surfactant at 0.5 mg · ml^{-1} required ~6 minutes (120 pulses) to reduce the ΔP_{min} to approximately zero. A higher concentration of surfactant reduced ΔP_{min} to near zero more quickly. Surfactant, however, at this concentration was "saturating" because at a lower concentration of 1 mg · ml^{-1}, reduced ΔP_{min} to approximately zero in the same time amount of time (figure 15). Therefore, the concentration of surfactant was chosen to be used throughout the experiments using the pulsating bubble

Figure 14. Reductions of data from bubble surfactometer tracings for suspensions of porcine surfactant at a concentration of 0.5 mg · ml^{-1} PL, n=3. Surfactant mixtures contained 0.15 M NaCl and 0.005 M $CaCl_2$. In this and subsequent figures, which express data which were acquired using the pulsating bubble surfactometer, pressure differences were read from the traces at maximum and minimum bubble sizes at certain times after pulsation was initiated. Pressure differences are plotted as a function of pulse number. Solid circles represent the pressure differences at maximum bubble size (ΔP_{max}) and the open circles represent pressure differences at minimum bubble size (ΔP_{min}). In all cases data from the first bubble formed is shown. Values are expressed as mean $\pm$ 1 SD; standard deviations are shown for every second reading and are not shown when they fall within the range denoted by the symbols

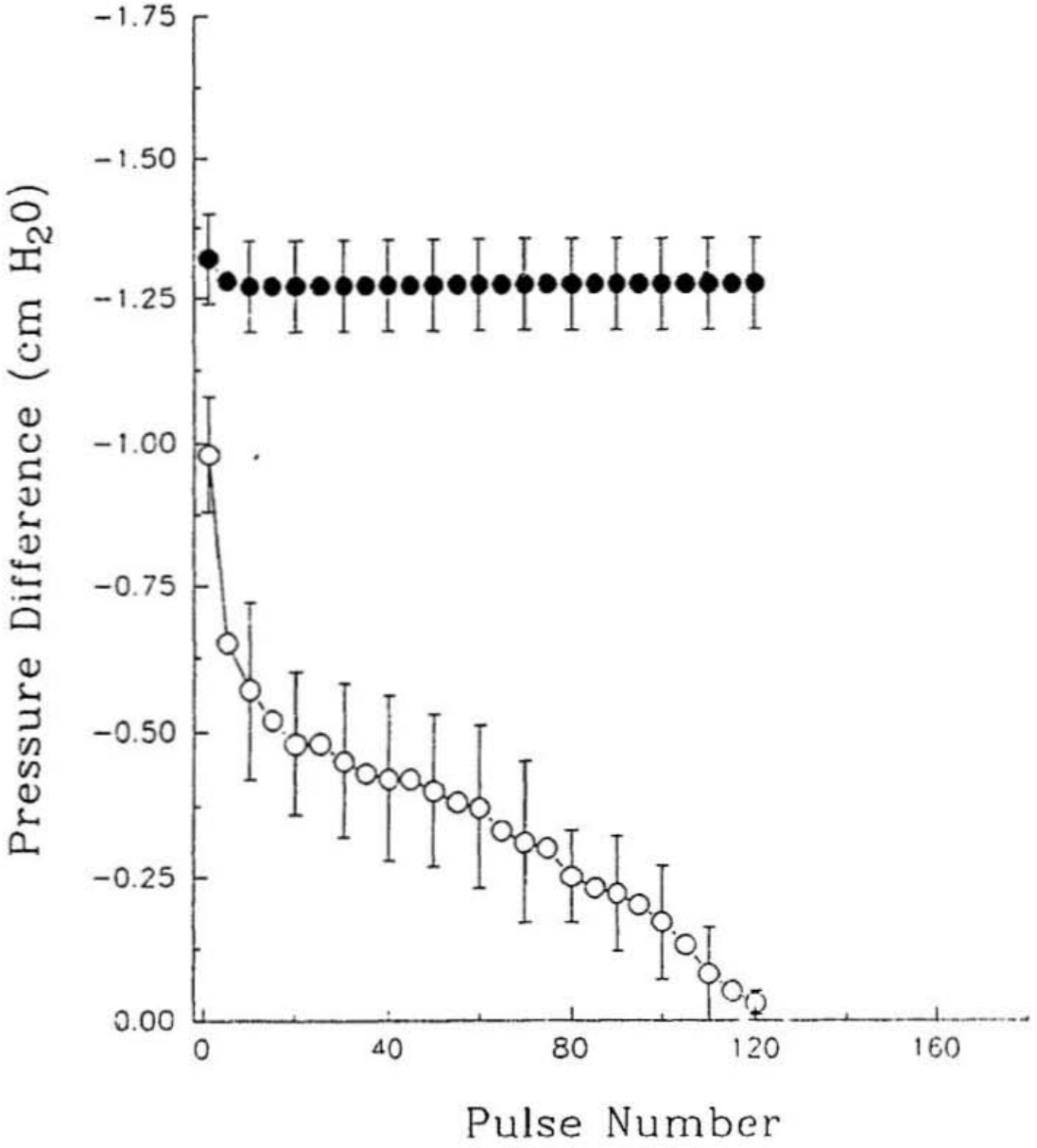
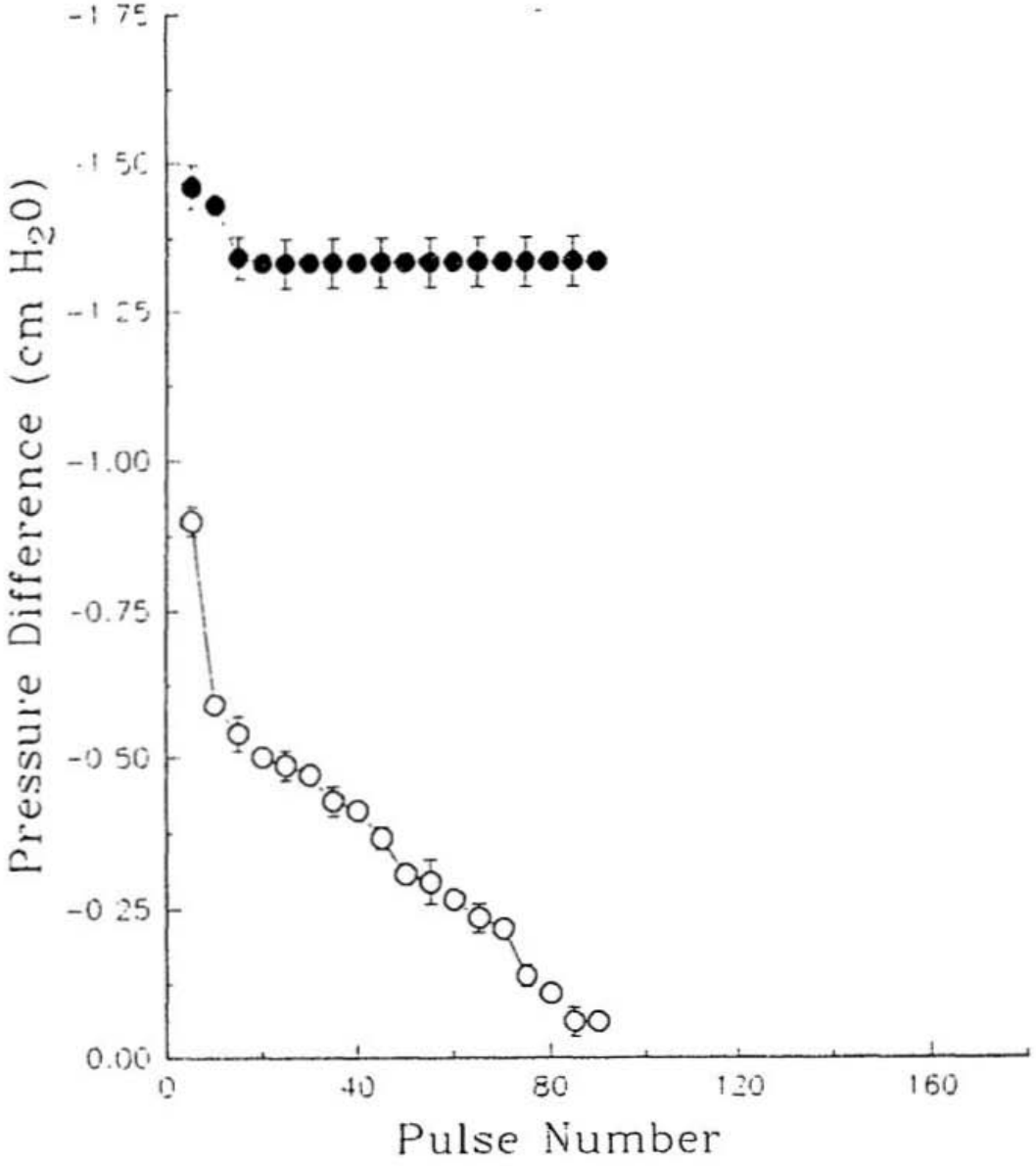

Pressure Difference (cm H_2O)
-1.75
-1.50
-1.25
-1.00
-0.75
-0.50
-0.25
0.00
0
40
80
120
160
Pulse Number

Figure 15. Pressure difference versus pulse number for porcine surfactant at a concentration of 1.0 mg · ml^{-1} PL, n=8. Surfactant suspension contained 0.15 M NaCl and 0.005 M $CaCl_2$.


-1.75
-1.50
-1.25
-1.00
-0.75
-0.50
-0.25
0.00
Pressure Difference (cm H_2O)
0
40
80
120
160
Pulse Number

surfactometer was 1.0 mg · ml^{-1}.

The effect of CRP on surfactant adsorption during dynamic compression and expansion was investigated (figure 16). While surfactant alone reduced $\triangle P_{min}$ to near zero in ~4 minutes (80 pulses), surfactant, when mixed with CRP at the same weight ratio as those previously reported, could not attain this low value of $\triangle P_{min}$ even after 8.5 minutes (170 pulses) (compare figures 15 and 16).

Figure 17 shows a series of typical tracings from the pulsating bubble surfactometer. Tracings from pulsated a bubble formed in a suspension of porcine pulmonary surfactant and a suspension of CRP + surfactant at a weight ratio of (0.5 : 1.0, CRP : PL) are presented. For comparison purposes, a tracing obtained from a bubble formed in a solution of CRP alone is included. Normal surfactant showed a trace in which the lowest pressure difference, $\triangle P_{min}$ occurred at or very near the point at which the bubble radius was at a minimum, which is indicated by a tick (figure 17a). This is a behavior which is characteristic of bubbles formed in suspension of well-functioning surfactant. Upon initial bubble formation, the pressure difference across the bubble interface decreased quickly because of the rapid adsorption of surfactant to the air-water interface. Pulsation of bubbles formed in solutions of surfactant, is believed to lead to a process of surface refinement of the surfactant film to leave film of insoluble material, enriched in DPPC. This enables the surfactant film, upon successive compressions, to the reduce the surface tension (measured as $\triangle P$) across the bubble interface to very low values. Therefore, the lowest $\triangle P$ occurs at minimum bubble radius.

A solution of water soluble CRP, however, showed a behavior which was opposite to that of surfactant (figure 17b). The initial pressure tracing shows that the $\triangle P$ is largest at minimum

Figure 16. Reductions of data from bubble surfactometer tracings for a mixture of porcine surfactant + CRP. CRP was at a concentration of 0.5 mg · ml^{-1} and porcine surfactant was at 1.0 mg · ml^{-1} PL, n=3. CRP + Surfactant mixture contained 0.15 M NaCl and 0.005 M $CaCl_2$.

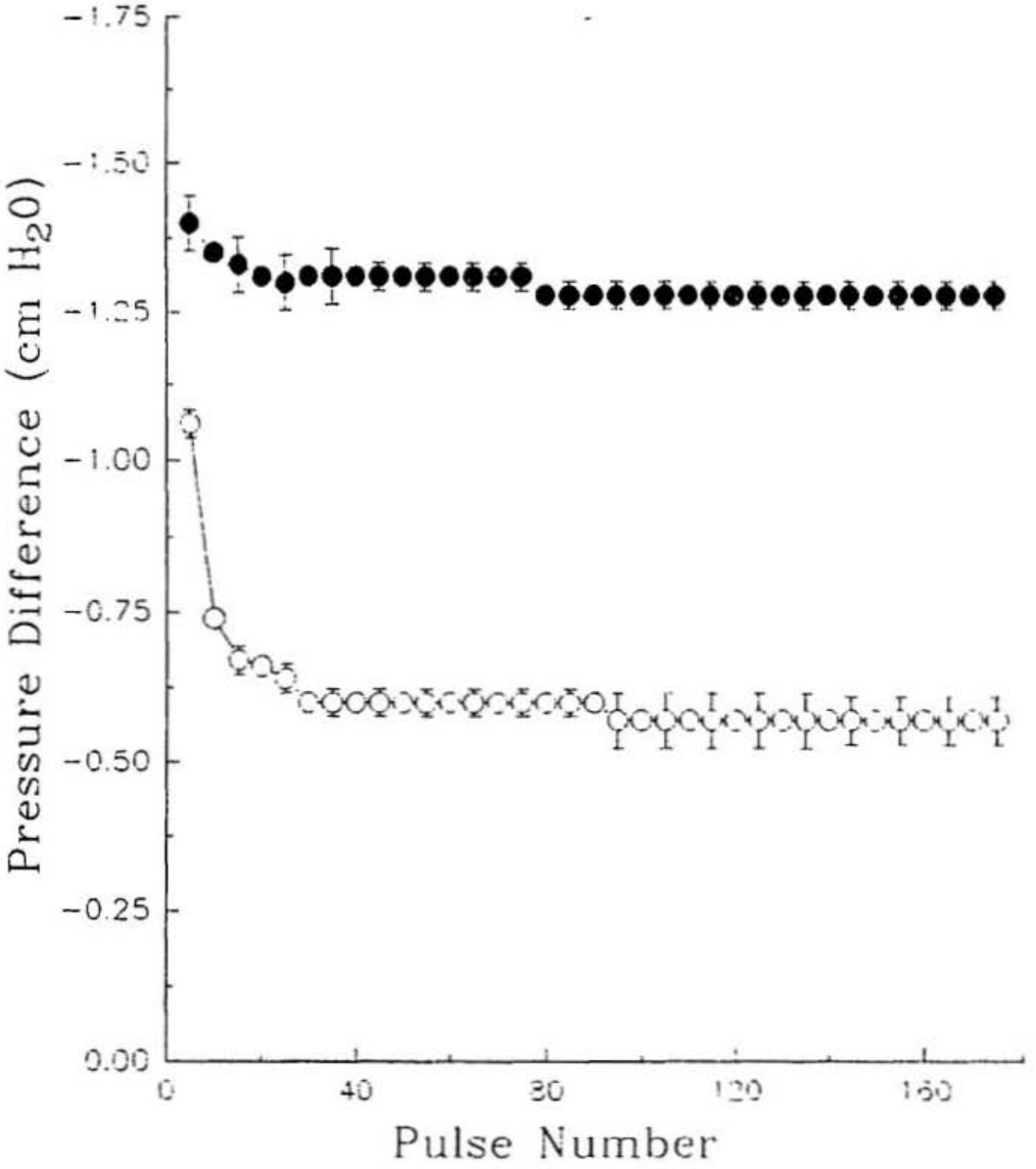
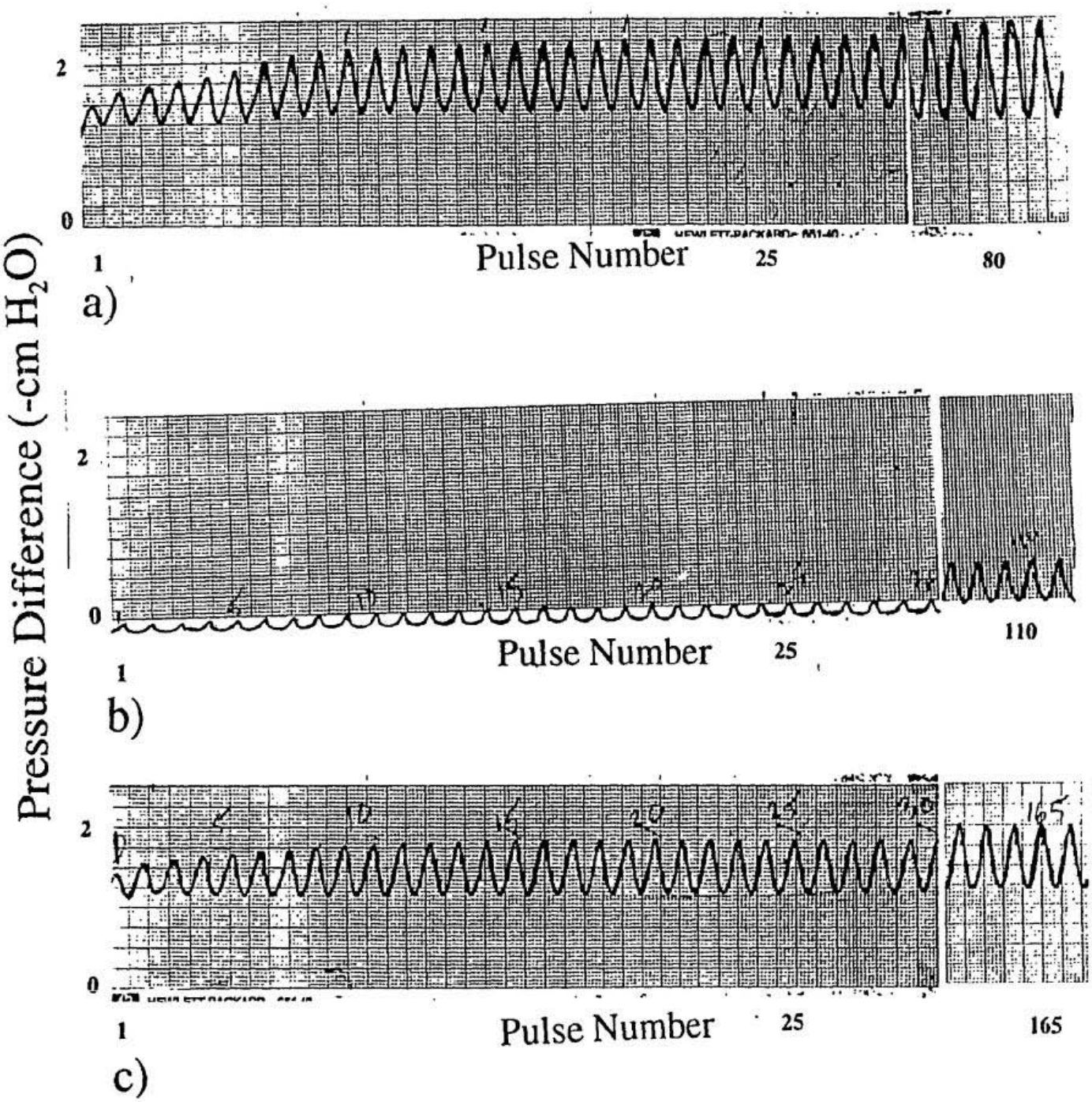

Pressure Difference (cm H_2O)
-1.75
-1.50
-1.25
-1.00
-0.75
-0.50
-0.25
0.00
0
40
80
120
160
Pulse Number

Figure 17. Typical pressure tracings of bubbles of porcine surfactant, CRP, porcine surfactant + CRP from the pulsating bubble surfactometer. Tracings are from the first bubble and represent ΔP, measured in -cm H_20 as the bubble is pulsated between maximum and minimum bubble radii. a) Porcine surfactant (1.0 mg · ml^{-1}); b) CRP (0.5 mg · ml^{-1}); c) CRP + porcine surfactant (0.5 mg · ml^{-1} : 1.0 mg · ml^{-1}). Minimum bubble radius is indicated by a tick and the bubble was pulsated at a rate of 20 pulses per minute. Subphase contained 0.15 M NaCl and 0.005 M $CaCl_2$.


Pressure Difference (-cm H_2O)
2
0
1
Pulse Number
25
80
a)
2
0
1
Pulse Number
25
110
b)
2
0
1
Pulse Number
25
165
c)

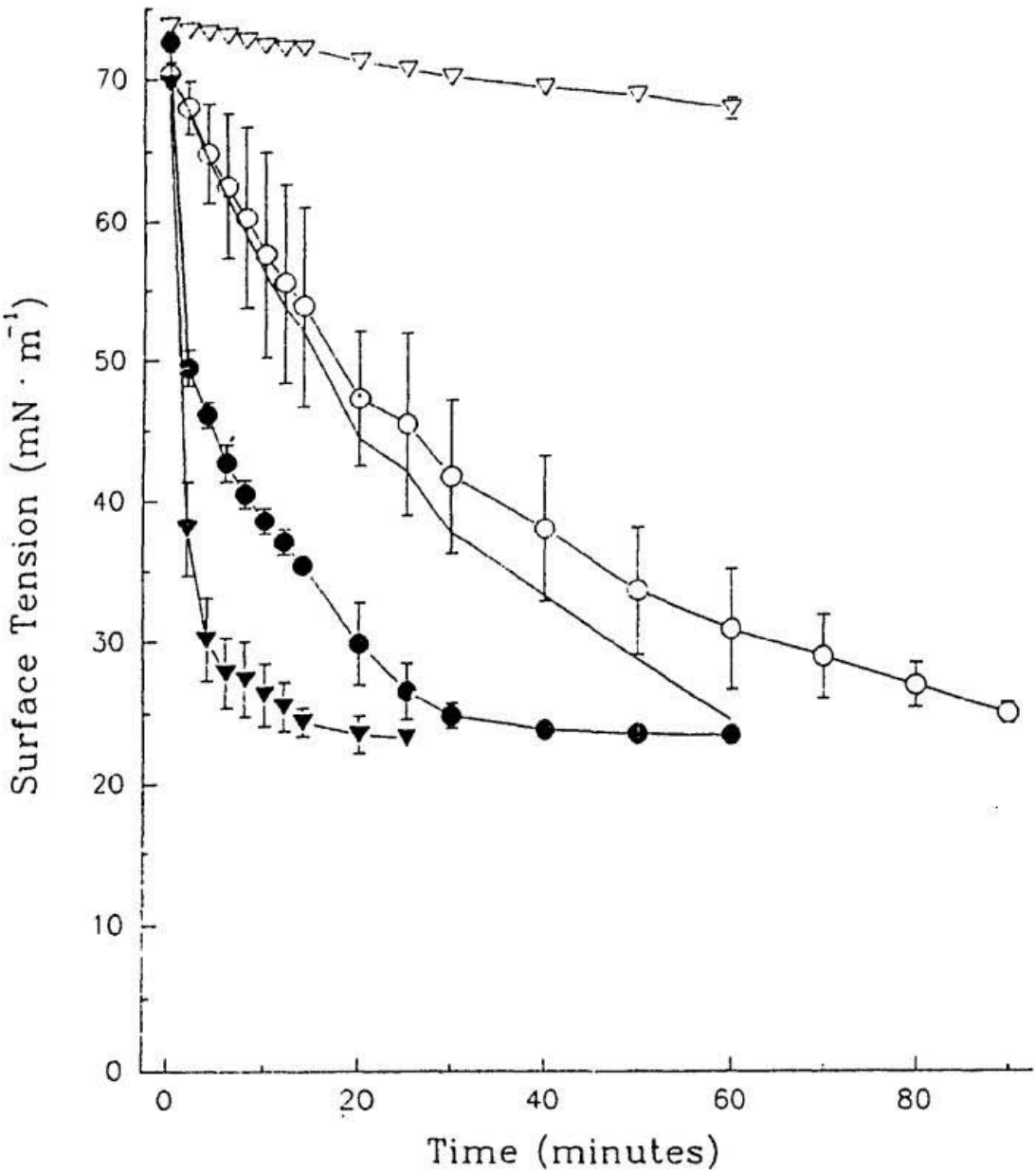
bubble radius, a behavior expected for a bubble with a constant surface tension. After a period of pulsation, a bubble formed in a solution of CRP underwent a reversal in the relation of ΔP versus bubble radius such that ΔP became lowest at minimum bubble radius. Keough et al. (1989) suggested that, with fibrinogen, which is water-soluble like CRP, some adsorbed protein material may become insoluble and remain in the surface during dynamic compression so that the density of fibrinogen in the interface increases and the surface tension decreases. The behavior of CRP in the pulsating bubble surfactometer suggests that CRP eventually becomes insoluble and remains at the surface during dynamic compression but this has only a minimal effect on ΔP_{min} (figure 17b). The tracing from a pulsated bubble of a mixture of CRP + porcine pulmonary surfactant (0.5 CRP : 1.0 PL, w/w) is shown (figure 17c). Pulsated bubbles of this mixture show that the phase or character is typical of surfactant, where ΔP_{min} occurred at or near the point where the bubble is at minimum radius.

The Effect of the Complement Protein C1q on the Adsorption and Surface Tension Lowering Ability of Pulmonary Surfactant

It is known that the hydrophillic surfactant protein SP-A increases the rate of adsorption of pulmonary surfactant. Estimates of the amount of SP-A in whole surfactant range from 4-7% of the total phospholipid content, by weight. Experiments were undertaken to determine what effect the complement protein C1q has on the adsorption rate of a dispersion of lipid extract of porcine surfactant which was void of SP-A, but which contained SP-B and SP-C.

The effect of 10% C1q (w/w) on the adsorption rate of lipid extract surfactant was investigated using the surface adsorption apparatus (figure 18). Whole porcine surfactant attained

Figure 18. Change in surface tension with time after injection of whole porcine surfactant, C1q, porcine lipid extract surfactant, and a mixture of C1q (10% C1q : PL, w/w) + porcine lipid extract surfactant into a stirred subphase. ▾, Porcine surfactant (39.0 $\mu g \cdot ml^{-1}$ PL, n=3); ○, porcine lipid extract surfactant (39.0 $\mu g \cdot ml^{-1}$ PL, n=5); ▿, C1q (3.90 $\mu g \cdot ml^{-1}$, n=3); ●, C1q + porcine lipid extract surfactant (39.0 $\mu g \cdot ml^{-1}$ C1q : 3.90 $\mu g \cdot ml^{-1}$ PL, n=3); -, the cumulative effects of C1q + porcine lipid extract surfactant (39.0 $\mu g \cdot ml^{-1}$ C1q : 3.90 $\mu g \cdot ml^{-1}$ PL) which have been added independently. All mixtures, were in water which contained 0.15 M NaCl and 0.005 M $CaCl_2$.


Surface Tension (mN · m⁻¹)
70
60
50
40
30
20
10
0
0
20
40
60
80
Time (minutes)

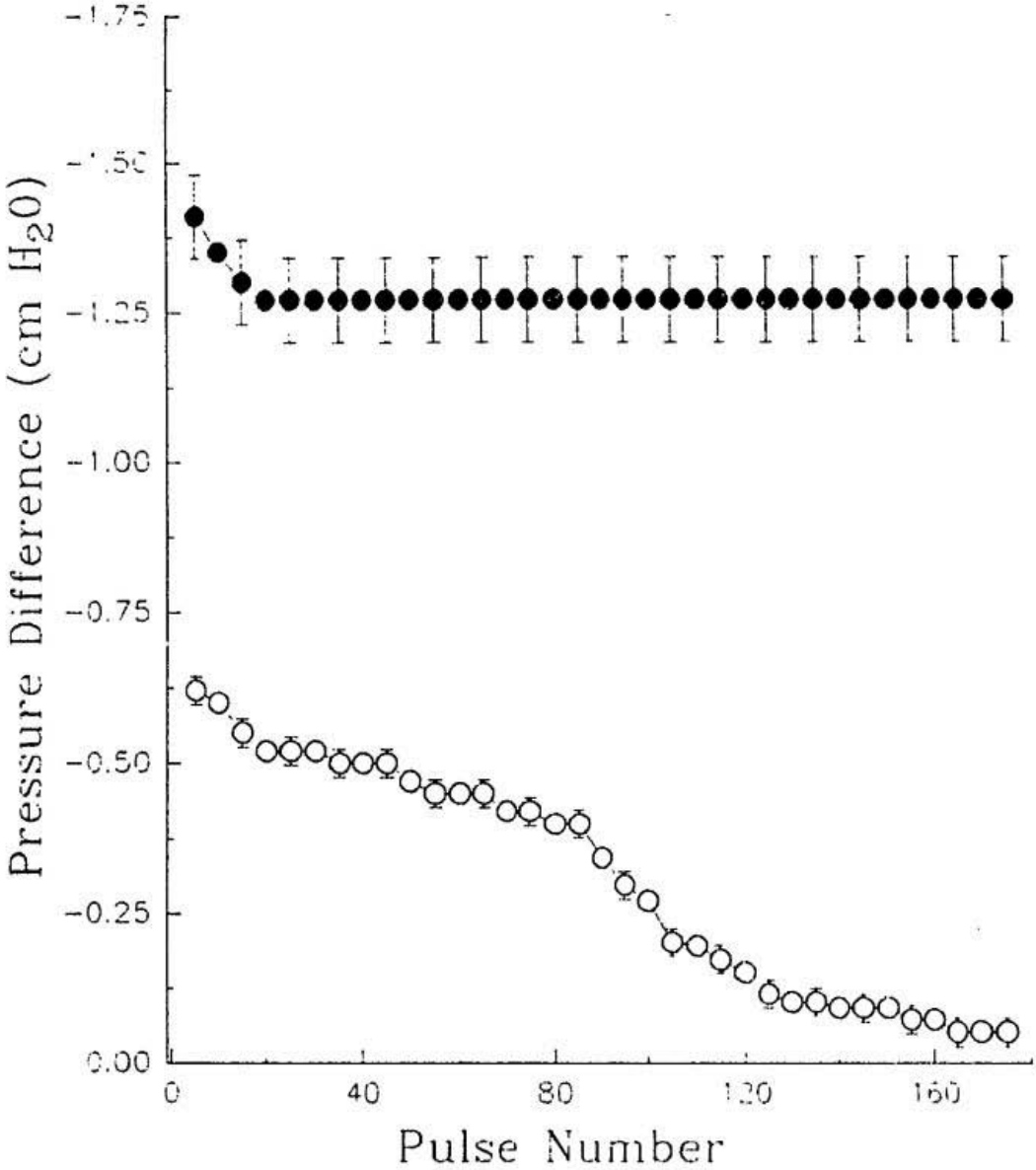
a minimum surface tension of 25 mN · m^{-1} within 5 minutes after injection. Subphase injections of an identical concentration of a dispersion of lipid extract of the same surfactant, which was void of SP-A, required 50 minutes to reduce the subphase surface tension to 33 mN · m^{-1}. Control injections of C1q (3.90 μg ·ml^{-1}) minimally reduced subphase surface tension. Lipid extract surfactant, which contained the hydrophilic surfactant proteins SP-B and SP-C, but not SP-A, when incubated with 10% C1q (w/w) and injected into the subphase, reduced subphase surface tension to approximately 25 mN · m^{-1} within 25 minutes after injection. The rate of adsorption of this mixture was greater than that for the lipid extract surfactant suspension alone. This adsorption rate was also greater than when the cumulative effects of C1q and lipid extract surfactant on subphase surface tension were added together. Therefore, Figure 18 shows that the complement protein C1q increased the adsorption rate of porcine lipid extract surfactant.

Effect of C1q on the Surface Adsorption During Dynamic Compression of Porcine Lipid Extract Pulmonary Surfactant

The effect of removing SP-A on the adsorption rate of porcine pulmonary surfactant during dynamic compression was investigated using the pulsating bubble surfactometer (figure 19). It required ~8 minutes (160) pulses for the lipid extract surfactant to reduce the surface tension of the bubble so that ΔP_{min} to approach zero. An identical concentration of the same surfactant, which contained SP-A, required ~4 minutes (80 pulses) to decrease ΔP_{min} to near zero (compare figures 15 and 19).

The effect of the addition of C1q on the surface behaviour of porcine lipid extract surfactant, which was void of SP-A, was investigated. The effect of adding 5% C1q (w/w) on

Figure 19. Pressure difference versus pulse number for a suspension of porcine lipid extract surfactant (1.0 mg · ml^{-1} PL) which was in water which contained 0.15 M NaCl and 0.005 M $CaCl_2$, n=5.


Pressure Difference (cm H_2O)
-1.75
-1.50
-1.25
-1.00
-0.75
-0.50
-0.25
0.00
0
40
80
120
160
Pulse Number

the adsorption rate of porcine lipid extract surfactant during dynamic compression and expansion is shown in Figure 20. Lipid extract surfactant required 8 minutes for ΔP_{min} to approach zero, however, when 5% C1q (w/w) was added to an identical concentration of the same surfactant, ΔP_{min} approached zero in ~6 minutes (120 pulses) (compare figures 19 and 20). The addition of a higher concentration of C1q (10% w/w) enabled the porcine lipid extract surfactant to reduce ΔP_{min} to near zero in ~4 minutes (80 pulses). This was approximately equal to the rate at which an identical concentration of the same surfactant, which contained SP-A, reduced ΔP_{min} (compare figures 15 and 21).

Control experiments, which involved the incubation of lipid extract surfactant with 10% (w/w) albumin, were performed and the results are shown in Figure 22. Unlike the addition of 10% (w/w) C1q to lipid extract surfactant, which increased the rate at which ΔP_{min} approached zero, the addition 10% (w/w) albumin did not increase the rate at which ΔP_{min} approached zero (compare figures 21 and 22). Instead, the addition of albumin decreased this rate so that ΔP_{min} was approximately -0.25 cm H_2O ~8 minutes (160 pulses) after bubble formation. This is consistent with the inhibitory effect of albumin on the surface adsorption of surfactant during dynamic compression which was observed by Keough et al. (1989).

A series of typical tracings from the pulsating bubble surfactometer are shown in Figure 23. Results from a suspension of whole porcine pulmonary surfactant, lipid extract surfactant alone, and lipid extract surfactant + two amounts of C1q and albumin are presented. For comparison purposes, tracings obtained from a bubble formed in a solution of C1q and albumin alone are included. A suspension of whole (figure 23a) or lipid extract surfactant (figure 23b), when present by itself or with C1q (figure 23c,d) or albumin (figure 23e) shows that the phase

Figure 20. Reductions of bubble surfactometer tracings for a suspension of C1q (0.050 mg · ml^{-1}) + porcine lipid extract surfactant (1.0 mg · ml^{-1}) which was suspended in water which contained 0.15 M NaCl and 0.005 M $CaCl_2$, n=5.

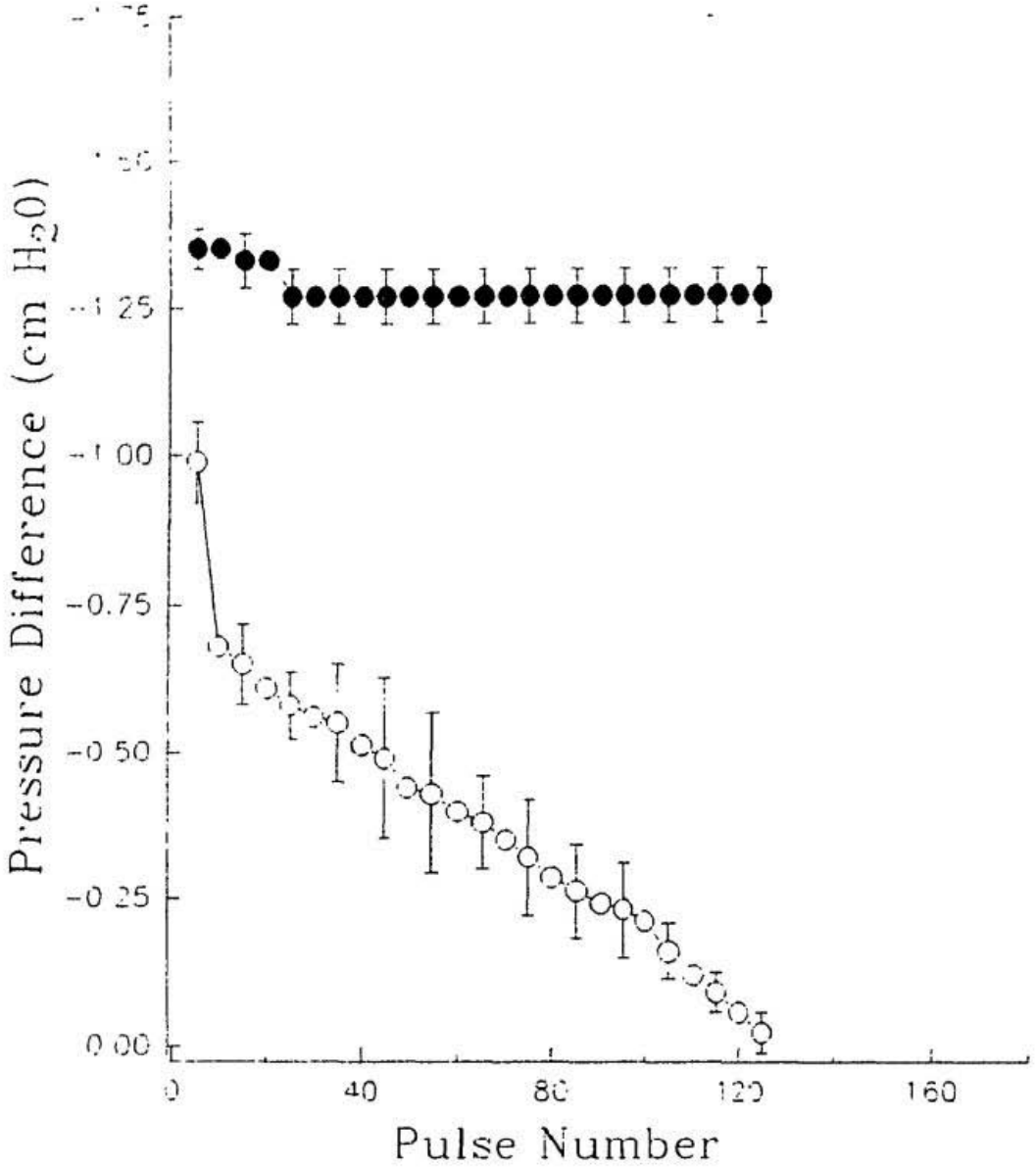

Pressure Difference (cm H2O)
-1.25
-1.00
-0.75
-0.50
-0.25
0.00
0
40
80
120
160
Pulse Number

Figure 21. Pressure difference versus pulse number for a suspension of C1q (0.10 mg · ml^{-1}) + porcine lipid extract surfactant (1.0 mg · ml^{-1}) which was suspended in water which contained 0.15 M NaCl and 0.005 M $CaCl_2$, n=5.

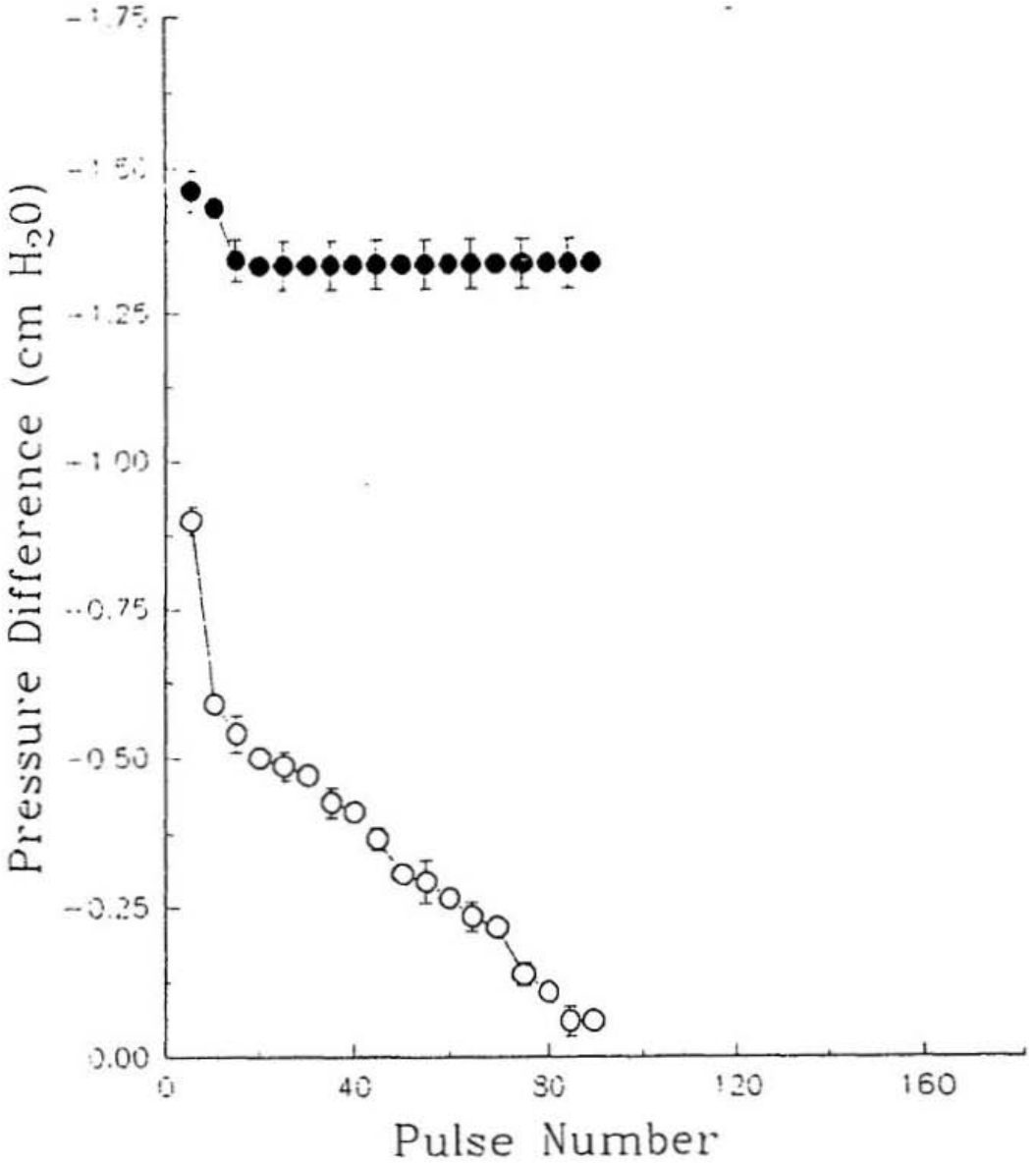

Pressure Difference (cm H2O)
-1.75
-1.50
-1.25
-1.00
-0.75
-0.50
-0.25
0.00
0
40
80
120
160
Pulse Number

Figure 22. Reductions of bubble surfactometer tracings for a suspension of albumin (0.10 mg · ml^{-1}) + porcine lipid extract surfactant (1.0 mg · ml^{-1}) which was suspended in water which contained 0.15 M NaCl and 0.005 M $CaCl_2$, n=5.

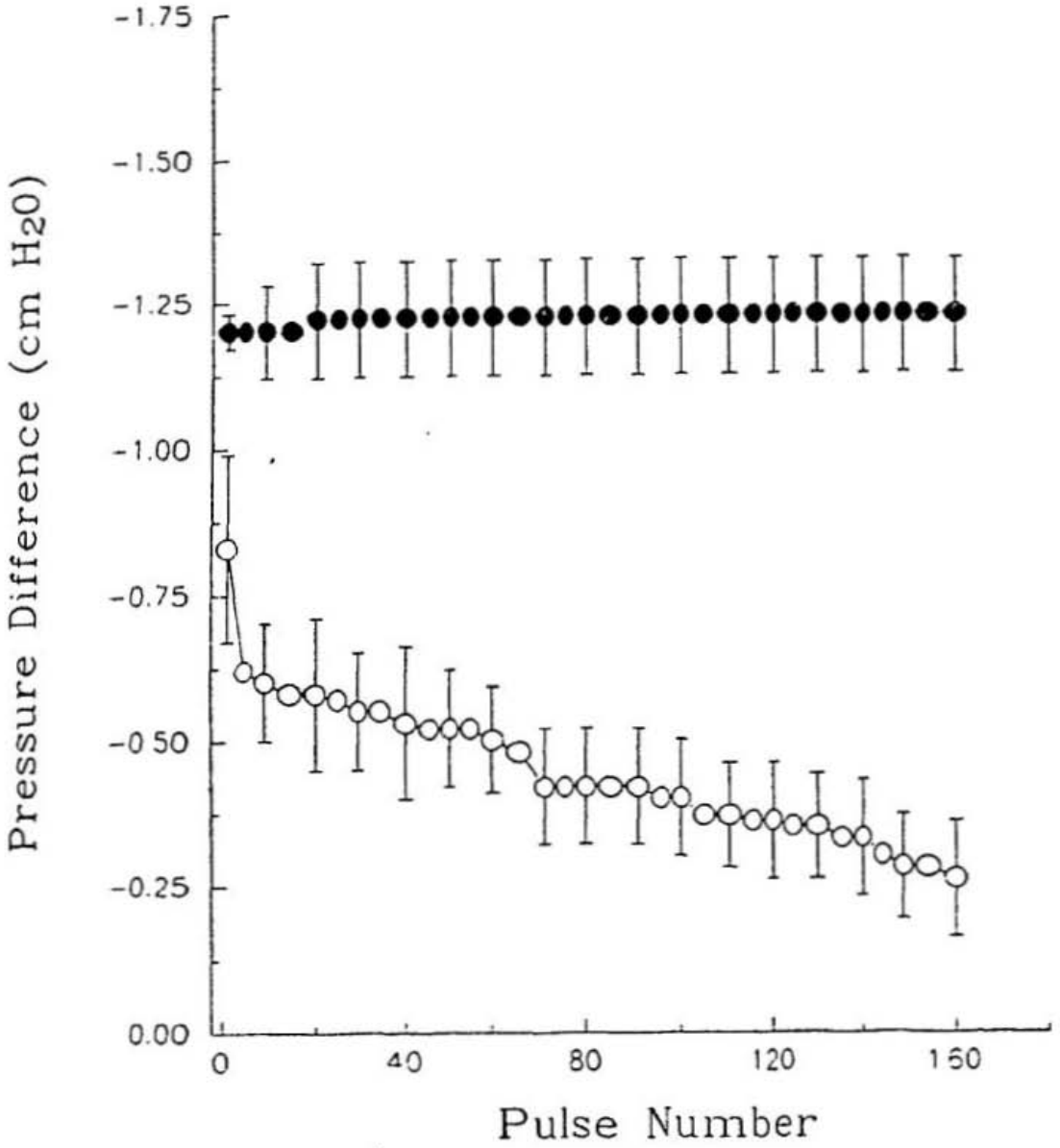
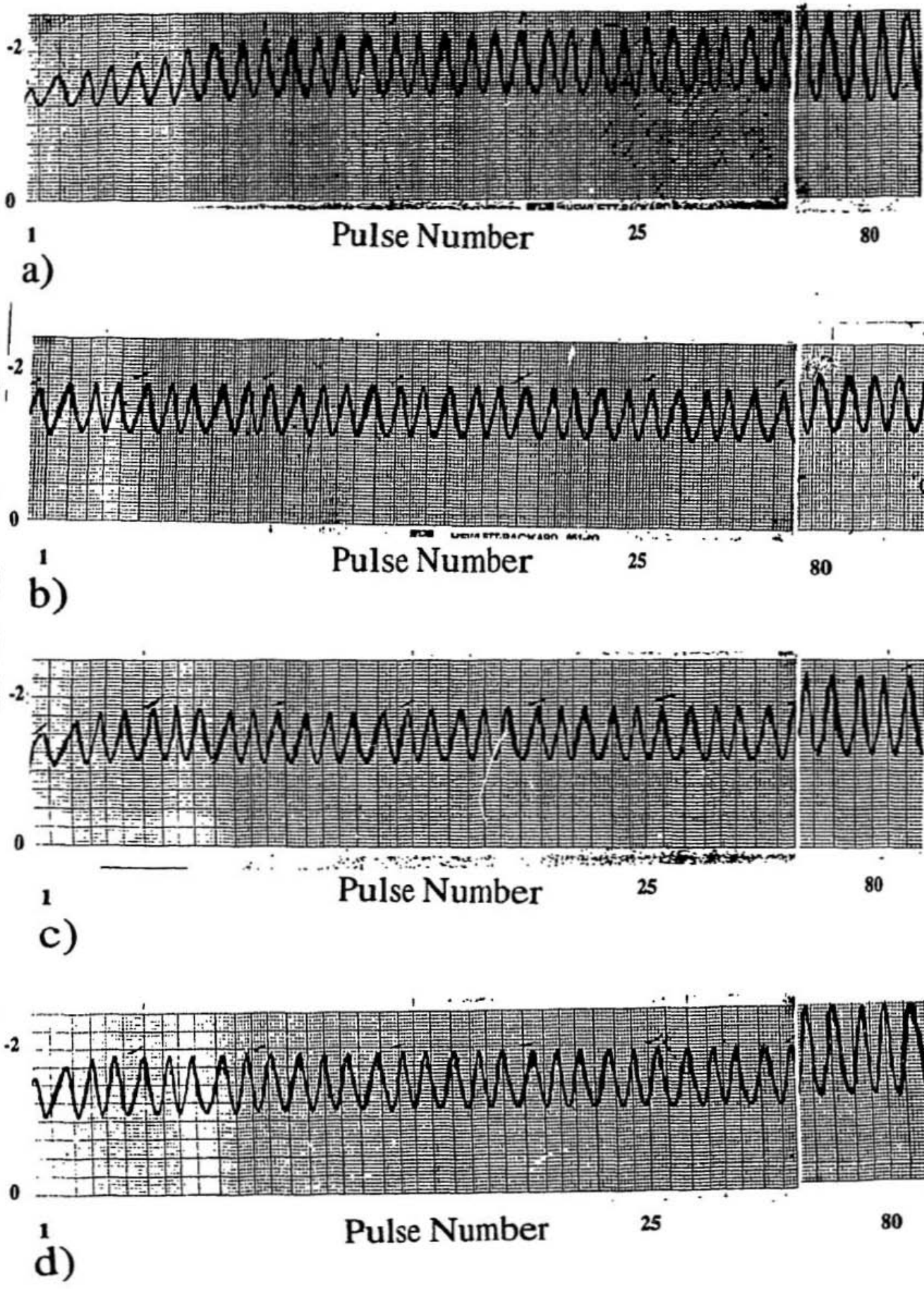
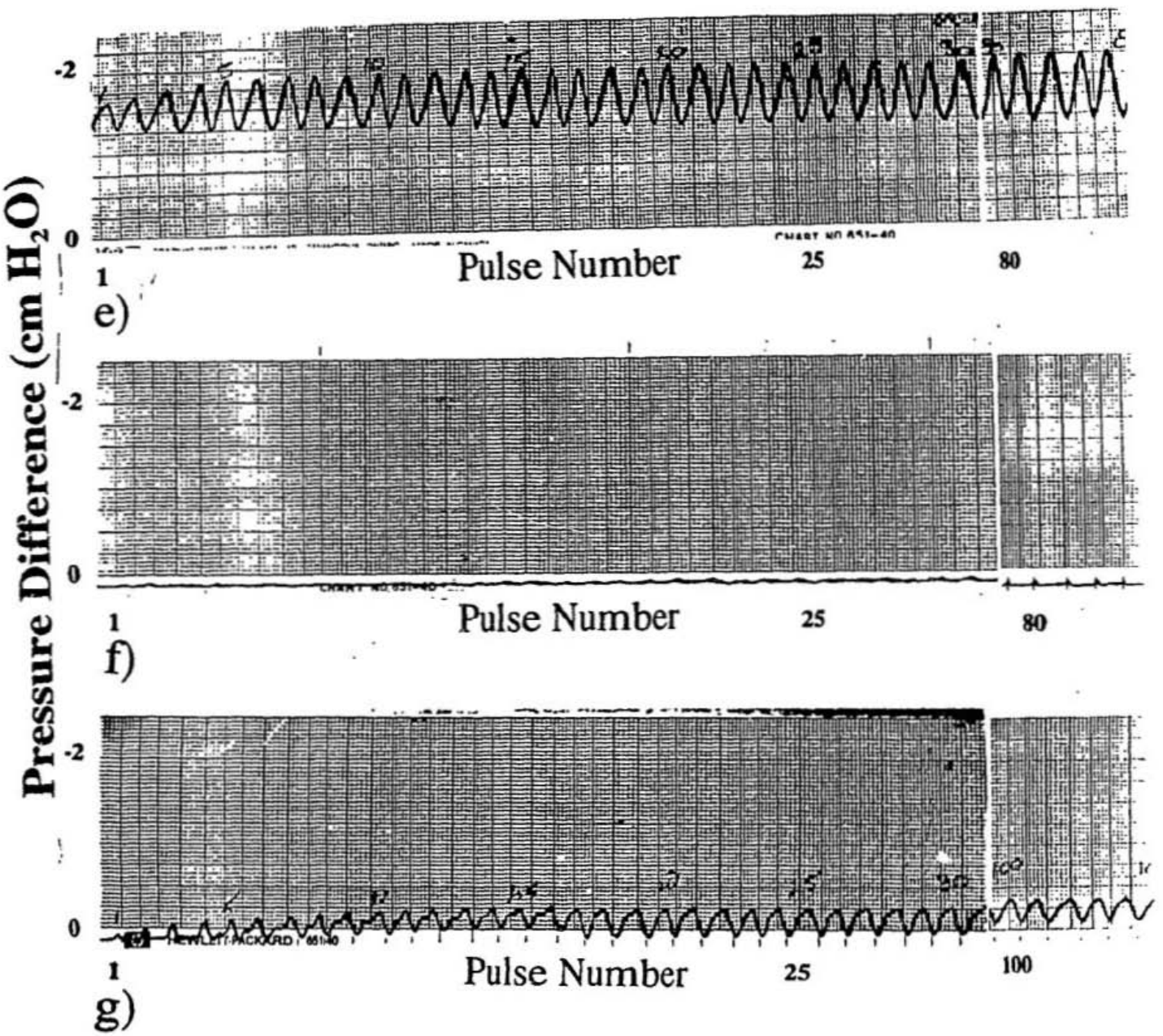

-1.75
-1.50
-1.25
-1.00
-0.75
-0.50
-0.25
0.00
0
40
80
120
160
Pressure Difference (cm H2O)
Pulse Number

Figure 23. Typical tracings of bubbles of whole porcine surfactant, lipid extract porcine surfactant, and of mixtures of lipid extract porcine surfactant + two amount of C1q and albumin and the serum proteins alone from the pulsating bubble surfactometer. Tracings are from the first bubble formed and represent ΔP, measured in -cm H_2O as the bubble was pulsated between maximum and minimum bubble radii. a) Whole porcine surfactant (1.0 mg · ml^{-1}); b) lipid extract porcine surfactant (1.0 mg · ml^{-1}); c) C1q (0.050 mg · ml) + lipid extract porcine surfactant (1.0 mg · ml^{-1}); d) C1q (0.10 mg · ml) + lipid extract porcine surfactant (1.0 mg · ml^{-1}); e) albumin (0.10 mg · ml) + lipid extract porcine surfactant (1.0 mg · ml^{-1}) f) C1q (0.10 mg · ml^{-1}); g) albumin (0.10 mg · ml). Minimum bubble radius is indicated by a tick and the bubble was pulsated at a rate of 20 pulses per minute. Subphase contained 0.15 M NaCl and 0.005 M $CaCl_2$.


Pressure Difference (cm H_2O)
-2
0
1
Pulse Number
25
80
a)
b)
c)
d)

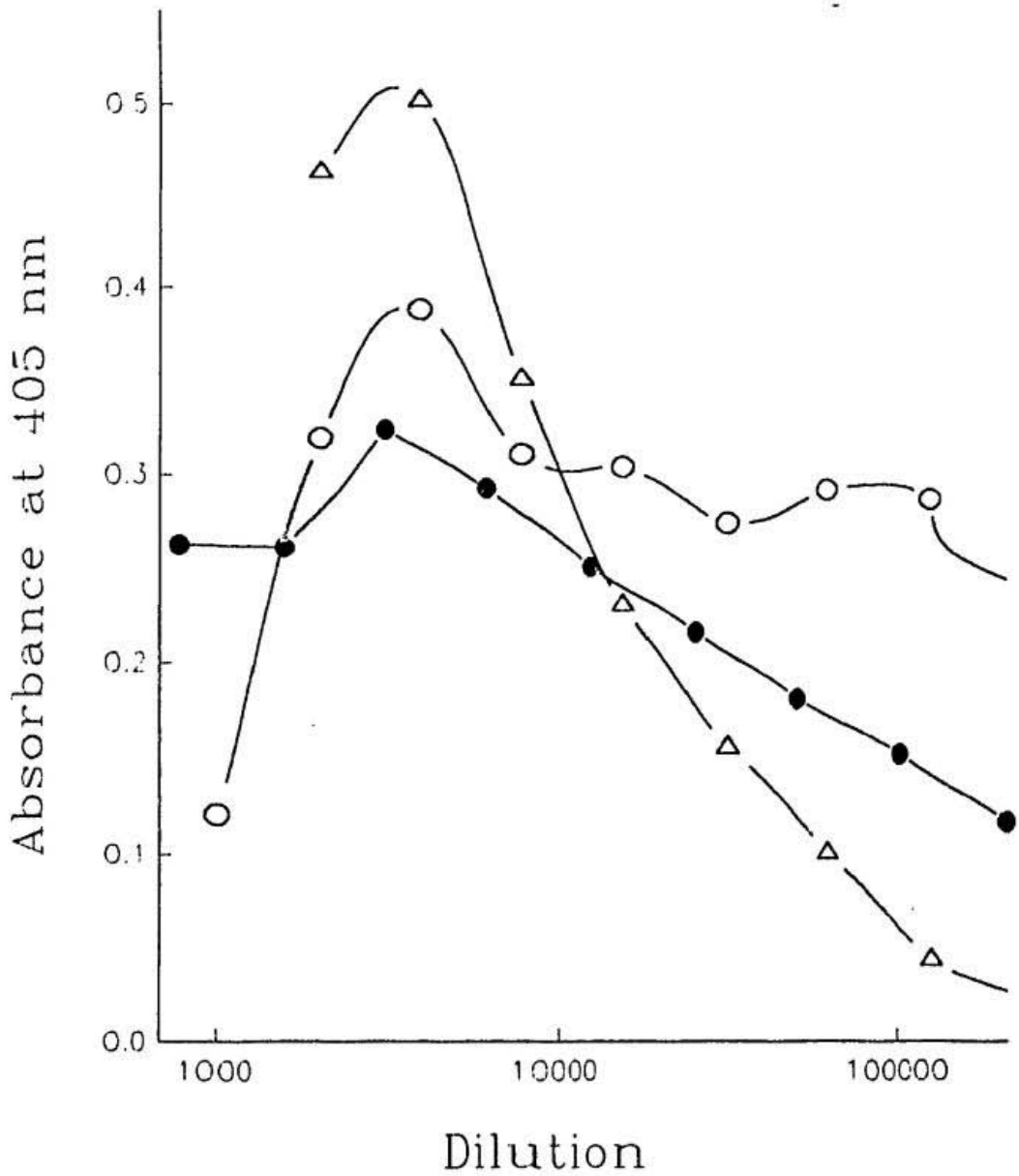

Pressure Difference (cm H_2O)
-2
0
1
Pulse Number
25
80
e)
-2
0
1
Pulse Number
25
80
f)
-2
0
1
Pulse Number
25
100
g)

or character of the suspensions is typical of surfactant, where ΔP_{min} occurs at or near the point at which the bubble is at a minimum radius. Figure (23f) shows that C1q alone, at a concentration of 0.10 mg · ml^{-1} has no effect on ΔP of the pulsated bubble. The tracing shown in figure (23g) indicates that albumin, at a concentration of 0.10 mg · ml^{-1} reduced the minimum surface tension (measured as ΔP) upon compression. This tracing also shows that, at this concentration of albumin, the phase or character is typical of a water soluble material which does not become insoluble at the interface.

Investigation of %C-reactive Protein/Total Protein with the Level of Lung Dysfunction and the APACHE II Score

The best antibody sandwich conditions for determining CRP concentration were established after consideration of two parameters: antibody and substrate concentration. Each of the two polyclonal antisera and the alkaline phosphatase conjugate were individually titred to determine the optimal dilution for this assay. This involved serially diluting each antiserum individually while maintaining the others, including the CRP standard, at a constant amount in excess. The two polyclonal antisera and the polyclonal antibody conjugate were used in the ELISA at a dilution which gave absorbances which were well above background (figure 24). This ensured that an excess of rabbit antihuman CRP was present to bind to the solid phase and an excess of goat antihuman CRP was present to bind to immobilized CRP. This process was repeated with the polyclonal antibody conjugate. It is interesting to note that the each of the three antisera showed a prozone-like effect at high concentrations. The prozone effect is frequently observed in haemagglutination titrations. It occurs when the response of wells which contain the highest

Figure 24. Absorbance versus dilution for polyclonal antisera of ○, rabbit anti-human CRP; ●, goat anti-human CRP, and ..., rabbit anti-goat alkaline phosphatase . ELISA procedures which were employed to determine these values were the same as those outlined in appendix 4, n=8 for each antibody species.


Absorbance at 405 nm
0.5
0.4
0.3
0.2
0.1
0.0
1000
10000
100000
Dilution

concentration of antisera fail to agglutinate test cells while higher dilutions agglutinate successfully. Several concentrations of the CRP standard were titred to determine which initial concentration of CRP became limiting at appropriate dilutions, which corresponded to wells of columns 8 to 12 of a (8 rows x 12 columns) ELISA plate.

The ELISA was calibrated by two means, one which used the sera of two acutely ill individuals in the ICU and one which used a commercially available human serum with a known concentration of CRP. In one control experiment, the CRP-depleted serum to which no CRP was added showed absorbance values which were equal to those of the blank wells. A known concentration of CRP was added to the CRP-depleted serum and the concentration of CRP, as determined by the ELISA, was 87% of that which was expected (determined independently of the ELISA). Also, a known concentration of CRP was added to another serum sample which was obtained from a patient in the ICU and had not been CRP depleted. The recovery of CRP was 107% of that which was calculated from adding the initial CRP concentration of the serum before addition of exogenous CRP, as determined by the ELISA, to the concentration of added CRP, as determined independently of the ELISA.

Assigned values of the commercially available human serum CRP standard were determined by the supplier by Nephalometry, TurbiTimeSystem, and Turbidimetry and were indicated to be 26.7 ± 4.0 mg $\cdot$ l^{-1}, 22.4 ± 3.4 mg $\cdot$ l^{-1}, and 21.0 ± 3.1 mg $\cdot$ l^{-1}, respectively, and represented means $\pm$ 95% confidence intervals. The CRP concentration of this serum, as determined by the ELISA was 24.8 ± 6.35 mg $\cdot$ l^{-1}. The concentrations of CRP in the test sera as determined by this assay were within the range of those which have been determined for healthy and ill individuals (e.g. Shine et al., 1981). Three sera samples were assayed repeatedly

for CRP by the ELISA to determine the reproducibility and similar values were found: sample (1) 294, 311, 281, 303, 273 mg · l^{-1}; sample (2) 74, 90, 81, 51, 72 mg · l^{-1}; sample (3) 54, 38, 42, 29, 23 mg · l^{-1}. Considering that the ELISA technique involves serially dilution of the analyte to extremely low concentrations, where one serial dilution represents a concentration difference of 2 these results suggest that the ELISA is both accurate and reproducible.

Figure 25 shows a plot of serum CRP as a proportion of total serum protein versus the APACHE II which was calculated from measurements which were performed in the first 24 hours of admission of 19 patients. A Pearson correlation coefficient of 0.24 was calculated for these data. This low correlation coefficient suggests that a weak correlation existed between elevation of serum CRP in relation to total serum protein and increased acute physiology score.

Figure 26 shows the serum CRP concentrations in two groups of ICU patients and in healthy volunteers. It shows that the levels of CRP in the sera of ICU patients at risk of ARDS and those who had this syndrome were higher than that of healthy volunteers. Since the CRP levels in the sera of the all healthy volunteers were below that of the ICU patients who are at risk of ARDS and are also below that of the ICU patients with ARDS, statistical analysis was inappropriate. A Wilcoxon rank sum test was performed, however, to determine if statistical significance did exist between the serum CRP levels of patients at risk of ARDS and those who had ARDS. The results are presented in Table 2a. The calculated critical T value was higher than the tabulated critical T value, therefore no significant difference existed between the serum CRP levels of these two groups.

The data presented in figure 26 indicated that the maximun serum CRP concentration obtained was 1.02 mg/ml. This is an extremely high level of CRP , however, the next highest

concentration of CRP in the sera of any of the ICU patients or of healthy volunteers was 430 μg/ml. Kew et al. (1990) found that patients at risk of ARDS and patients with ARDS had serum CRP concentrations of up to approximately 400 μg/ml. Therefore, the levels of CRP detected in the sera of acutely ill individuals in this thesis was similar to that which has been published elsewhere, save for one extreme measurement.

Serum CRP expressed as a percentage of total serum protein of three groups; healthy volunteers, ICU patients at risk of ARDS, and ICU patients with ARDS is shown in figure 27. This figure shows that patients at risk of developing ARDS and those who have developed this syndrome possessed elevated levels of serum CRP in relation to total serum protein compared to that of normal healthy individuals. Statistical analysis was not used to evaluate differences between the CRP levels expressed as a percentage of total serum protein of the healthy volunteer group and the two groups of ICU patients because the %CRP/total serum protein of all healthy volunteers was below that of the ICU patient at risk of ARDS and those who had ARDS. A Wilcoxon rank sum test was performed to determine if the %CRP/total serum protein of the patients who were at risk of ARDS was difference from that of patients who had ARDS in the ICU, see Table 2b. The calculated critical T value is larger than the tabulated critical T value, therefore the %CRP/total serum protein of these two groups was not significantly different.

Figure 25. CRP expressed as a percentage of total serum protein versus APACHE II score of 19 ICU patients. A Pearson correlation coefficient of 0.24 was calculated for the level of correlation of these two variables. CRP concentration was determined by an ELISA (appendix 4) and total serum protein was determined by a modification of the method of Lowry et al. (1951) (appendix 3). Samples were obtained within the first 24 hours of ICU.

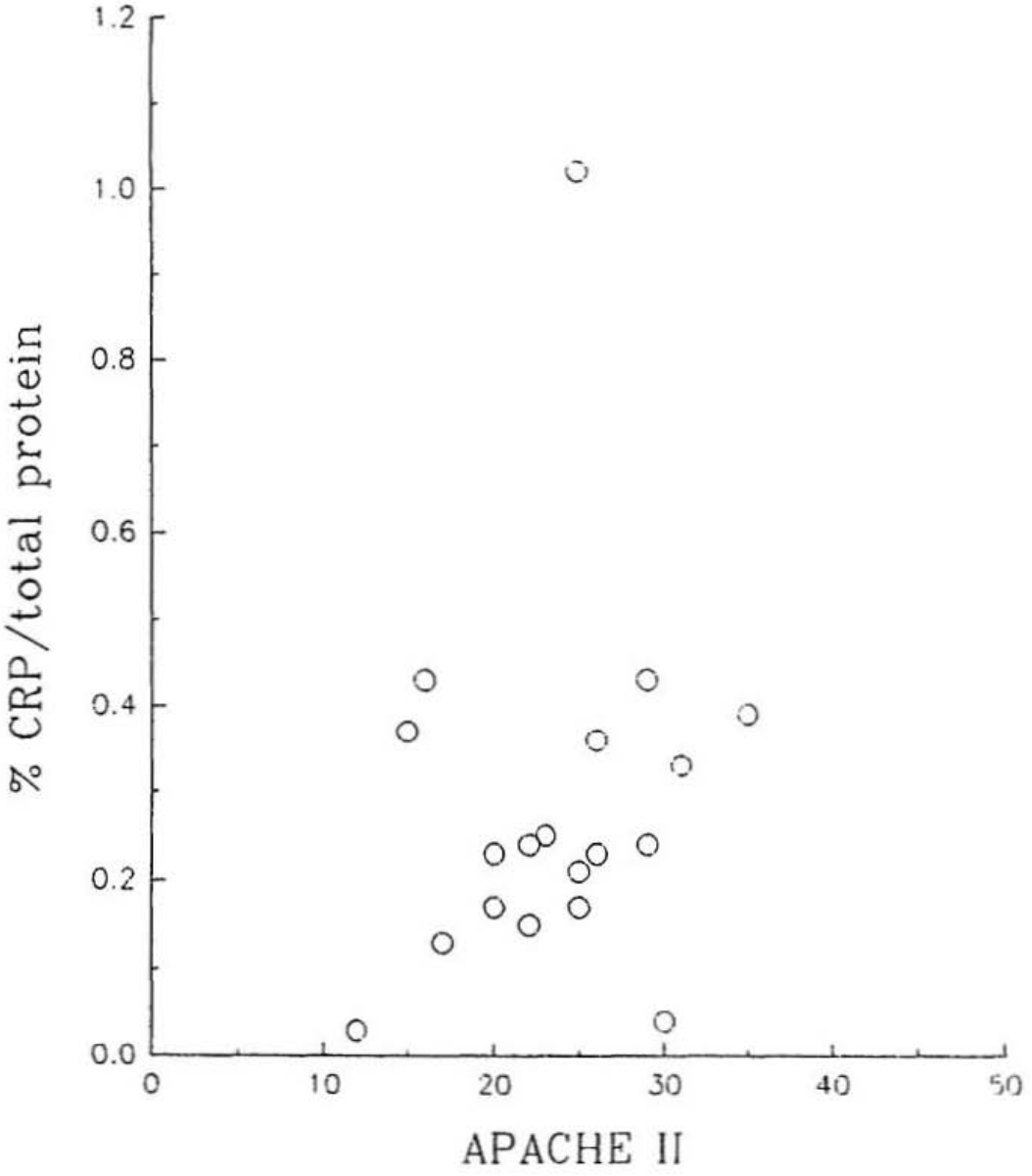

% CRP/total protein
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
APACHE II

Figure 26. Serum CRP concentrations of ICU patients who were divided into two groups; those who were at risk of ARDS (n=13) and those who had ARDS n=6). Also included is the serum CRP concentrations of 9 healthy volunteers, the symbols of which overlap. These data were statistically analysis using a Wilcoxon rank sum test, see Table 2a. CRP concentration was determined an ELISA (appendix 4) and total serum protein was determined by a modification of the method of Lowry et al. (1951) (appendix 3).

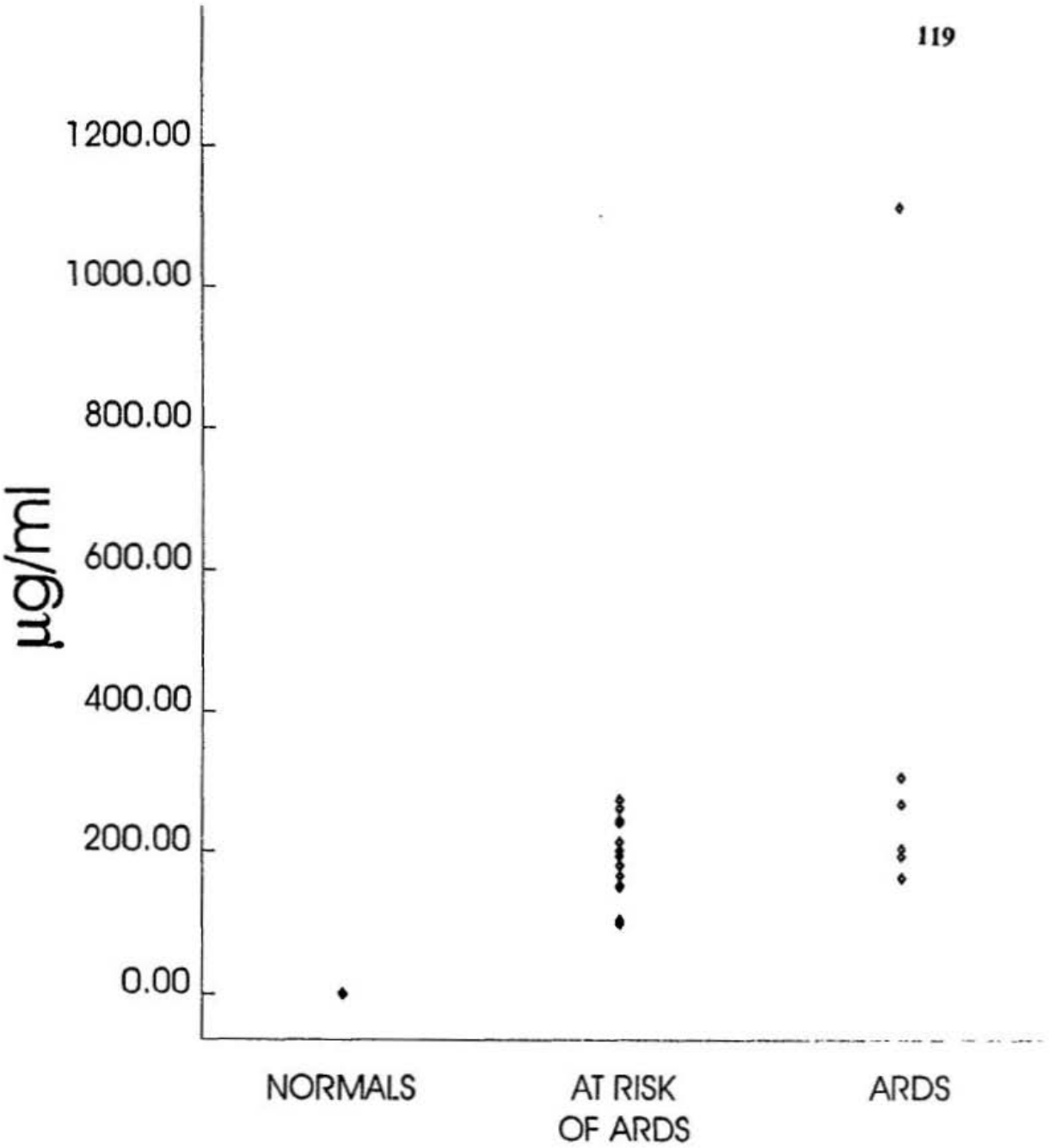
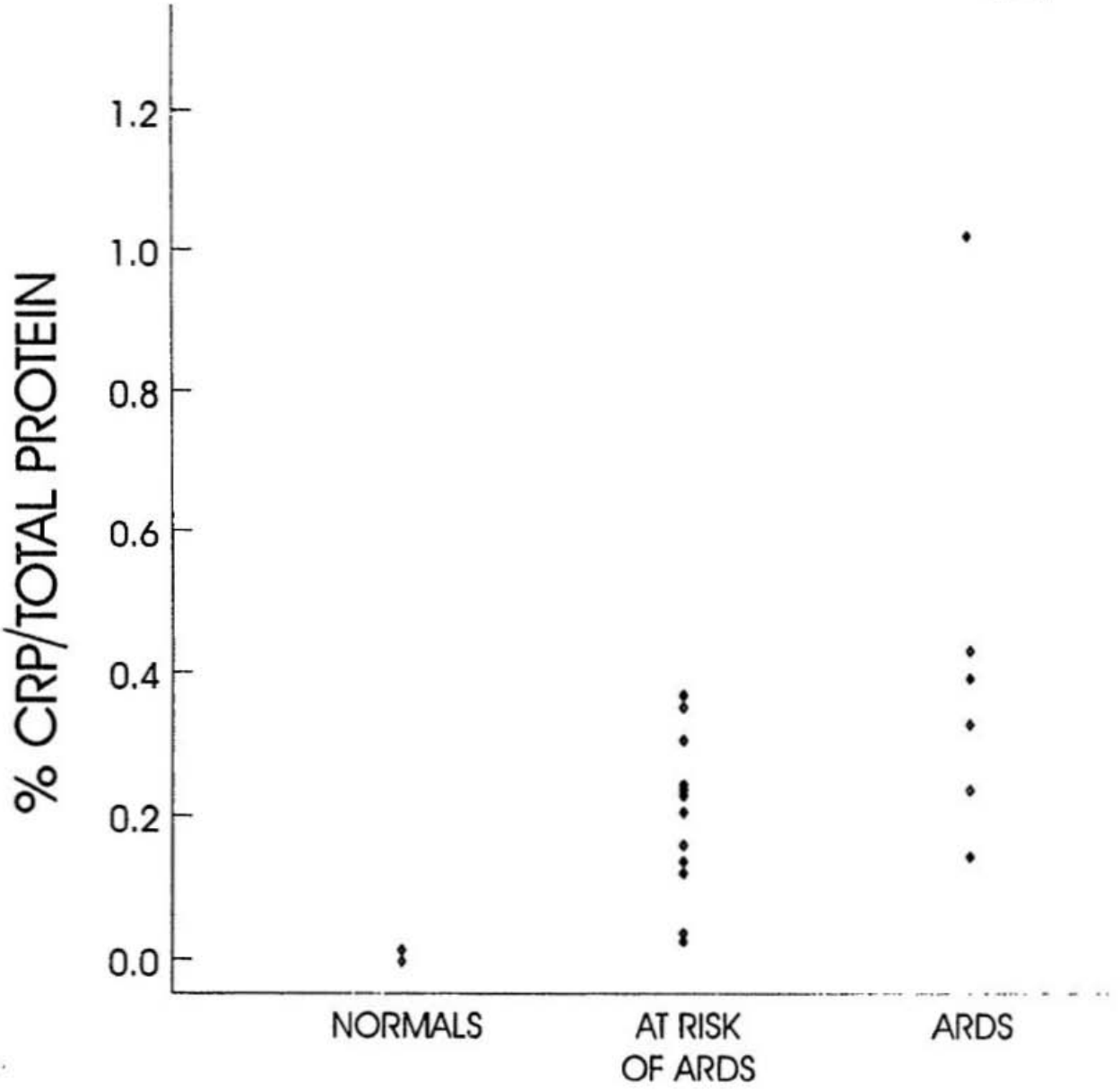

1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
µg/ml
NORMALS
AT RISK OF ARDS
ARDS

Figure 27. CRP expressed as a percentage of total serum protein for ICU patients who were divided into two groups; those who were at risk of ARDS (n=13) and those who had ARDS n=6). Also included is the %CRP/total serum protein of 9 healthy volunteers, the symbols of which overlap. These data were statistically analysis using a Wilcoxon rank sum test, see table 2b. CRP concentration was determined an ELISA (appendix 4) and total serum protein was determined by a modification of the method of Lowry et al. (1951) (appendix 3).


% CRP/TOTAL PROTEIN
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NORMALS
AT RISK OF ARDS
ARDS

a) $n_1 = 6$, ICU patients with ARDS
$n_2 = 13$, ICU patients at risk of ARDS
calculated critical T value = 77.0
tabulated critical T value at a 95% confidence level = 37
variable measured is serum CRP (μg/ml)

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b) $n_1 = 6$, ICU patients with ARDS
$n_2 = 13$, ICU patients at risk of ARDS
calculated critical T value = 74.5
tabulated critical T value at a 95% confidence level = 37
variable measured is serum %CRP/total serum protein

Table 2. Wilcoxon rank sum tests.

DISCUSSION

Mechanism by which C-reactive Protein Inhibits Pulmonary Surfactant Adsorption

Several of these studies were designed to determine what effect CRP has on the ability of natural porcine pulmonary surfactant to adsorb to the air-water interface and to help elucidate the mechanism by which it occurs and how it might be overcome.

Previous studies have demonstrated that fibrinogen and fibrin monomers and globulin inhibit the adsorption of pulmonary surfactant to the air-water interface. Fuchimukai et al. (1987), using a pulsating bubble surfactometer, showed that fibrinogen was a more potent inhibitor of the adsorption of artificial surfactant than albumin, serum, serum lipids, or bilirubin. Holm et al. (1988), using a surface adsorption apparatus, similar to that described by King and Clements (1972), found that fibrinogen, albumin, and haemoglobin possessed similar capacities to inhibit surfactant adsorption. Keough et al. (1989), also investigated the effect of fibrinogen on surfactant adsorption and compared it with the effect of albumin and globulin. They showed that fibrinogen was a more potent inhibitor of surfactant adsorption than albumin, however, it was not as potent as globulin. Cockshutt et al. (1991) showed that globulin, along with albumin, inhibited the adsorption of artificial surfactant. Seeger et al. (1993), using the pulsating bubble surfactometer, showed that fibrinogen was a more potent inhibitor of the adsorption of calf lung lipid extract on the surface activity of three kinds of artificial surfactant than albumin or haemoglobin. These results are consistent and suggest that fibrinogen and globulin are potent inhibitors of surfactant adsorption.

Our data agrees with that of others that calcium increases the rate of adsorption of

pulmonary surfactant to the air-water interface (e.g. Kobayashi & Robertson, 1983; Hawgood et al., 1985; Notter et al., 1986; Schürch et al., 1992). The mechanism by which calcium enhances surfactant adsorption has not yet been fully elucidated, its effect could be exerted through interactions with surfactant phospholipids, or with SP-A, or both.

Haagsman et al. (1990) have shown that SP-A binds two to three calcium ions per 35 KDa subunit in the carboxy-terminal ligand binding portion and that occupancy of a single high-affinity binding site by calcium induces a significant conformational change in SP-A. Haagsman et al. (1990) have also demonstrated that occupancy of both low- and high-affinity calcium binding sites was necessary for SP-A self-association. SP-A has been shown to induce a calcium-dependent aggregation of phospholipid vesicles prepared from surfactant extracts and from simple phospholipid mixtures (King & MacBeth, 1981; Hawgood, 1985). The formation of tubular myelin has been shown to be reversibly dependent on the presence of calcium (Gil & Reiss, 1973; Benson, 1984) and phospholipid aggregation has been speculated to be a prerequisite for the formation of tubular myelin (Efrati et al., 1987). More recent work by Suzuki et al. (1989) have supported the role of SP-A and calcium in the formation of tubular myelin. Lowering the pH from 7 to 4.4, in the absence of calcium, resulted in increased aggregation of liposomes composed of DPPC and PG, in the presence of SP-A (Efrati et al., 1987). They have shown that this process produced structures which were similar to tubular myelin, and that it increased the rate of adsorption of these structures to the air-water interface. The pH at which the protein-induced changes in lipid aggregation and increased rate of adsorption occurred was similar to the pI of SP-A (Efrati et al., 1987). The similarity of decreasing pH and addition of calcium suggest that charge neutralization of the carboxy-terminal of SP-A may be necessary to facilitate SP-A

aggregation (Efrati et al., 1987). It is likely that a significant conformation change or charge neutralization or a combination of both may be necessary to facilitate the self-association of SP-A. This, in the presence of calcium may promote the formation of tubular myelin-like structures which are associated with rapid adsorption of surfactant to the air-water interface.

The effect of calcium on lipid adsorption may not only involve its effect on SP-A aggregation but it may also involve an interaction with the hydrophobic surfactant-associated proteins, SP-B and SP-C, or surfactant phospholipids. It has previously been shown that calcium is required for optimal adsorption of lipid extract surfactant which is void of SP-A (Kobayashi & Robertson, 1983; Weber & Possmayer, 1984). The extent of ion binding to phospholipid head groups, especially to acidic phospholipids such a PG, is one important determinant of lipid bilayer aggregation (Ekerdt & Papahadjopoulos, 1982). Calcium has been shown to neutralize surface charge and decrease surface hydration of lipid vesicles and thus reduce swelling of lipid structures in water. Decreased surface hydration increases the tendency of lipid vesicles to orient at the air-water interface (Rand, 1981). King and Clements (1972) suggested that adsorption of pulmonary surfactant is rate limited by the energy barrier associated with hydrocarbon-water interactions occurring during the release of phospholipids as aggregates from the subphase into the air-water interface. Calcium binding to SP-A directly or to anionic phospholipids or to both would potentially decrease lipid bilayer stability (Ekerdt & Papahadjopoulos, 1982). Therefore, the binding of calcium to SP-A, hydrophobic surfactant proteins, or surfactant phospholipids, or a combination thereof may facilitate the adsorption of pulmonary surfactant to the air-water interface via the formation of tubular myelin-like structures or via a pathway which is independent of such unique structures.

Several investigators have suggested that plasma proteins impede the surface tension lowering ability of pulmonary surfactant by competing with surfactant for space at the air-water interface. Plasma proteins may integrate into the interface thereby impeding the surface tension lowering ability of surfactant (Holm et al., 1983; Keough et al., 1989; Seeger et al., 1993). Experiments, performed by Holm et al. (1988), using a surface adsorption apparatus, showed that the administration of albumin prior to or with the injection of surfactant resulted in substantial inhibition of surface adsorption. The injection of albumin into the subphase after surfactant film formation, however, had a much reduced inhibitory effect on surfactant function (Holm et al., 1988). At the concentrations used in the experiments presented here, water soluble CRP and fibrinogen adsorbed to the air-water interface at similar rates. The results presented here show that CRP inhibits the adsorption of porcine pulmonary surfactant to the air-water interface. Therefore, in the presence of surfactant, CRP, like fibrinogen, may adsorb with surfactant, enter the interface, and compete with surfactant for space. This may be one of the mechanisms by which CRP decreases the adsorption rate of surfactant. When mixed with surfactant, however, CRP had a higher capacity to inhibit the adsorption of surfactant to the air-water interface than fibrinogen. In fact, to approach the inhibitory capacity of CRP on surfactant adsorption in the presence of calcium, it was necessary for fibrinogen to be present with surfactant at twice the molar ratio and 5 to 6 times the weight at which CRP was present. Therefore, there may be an additional mechanism by which CRP decreases surfactant adsorption to the air-water interface. This is supported by the observation that, in the absence of surfactant, globulin adsorbed to the interface more quickly than CRP or fibrinogen, yet CRP was a more potent inhibitor of surfactant adsorption than globulin.

It has also been suggested that the inhibition of surfactant adsorption by plasma proteins might be, at least partially, the result of molecular interactions between plasma proteins with components of surfactant in the subphase (Holm et al., 1983; Fuchimukai et al., 1987; Seeger et al., 1993). These interactions might increase the stability of surfactant structures in the subphase, thereby preventing surfactant phospholipids from spreading as they approach the interface or respreading after surfactant film collapse (Fuchimukai et al., 1987; Seeger et al., 1993).

Our results support previous observations that in the absence of plasma proteins, optimal adsorption of surfactant requires calcium (see above discussion). Therefore, if a protein inhibits surfactant adsorption primarily by competing with surfactant for surface space, then one would expect surfactant to more effectively resist the deleterious effects of that protein in the presence of calcium then in its absence. Such an occurrence has been shown for the inhibitory effect of albumin on surfactant adsorption (Holm et al., 1983). It has been shown here that, in the presence of CRP, however, calcium did not enable surfactant to more effectively resist adsorption inhibition. In fact, calcium was required for the potent inhibitory effect of CRP on surfactant adsorption. This suggests that the potent inhibitory effect of CRP on surfactant adsorption is primarily the result of a calcium-dependent interaction between CRP and a surfactant component(s) in the subphase.

Recent studies using solid phase-immobilized lipids have shown that the reactivity of CRP for phosphatidylcholine was higher than that for phosphatidylethanolamine or phosphatidylglycerol (A. Szalai, personal communication). At the concentration used in the presented experiments, phosphocholine almost completely eliminated the inhibitory capacity of CRP with respect to surfactant adsorption. The addition of lower concentrations of phosphocholine to mixtures of

surfactant + CRP caused a concentration dependent reduction of the inhibition of surfactant adsorption by CRP. Also, the addition of phosphocholine to a mixture of CRP and surfactant, which was already in the subphase, was shown to partially reverse the inhibitory effect of CRP on surfactant adsorption. These results are consistent with the idea that a specific interaction occurs between CRP and the phosphocholine headgroup of surfactant lipids. Since phosphatidylcholine is the major surfactant lipid, this is not an unreasonable suggestion.

CRP may also bind to phosphatidylglycerol and phosphatidylethanolamine, two other phospholipids which are present in pulmonary surfactant, albeit at lower concentrations than phosphatidylcholine. Phosphocholine was able to decrease the inhibitory effect of CRP on surfactant adsorption while the other two CRP ligands were relatively ineffective in this respect. If CRP does interact with these phosphatidylglycerol and phosphatidylethanolamine, it is likely that the binding would not be as strong as a binding to phophatidylcholine because of the relative abundance of phosphatidylcholine and the high affinity with which it binds to CRP compared to the other two surfactant phospholipids.

Phospholipids may not be the only "targets" of inhibitory proteins. It has been suggested fibrinogen may impede the surface activity of pulmonary surfactant by interacting with a surfactant protein (Seeger et al., 1993). Studies with SP-B peptides suggested that they may partition within the phospholipid bilayer in contact with both polar head groups and acyl chains (Cochrane & Revak, 1991), thus being partially exposed to the aqueous environment. Recently, it has been suggested that the tertiary structure of SP-B may contain kringle-like structures which are known to interact strongly with fibrinogen (Johanson et al., 1991). Seeger et al. (1993) suggested that a specific interaction between fibrinogen or its degradation products, and SP-B may

be one mechanism by which such proteins impair the adsorption of surfactant to the air-water interface.

Although the precise biological role of CRP remains unclear, its functional similarities to antibodies in terms of its ability to act as a precipitin, an agglutinin, an opsonin, and as an activator of the classical complement system are well documented (e.g. Kaplan & Volanakis, 1974). CRP has been shown to bind to phosphocholine (e.g. Volanakis & Kilpatrick, 1971) and Kilpatrick & Volanakis (1974) have shown that CRP also binds to C1q. The CRP binding sites for phosphocholine (Liu et al., 1987) and the sites at which C1q binds CRP have been delineated (Jiang et al., 1992). Binding of CRP to phosphocholine residues of C-polysaccharide has been shown to activate early part of the classical complement cascade (Kaplan & Volanakis, 1974) via an interaction with C1q (Claus et al., 1977). Therefore, CRP can bind to phosphocholine and C1q simultaneously, presumably via distinct binding sites. Although SP-A can not substitute for C1q in the formation of haemolytically active C1 (Tenner et al., 1991), it can bind to C1q receptors of U937 cells and upregulate C1q receptor production of these cells (Malhotra et al., 1992). Therefore, it is possible that CRP may bind to the hydrophilic surfactant protein SP-A, although more research needs to be undertaken to explore this possibility. If an interaction between CRP and SP-A does occur, it is likely not to be as strong as that between CRP and phosphatidylcholine since the addition of a sufficient quantity of phosphocholine almost completely eliminated the inhibitory effect of CRP on surfactant adsorption.

The pressure tracing of a bubble formed in a solution of CRP alone showed a behavior which was similar to that which Keough et al. (1989) have shown for albumin, fibrinogen, and globulin, ΔP is largest at minimum bubble radius. This eventually reversed such that ΔP_{min}

occurred at minimum bubble radius. It has been shown here that, even after 8 minutes (160 pulses), CRP reduced the ΔP of the bubble air-water interface at minimum bubble radius by a only small degree. When the bubbles were initially formed in a mixture of CRP + surfactant, the initial pressure difference across the bubble interface at minimum bubble size was higher than that of a suspension of surfactant alone. This difference, however, is not large. This indicates that a small amount of CRP adsorbs, along with surfactant, to the bubble surface. Therefore, CRP may prevent surfactant from entering the air-water interface and lowering the surface tension by competition for surface space. Keough et al. (1989) stated that if an inhibitory protein competitively adsorbed with surfactant for surface space, then the initial pressure tracing should resemble those of the protein alone. The experiments of presented in this thesis show that the initial pressure tracing of mixtures of CRP + surfactant, however, resembled that of surfactant alone. This suggests that, at the concentration of CRP and pulmonary surfactant used in these experiments, the inhibitory effect of CRP on the surface tension lowering ability of surfactant upon compression most likely does not occur primarily because of competition with surfactant for surface space. Two mechanisms may be primarily responsible for the inhibitory effect of CRP on surfactant adsorption during dynamic compression. A small amount of CRP which has adsorbed to the air-water interface may be remove from the interface during dynamic compression. As this occurs CRP may remove some surfactant phospholipids (DPPC in particular) from the interface thus inhibiting the surface tension lowering ability of the adsorbed surfactant film upon compression. Alternatively, CRP, in the subphase, may prevent surfactant from spreading or respreading at the air-water interface thus inhibiting the initial opening and impeding the surface tension lowering ability of surfactant upon compression. A combination of

both mechanisms may be involved. These results suggest that the mechanism by which CRP inhibits surfactant adsorption during dynamic compression occurs primarily because of an interaction with CRP and the phosphatidylcholine component of surfactant. They also suggest that the inhibitory effect of CRP on the performance of pulmonary surfactant in the pulsating bubble surfactometer is not exclusively due to physical competition with surfactant for surface space. These results are in keeping with those obtained from the surface adsorption apparatus, and are consistent with the main mode of inhibition of surfactant by CRP as being through its binding to the lipids of surfactant. The fact that the CRP inhibition can be reversed by phosphocholine may have therapeutic implications.

The Effect of C1q on the Adsorption of Lipid Extract Pulmonary Surfactant

Several investigators have shown that the surfactant protein SP-A facilitates the adsorption of pulmonary surfactant to the air-water interface. SP-A has been shown to alter the state of phospholipid dispersions in the presence of calcium, and the hydrophilic surfactant proteins SP-B and SP-C (e.g. King & MacBeth, 1981; Hawgood, 1985). The presence of SP-A has been shown to enhance the rate of formation of adsorbed films of surfactant mixtures which were composed of phospholipids, SP-B and SP-C (King & MacBeth, 1981; Suzuki, 1982; Notter et al., 1983; Wright et al., 1984; Hawgood et al., 1985; Efrati et al., 1987). At calcium concentrations which are comparable to that found in the alveolar subphase, liposomes of DPPC and PG, in the presence of the surfactant proteins SP-B and SP-C, but not SP-A, aggregate minimally and form films at the air-water interface more slowly than in the presence of SP-A (Neilson, 1984). Efrati et al. (1987) have speculated that phospholipid aggregation is a prerequisite for tubular myelin formation, and Suzuki et al. (1989) and Williams et al. (1991) have further supported the role of

SP-A in the formation of this unique structure. Using freeze-fracture electron microscopy of mixtures of pulmonary surfactant, Sen et al. (1988) have demonstrated that tubular myelin-like structures exist at and very near the air-water interface. The presence of tubular myelin-like structures has been shown to be associated with rapid adsorption of pulmonary surfactant (Magoon et al., 1983; Notter et al., 1986; Benson et al., 1984; Efrati et al., 1987). Therefore, circumstantial evidence suggests that the presence of SP-A is a prerequisite for tubular myelin formation, and tubular myelin is believed to facilitate the adsorption of pulmonary surfactant to the air-water interface.

Some authors, however, have shown that the presence of SP-A and tubular myelin are not absolutely necessary for the rapid adsorption of lipid-extract pulmonary surfactant. Metcalfe et al. (1980) have shown that calf lung lipid extract adsorbed to the air-water interface as rapidly as whole calf lung surfactant. Later, Notter et al. (1986) have shown that calf lung lipid extract, which is void of SP-A, adsorbed rapidly to the air-water interface. Aqueous suspensions of this surfactant did not contain structures which resembled tubular myelin. Notter et al. (1986) have shown, however, that surfactant suspensions which adsorbed rapidly contained thin-walled surfactant liposomes along with less ordered open microstructures. They speculated that, although tubular myelin was not present, these open structures facilitated the delivery and spreading of surfactant lipids to the air-water interface. We do not know what type of microstructures were present in the surfactant suspensions studied here.

We have shown that in the presence of calcium, lipid extract surfactant, which is void of SP-A, did not adsorb as rapidly as whole surfactant. The concentration of SP-A in pulmonary surfactant has been estimated to be 5% to 10% of the phospholipid concentration, by weight. The

addition of C1q to lipid extract surfactant, at 10% of the phospholipid content, by weight, has been shown here to increase the adsorption rate of surfactant lipids to the air-water interface, compared to the lipid extract alone. It has also been shown that this mixture of C1q and lipid extract surfactant adsorbed more quickly than that which would have occurred if the C1q and lipid extract surfactant were injected independently. This suggests that C1q interacts with components in the lipid extract surfactant in the subphase and facilitates the delivery and spreading of surfactant lipids at the air-water interface. Also, C1q did not reduce the equilibrium spreading pressure of adsorbed surfactant films. In these ways C1q is functionally homologous to SP-A.

The results of experiments with the pulsating bubble surfactometer support the suggestion that C1q increases the adsorption rate of lipid extract surfactant by an interaction with surfactant components. They also demonstrate that C1q, unlike globulin, increases the adsorption rate of lipid extract surfactant. Also, C1q does not interfere with the ability of the adsorbed surfactant film attaining a low surface tension upon compression.

Bubbles which were formed in solutions of lipid extract surfactant + 5 and 10% C1q had higher pressure differences across the bubble interface upon initial bubble formation than bubbles which were created in lipid extract surfactant alone. C1q, by itself, however, did not measurably reduce the subphase surface tension. Also, bubbles in a dispersion containing 5% C1q and lipid extract surfactant had higher pressure differences across the bubble interface upon initial bubble formed in suspensions of lipid extract surfactant + 10% C1q.

The effect of 5% and 10% C1q on the adsorption of lipid extract surfactant to the air-water interface suggests that an interaction occurs between C1q and components of lipid extract

surfactant. It required 160 pulses (8 minutes) for the lipid extract surfactant, which is void of SP-A but contained the hydrophobic surfactant proteins SP-B and SP-C, to attain a ΔP_{min} of approximately zero. Ten percent C1q increased the adsorption rate (and rate of surface refining, see below) of lipid extract surfactant such that this mixture adsorbed as rapidly as whole surfactant, which contains SP-A. The addition of 5% C1q to lipid extract surfactant produced an adsorption rate which was intermediate between that of the lipid extract surfactant alone and that of lipid extract surfactant + 10% C1q. The increased adsorption rate of lipid extract surfactant which was dependent on the concentration of C1q, suggests that an interaction occurs between C1q and lipid extract surfactant components.

The experiments presented here show that the effect of C1q on the adsorption of lipid extract pulmonary surfactant may be unique, compared to that of other serum proteins. C1q, via a tertiary and quaternary structural homology with SP-A, may increase the rate of adsorption of lipid extract surfactant to the air-water interface. An identical amount of globulin, however, decreased the adsorption rate of the lipid extract surfactant. Others have shown that globulin and other serum proteins such as albumin and fibrinogen, inhibit the adsorption of surfactant to the air-water interface during dynamic compression (e.g. Keough et al., 1989). In this respect, the behaviour of C1q is different from that of other serum proteins.

Another mechanism, however, may be partially responsible for the more rapid lowering of the ΔP_{min} across the interface of bubbles formed form lipid extract surfactant in the presence of C1q than in its absence. Schurch et al. (1989) have shown that repeated cycling of films of pulmonary surfactant produced films with pressure-area hysteresis curves which more closely resembled that of DPPC than the original surfactant film. They interpreted this as resulting from

the selective exclusion of non-DPPC film components. It has been previously suggested that SP-A may increase the removal of non-DPPC components from surfactant films during compression (e.g. Cockshutt et al., 1990). Therefore, in our experiments, it is possible that C1q may not only be increasing the rate of adsorption of lipid extract surfactant but it may also facilitate the rate at which non-DPPC film components are removed from the interface, in a similar manner as that which has been speculated to occur for SP-A. This would more quickly produce a DPPC-rich surfactant film which has been shown to be necessary for the low surface tension which pulmonary surfactant is capable of attaining. Therefore, a molecular interaction(s) would be necessary if C1q does increase the rate at which non-DPPC components are removed from surfactant films, which are void of SP-A. It is not possible from these experiments to determine the quantity of C1q at the bubble interface, although from control experiments, the amount of C1q at the air-water interface would not appear to be very large since it did not reduce the surface tension of the bubble interface by a substantial amount. Even if interactions do occur between C1q and components(s) of the lipid extract surfactant in the air-water interface any C1q molecules at the bubble interface must not interfere with the removal of non-DPPC surfactant components. Also, C1q itself must be effectively displaced from the interface, and it must not remove DPPC from the surface.

The precise mechanism by which C1q facilitates the adsorption of lipid extract surfactant is not clear. C1q may interact with surfactant lipids, hydrophobic surfactant proteins, or both, in the subphase thereby increasing the rate of adsorption of lipid extract surfactant. Such interactions may facilitate the delivery of surfactant lipids to the air-water interface or it may produce surfactant structures which more easily spread or respread at the air-water interface. In

addition to this mechanism, C1q may also increase the rate at which non-DPPC surfactant film components are removed from the bubble surface. If this second mechanism does not occur, C1q must be effectively squeezed-out of the interface without removing any of the DPPC component of the surfactant film. Perhaps some of the beneficial effects of SP-A with respect to the surface adsorption and surface tension lowering ability of pulmonary surfactant are related in aspects of its structure which are reflected in C1q.

Serum C-reactive Protein Levels and Adult Respiratory Distress Syndrome

Many of the etiologies of ARDS such as infection, sepsis, and trauma (e.g. Bernard & Brigham, 1985) have also been shown to produce the acute phase response. Therefore, it is likely that serum CRP will be elevated in a number of instances where patients have ARDS or at risk of developing this syndrome. Indeed, Kew et al. (1990) have shown that, in serum of sera of patients with ARDS, CRP is elevated compared to normals and it is also elevated in the sera of patients who are at risk of ARDS compared to that of normals. Our data supports the work of Kew et al. (1990) in showing that the level of serum CRP is elevated in patients with ARDS and patients who are at risk of this syndrome compared to that of normal healthy volunteers. We were not able to confirm, however, that serum CRP levels of patients with ARDS is elevated compared to patients who were at risk of this syndrome. This may be a result of the small sample size of this study.

Pulmonary edema is the hallmark of ARDS. It has been suspected since the 1960's that the impaired surfactant function results from the presence plasma proteins in the lung and this is thought to contribute to the pathophysiology of RDS (e.g. Taylor & Abrams, 1966) and ARDS (Ashbaugh et al., 1967). The pulmonary vascular damage which occurs during the development

of ARDS, varies from increased permeability of the pulmonary vasculature to areas of haemorrhage (e.g. Weiner-Kronish et al., 1990). Therefore, the relative concentrations of serum proteins in the serum should be reflected in the concentration of the these proteins in the alveolar space. During the acute phase response, the concentration of CRP in the serum can increase up to 1000-fold, therefore, it is likely that CRP will also be increased in the alveolar fluid of ARDS patients and those who are at risk of this syndrome. This premise was investigated by Li et al. (1989) who showed that the concentration of CRP in the BAL of patients with ARDS was significantly elevated compared to that of normals. They (Li et al., 1989) found that this level of CRP in patients with ARDS can increase from0.4% of total lavage protein in normal adults to approximately 10% of total lavage protein in patients with ARDS. The ratio of total protein to total phoshphlipid in BAL obtained from pateints with ARDS has been determined to be approximately 6 : 1 by weight (T. J. Gregory, personal communication). Therefore, a CRP : surfactant phospholipid ratio of approximately 0.6 : 1 (w/w) may exist in the edematous lung *in vivo*.

Tillett and Francis (1930) were the first to discover CRP in the sera of patients with pneumonia. Since then, many investigators have been interested in the acute phase response and how knowledge of changes in the concentration of acute phase proteins could provide clinically sueful information. ARDS is a well known cause of acute respiratory failure that can occur after a variety of pulmonary or systemic insults. This includes pulmonary and non-pulmonary sepsis, shock, thoracic and non-thoracic trauma (e.g. Rogers & Levin, 1988). In fact, sepsis is the most common clinical condition associated with ARDS with 20-40% of patients with sepsis developing ARDS (wiener-Kronish et al., 1990). It has been shown that the degree of elevation of CRP

corresponds reasonably well with the severity of infection (e.g. Sabel & Hanson, 1974; Kushner & Feldman, 1978; Sabel & Wadsworth, 1979; Macintry et al., 1982) and with the severity of tissue damage (Kushner et al., 1978; deBeer et al., 1982).

Serum C-reactive Protein Levels and Chronic Health Evaluation

Griner (1972) has suggested that there is a need for a system which standardizes information of patients admitted and treated in I.C.U.s. This would facilitate both the evaluation of intensive care and assessment of new therapies (Knaus et al., 1981, and references cited within). A widely accepted index for determining the severity of illness is a scale which assesses the probability of mortality (Kricker, 1976). The first widely accepted scoring system to classify groups of acutely ill patients based on severity of illness was the APAHCE I scoring system (Knaus et al., 1981). Simplification of the APACHE I system with the intention of providing a more clincially yet stastically accurate and valid patient classification system, the APAHCE II scoring system (Knaus et al., 1985).

Our results show that a weak correlation exists between the level of CRPin the sera of I.C.U patients and the APAHCE II scores which the patients receive. A larger sample size would more accurately represent the actual population and allow the use of more powerful parametric stastical analysis. Therefore, these results suggest that a larger investigation be undertaken to more accurately determine if the measurement of CRP levels should included in a scoring system for I.C.U. patients such as the APAHCE II.

CONCLUSION

The results which are presented here show that CRP, at physiologically plausable concentrations, is a potent inhibitor of pulmonary surfactant adsorption. This inhibitory effect required the presence of calcium, and was eliminated and reversed by the addition of the water soluble CRP ligand phosphocholine, at a molar ratio of 300 : 1, phosphocholine : CRP. Phosphocholine at this molar ratio, approximately 2 mM, did not influence the adsorption characteristics or the equilibrium spreading pressure of porcine pulmonary surfactant. Therefore, an interaction may occur between CRP and the headgroup of dipalmitoylphosphatidylcholine, predominantly in the subphase and possibly at the air-water interface. The results presented here suggest that CRP may contribute substantially to the lung dysfunction which is associated with ARDS. They also suggest that the addition of a small amount of phosphocholine to patients with ARDS may be a beneficial therapeutic treatment for this syndrome.

These data show that the complement protein C1q, increases the adsorption os lipid extract pulmonary surfactant which is void of SP-A. It is likely that this effect of C1q is due to a similar molecular mechanism as that which occurs between SP-A and and the other components of pulmonary surfactant.

It has been shown that serum CRP levels were significantly elevated in patients with ARDS and in patients at risk of ARDS compared to normals. It has also been shown that a weak correlation exists between the level of CRP in the serum of ICU patients and the APACHE II score. Therefore, further research should be undertaken to more accuralted determine the clinical usefulness of measuring serum CRP levels in patients in the ICU.

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APPENDCES (Methods and Protocols)

(1) Modification of the Method of King and Clements (1972) for Surfactant Recovery by Lavage

All procedures were performed at 4°C.

1. Lavage was centrifuged at 800 x g_{av} for 10 minutes. (Sorvall RC-3; Sorvall HG-4L rotor; 1650 rpm).

2. The supernatant of (1) was centrifuged at 8000 x g_{av} for 1 hour. (Sorvall RC2-B; Sorvall GS-3 rotor or a Beckman J2-21; Beckman JA-10 rotor; 7000 rpm).

3. White portions of the pellets from (2) were homogenized with the aid of a glass tissue homogenizer (with a ground glass pestle) and suspended in the following (M): NaCl, 0.15; $MgCl_2$ 0.035; NaBr, 1.64; Tris-HCL 0.005; pH=7.35. The pellets, homogenized in the sodium bromide solution, were centrifuged overnight at 81 500 x g_{av}. (Beckman L5-50B; Beckman SW-28 rotor; 25 000 rpm). If pellicles did not form because of a high lipid content of the sodium bromide gradient the lipid content was diluted by one half and centrifuged again.

4. The resultant pellicles were homogenized and suspended in the following (M): NaCl, 0.15; Tris-HCL, 0.005; pH=7.35. The suspension was centrifuged at 61 900 x g_{av} for 2 hours. (Beckman L5-50; Beckman 60 Ti rotor; 31 000 rpm).

5. Pellets from (4) were homogenized and suspended in a minimal volume of 0.15 M NaCl, placed in Nalgene Cryovials (~1 ml per tube) and stored at -70°C.

(2) Phosphorus Determination by a Modification of the Method of Bartlett (1959) as Described by Keough and Kariel (1987).

Materials:

chromic acid washed glass tubes

phosphorus standard (2 $\mu g \cdot ml^{-1}$, KH_2PO_4)

perchloric acid (70%)

ANSA (1-amino-2-napthol-4-sulfonic acid), (0.25 %)

ammonium molybdate (5%)

anti-bumping granules

Method:

1. 1 ml of perchloric acid and anti-bumping granules were added to all tubes.

2. The phosphorus standard (2 μg/ml) and surfactant samples (1-10 μg/ml phosphorus) were added to the respective tubes. The tubes were vortexed well.

3. The samples were digested by boiling for 12 minutes and were allowed to cool. Water was added so that the final volume of each tube was 9 ml.

4. 0.5 ml ammonium molybdate was added to all tubes and vortexed well.

5. 0.5 ml ANSA was added to all tubes and vortexed well.

6. The tubes were immersed in boiling water for 12 minutes, removed and were allowed to cool.

7. Optical density was measured with a spectrophotometer (LKB Biochrom, 4049 Novaspec) at 815 nm which had been calibrated with water. Samples were assayed in triplicate and the phosphorus concentration of the sample was determined from the average of these values. The phosphorus concentration of the surfactant suspensions was converted into phospholipid

concentration by multiplying by 25.

(3) Protein Determination by a Modification of the Method of Lowry et al. (1951).

Materials:

disposable test tubes

protein standard (crystaline bovine serum albumin)

sodium dodecyl sulphate (SDS), (10%)

Folin & Ciocalteau's phenol reagent (2.0 N)

reagent A: 100 g of Na_2CO_3 in 0.5 M NaOH, H_2O was added for a final volume of 1 l and was protected from light.

reagent B: 1 g of $CuSO_4 \cdot 5H_2O$ was added to H_2O with a final volume 100 ml. and was protected from light.

reagent C: 2 g of sodium potassium tartrate was added to H_2O with a final volume of 100 ml.

Method:

1. Protein standard (2.0 mg/ml) and samples (1-10 mg/ml total protein) were added to respective glass culture tubes.

2. If required, 100 μl of 10% SDS was added to all tubes.

3. The volume of liquid in each tube was brought to 1.0 ml by adding an appropriate amount of H_2O and vortexed well.

4. 15 ml of reagent A, 0.75 ml of reagent B and 0.75 ml of reagent C were mixed and 1 ml of the resultant solution was added to each tube and vortexed well.

5. The tubes were incubated for 15 minutes at room temperature.

7. While the tubes were incubating, 5.0 ml of 2 N Folin & Ciocalteau's phenol reagent was mixed with 50 ml of H_2O.

8. At the conclusion of the incubation period, 3.0 ml of the solution which was made in (7) was forcibly pipetted into each tube and immediately vortexed before preceeding to the next tube. This step was executed as quickly as possible.

9. Samples were incubated at room temperature for 45 minutes then the optical density is determined spectrophotometrically (LKB Biochrom, 4049 Novaspec) at 540 nm. Samples were assayed in triplicate and the sample protein concentration was determined from the average of these values.

(4) Enzyme-linked Immunosorbent Assay (ELISA): a Polyclonal Double Antibody Sandwich

Materials:

96 well (8 rows x 12 columns) flat bottom polystyrene microtitration plates (Linbro Titertek, Horsham, PA)

8 channel ELISA manual plate washer (Corning, New York, NY)

8 channel 100 μl pipette (Costar, Cambridge, MA)

ELISA plate reader (BIO-RAD, Richmond, CA)

phosphate buffered saline: (PBS)

NaN_3 0.20 g; NaCl 8.0 g; KH_2PO_4 0.20 g; Na_2HPO_4 1.15 g; KCl 0.20 g; add H_2O for a final volume of 1 l; pH=7.4

coating buffer: (carbonate-bicarbonate)

Na_2CO_3 1.59 g; $NaHCO_3$ 2.93 g; NaN_3 0.20 g; add H_2O for a final volume of 1 l; pH=9.6

blocking solution: PBS-gelatin (0.5%)

diluent solution: PBS-gelatin (0.25%)-Tween (0.05%)

washing solution: PBS-Tween (0.05%)

substrate buffer: (diethanolamine buffer)

NaN_3 0.20 g; $MgCl_2 \cdot 6H_2O$ 0.10 g; 97 ml of diethanolamine (approx. 98%); add H_2O for a final volume of 1 l; pH=9.8

alkaline phosphatase substrate:

p-nitrophenol phosphate, (40 mg per tablet)

protein standard: human CRP, (> 95% pure by SDS-PAGE) (Calbiochem, La Jolla, CA)

polyclonal antisera:

rabbit anti-human CRP (Sigma, ST. Louis, MO)

goat anti-human CRP (Sigma, ST. Louis, MO)

immunoglobulin:

rabbit anti-goat (IgG) alkaline phosphatase (Sigma, ST. Louis, MO)

Method:

1. Microtitration plates were coated with 100 μl/well of rabbit anti-human CRP (1 : 4000 dilution) in carbonate coating buffer and incubated overnight at 4°C. All subsequent procedures were performed at room temperature.

2. The solution of (1) was removed by inverting the plates and shaking. The plates were dried by tapping on a paper towel. The plates were blocked with PBS-gelatin (200 μl/well) and incubated for 1 hour.

3. The plates were washed 3 times and dried. 100 μl of serum or CRP standard was added to the appropriate wells, serially diluted and incubated for 1 hour.

4. The plates were again washed 3 times and dried. 100 μl of goat anti-human CRP (1 : 4000 dilution) was added to each well, and incubated for 1 hour.

5. After washing the plates 3 times and drying, 100 μl of rabbit anti-goat alkaline phosphatase (1 : 5000 dilution) was added to each well and the plates were again incubated for 1 hour.

6. While the plates were incubating the substrate solution was made by adding 1 40 mg tablet of phosphatase substrate to 40 ml of substrate buffer. This was stored in the dark. The plates were then washed 5 times and dried. 100 μl of substrate solution was added to each well and stored in the dark until the reaction was completed. The reaction was determined to be complete when color development in each well which contained the samples and standard was more intense than

the blank wells. Due to viariability in the handling of each plate, with respect to rate of color development, each plate contained 3 rows in which a standard was assayed.

7. Colour development was stopped by adding 2 M NaOH to each well.

8. Absorbance was measured at 405 nm and the background was subtracted.

(5) Instructions for Opperating the Pulsating Bubble Surfactometer (Enhorning, 1977)

1. The power was turned on and the heater switch was engaged (temperature was set at 37°C).

2. The piston chamber, which is in connected hydraulically to the sample which is being assayed was cleaned by flushing the water bath 3 times with distilled water which has been boiled and placed in a Travenol Viaflex 1000 ml capacity fliud container and connected to the water flushing mechanism. The water was removed using a Pastieur pipette. The water bath was filled to the full mark which is a point which is just below a piece of filter paper which is held down on the top of the water bath chamber by a spring. The filter paper is used to absorb excess H_2O. The bath chamber was raised and covered by rotating the plastic lid over the chamber.

3. With the pressure transduces in the off position, the recorder turned on to the low speed (25 mm/s) and the pen was placed in the zero position on the chart paper with the pen position knob.

4. With the pressure transducer on, the calibration knob was pressed (the signal it produces is proportional to -2 cm H_2O). The sensitivity knob was used to place the pen at the -2 cm of water on the chart paper. The pressure transducer and the chart recorder were turned off and the water was removed.

5. The water bath chamber was lowered and locked into place.

6. Using a 50 μl Hamilton syringe, a sample chamber was filled with the surfactant, protein, or surfactant + protein mixtures to be tested while making sure that there were no air bubbles in the chamber. With the bubble adjustment knob in the full counterclockwise position, the sample chamber was placed over the piston and clamped in place.

7. The bubble adjustment knob was rotated fully clockwice and the excess fluid on the top of the sample chamber was absorbed with tissue paper. The water bath was raised and covered. The

sample was allowed to equilibrate to 37°C.

8. The chart recorder was turned on, then the pressure transducer and the pulsator, and the bubble adjustment knob was rotated so that the initial bubble radius was 0.55 mm. The bubble was subsequently pulsated between minimum and minimun bubble radii at a rate of 20 pulses/minute.

9. After the desired number of cycles, the recorder, pulsator and pressure transducer were turned off. The water bath was lowered, the sample chamber was removed. The water bath was raise the piston chamber was washed 3 times (see above). These steps were executed during every experimental test.