1991

An In Vitro Examination Of Surface Tension Reduction By Pulmonary Surfactant In The Presence And Absence Of Inhibitory Agents

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AN IN VITRO EXAMINATION OF SURFACE TENSION REDUCTION BY PULMONARY SURFACTANT IN THE PRESENCE AND ABSENCE OF INHIBITORY AGENTS

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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The University of Western Ontario
London, Ontario
July, 1991

ISBN 0-315-66288-3

ABSTRACT

The surface activity of Lipid Extract Surfactant (LES), a clinical preparation used for the treatment of Neonatal Respiratory Distress Syndrome, and the effects of addition of surfactant-associated protein A (SP-A) and palmitic acid were assayed *in vitro* in the presence and absence of challenge by inhibitory agents. Measurements of surface activity were made with the pulsating bubble surfactometer and the captive bubble technique. The addition of SP-A to LES enhances the surface activity of the preparation, especially at low surfactant concentrations. SP-A accelerates the adsorption of surfactant lipids from the aqueous subphase to the air-liquid interface and causes enrichment of the monolayer film in dipalmitoylphosphatidylcholine.

Blood proteins are potent inhibitors of LES. The addition of SP-A completely reverses the inhibition by fibrinogen, albumin and alpha-globulin. As little as 0.5% (w/w of the surfactant lipid concentration) of SP-A causes reversal of inhibition. This effect is absolutely dependent upon the presence of calcium in the assay mixture. The addition of SP-A to another clinical preparation, Survanta, does not result in enhancement of surface activity or reversal of blood protein inhibition.

The addition of palmitic acid to LES enhances the surface activity, and accelerates adsorption. This effect requires relatively high concentrations of palmitic acid ($\sim$8% w/w). Addition of palmitic acid causes a partial reversal of

inhibition by blood proteins. Lysophosphatidylcholine (lyso-PC) also inhibits LES. This inhibition is reversed by the addition of palmitic acid, but not SP-A.

The presence of small amounts of lyso-PC in preparations of LES sensitizes the surfactant to inhibition by fibrinogen. This effect is observed regardless of whether the lyso-PC is endogenous, exogenous, or endogenously generated *in vitro*.

The results of these experiments demonstrate the importance of SP-A in the surface activity of surfactant and the interactions of palmitic acid and lyso-PC with LES. The addition of SP-A and/or palmitic acid should be considered for some applications of surfactant replacement therapy.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada through operating grants to F.P. and a Studentship to A.M.C.. I would also like to thank the family of Nellie L. Farthing for the kind donation through the Memorial Fellowship.

I thank Dr. Fred Possmayer for his excellent guidance and generous support. Fred has shown me not only the essentials of basic research, but also the existence of the marriage between science and culture.

I would like to thank all of those people who helped me with my research, provided guidance, or otherwise broadened my mind. In particular I would like to acknowledge Dr. Sam Schürch, Dr. John Silvius, Stanley Cheng, Dr. Shou-Hwa Yu, Dr. Bill McMurray, Dr. Jeffrey Weitz, Michael Clarke, and Dr. Octávio Filgueiras. In particular, I would like to thank those people in the Possmayer lab that kept my life interesting and provided invaluable help, Ruud Veldhuizen, Mary Ann Ormseth, Kevin Inchley, Barb McDougall, Dr. Li-Juan Yao, Jing Xu, Ian Connelly, Carol Ford, Elizabeth Marek, Riad Qanbar, and Kathy McDougall. I would also like to thank friends who provided emotional support, especially, Terry Moore, Vaughan Hurry, Michelle Rouleau, and Ruud. My heart-felt thanks to my parents, Julia and Philip, for everything.

I would like to acknowledge the continual support and encouragement of Dr. Doug Campbell, hard-rocking scientist, environmentalist and marital unit.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ARDS	Adult Respiratory Distress Syndrome
BSA	bovine serum albumin
C_{15}	film compressibility at 15 mN/m
DPPC	dipalmitoylphosphatidylcholine
EDTA	ethylene-diamine-tetraacetic acid
γ	surface tension
LB	lamellar body
LES	Lipid Extract Surfactant
lyso-PC	lysophosphatidylcholine
NRDS	Neonatal Respiratory Distress Syndrome
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
R_{max}	maximum bubble radius
R_{min}	minimum bubble radius
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
S.D.	standard deviation
S.E.M.	standard error of the mean
SM	sphingomyelin

SP-	surfactant-associated protein

CHAPTER 1 - INTRODUCTION

1.1 General Introduction

Pulmonary surfactant is a complex mixture of lipids and proteins found in the alveolar spaces and terminal airways of the lung which reduces the surface tension across the air-liquid interface. The absence of a surface active material in the lung, such as is observed in premature infants delivered prior to the completion of lung maturation, leads to collapse of the alveoli (atelectasis) and the transudation of serum into the alveolar space (Avery & Mead, 1959; Guyton et al, 1984).

Despite the complex nature of surfactant composition, it is thought that a monomolecular layer enriched in the disaturated phospholipid dipalmitoylphosphatidylcholine (DPPC) is responsible for reduction of surface tension to very low values upon expiration (Clements, 1977; Notter, 1984). However, the properties of DPPC which make it ideal for the reduction of surface tension under physiological conditions, i.e. its high gel-liquid crystalline phase transition and its ability to pack tightly upon compression of the surface film, prevent the molecule from adsorbing and spreading quickly at the air-liquid interface (Bangham, 1987). The other components of surfactant, including the unsaturated and anionic phospholipids and the surfactant-associated proteins, are required to generate and maintain this monolayer highly enriched in DPPC.

1.2 Historical Perspective

The role of surface tension in alveolar mechanics was first suggested by von Neergard in 1929, who demonstrated that much larger pressures were required to expand air-filled than fluid-filled lungs (von Neergard, 1929). These observations were confirmed by Radford 25 years later (Radford, 1954). In 1955 Pattle reported the presence of stable bubbles in the fluid of edematous and healthy lungs (Pattle, 1955). He concluded that the alveolar surfaces must be lined with a material which reduces the surface tension to very low values.

More direct evidence for the presence of a surface active material in the lung came from Clements in the late 1950's (Clements, 1957). Clements demonstrated using a combined Langmuir trough and Wilhelmy plate that lung extracts decreased the surface tension when the surface film was compressed. Klaus et al were the first to describe the composition of pulmonary surfactant in 1961 (Klaus et al, 1961).

The discovery which instigated the surge of research into pulmonary surfactant came in 1959, when Avery and Mead demonstrated that neonatal respiratory distress syndrome (NRDS) was associated with a deficiency of surfactant (Avery & Mead, 1959). Since this disease is associated with premature delivery, research into the ontogeny and control of surfactant synthesis ensued.

1.3 Surface Tension

Surface tension occurs at the interface between phases, and is the result of attractive forces between the molecules in solution. Water molecules undergo positive interactions with each other via a combination of hydrogen bonds and van der Waal's forces. When a molecule in the bulk phase, surrounded by other water molecules, moves to the interface with air it loses a number of these positive interactions. Thus, energy is required to move molecules from the bulk phase to the surface. Surface tension is defined as the force required to expand a surface of given dimensions by a unit of length, and is therefore reported in units mN/m. The surface tension of water, or aqueous solutions, is high, approximately 70 mN/m at 37°C.

Substances which reduce the surface tension at an interface are known as surfactants. Surfactant molecules interact positively with the water molecules at the surface replacing interactions with other water molecules. The surfactant molecules also interact positively with one and other, such that the energy required to maintain this surface film is less than if water occupied the interface.

Phospholipids, and in particular DPPC, are excellent surfactants because they are amphipathic molecules. The polar headgroup of the lipid interacts favourably with water molecules, whereas hydrophobic interactions between the acyl chains stabilize the interfacial monolayer. DPPC has the ability to pack tightly upon lateral compression producing a further positive association between the acyl chains, and the resulting reduction of surface tension to less than 1 mN/m. Unsaturated phospholipids, like egg PC, reduce the surface tension to an

equilibrium value of ~25 mN/m. However, upon compression, the monolayers collapse since the kinked acyl chains do not pack to form a stable film. Thus, these films do not reduce the surface tension to very low values. Some investigators would argue that the formation of such a surface film actually eliminates the air-liquid interface, replacing it with an air-solid/solid-liquid three phased system (Bangham, 1987).

1.4 Methods for Measuring Surface Activity

Surface activity can be measured both with a number of *in vitro* techniques and by methods which incorporate the lungs of animals, either *in situ* or excised (Robertson & Lachmann, 1988). Although animal models generate comprehensive data reflecting the sum of conditions found in the lung, this information is often too complex to elucidate precise molecular mechanisms. For this reason, a number of *in vitro* models have been developed and employed. The major *in vitro* techniques are the Wilhelmy dipping plate and Langmuir trough, and the pulsating bubble surfactometer. Surface adsorption (film formation at the interface from molecules in the hypophase) is often evaluated by measuring the traction force exerted by surface tension on a platinum (Wilhelmy) plate dipping into the sample. The film can be removed by aspiration, and the time required to re-establish equilibrium can be measured. An extension of this simple apparatus is the Langmuir-Wilhelmy surface balance. Surface tension is measured as

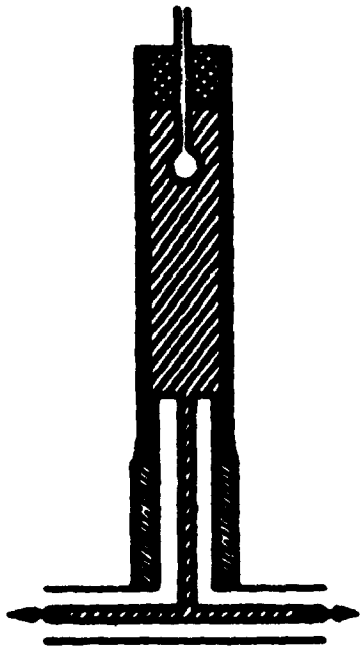
before, but the surface area of the trough is controlled by a movable barrier. A film, either spread from organic solvent or adsorbed from the aqueous phase, can be compressed dynamically, and the surface tension can be monitored during the procedure. Another parameter which can be measured with this technique is the "solidity" of the film, which is assessed by measuring the ease of rotation of a paddle or dipping plate in the surface film (Bangham et al, 1979).

Studies with Wilhelmy balance are limited by the large volume of the trough, the slow compression times, the large compression required to achieve low surface tensions, and the potential leakiness of the system. Another *in vitro* technique which attempts to overcome these problems is the pulsating bubble surfactometer developed by Enhorning (Enhorning, 1977). In this artificial model of the alveolus, a bubble of ambient air is drawn through a narrow capillary into a small chamber containing a concentrated surfactant suspension (Figure 1.1A). The pressure across this bubble is measured by a pressure transducer. From the Law of Young and Laplace which relates surface tension, radius and pressure of spherical bubbles ($\Delta P = 2\gamma/r$; where ΔP is the pressure across the bubble, γ is the surface tension, and r is the radius of the bubble), the surface tension can be calculated. The volume of the bubble is oscillated or pulsated (between fixed radii of 0.4 and 0.55 mm) by the movement of a piston resulting in a 50% reduction in total surface area of the bubble. In this fashion, a bubble can be formed and cycled dynamically at rates comparable to those of normal breathing.

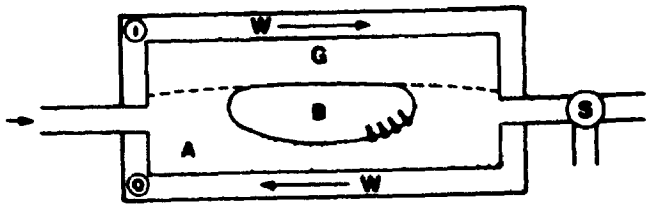
Another *in vitro* technique that has been developed recently is the captive

Figure 1.1 A schematic representation of the pulsating bubble surfactometer and the captive bubble. A. The pulsating bubble surfactometer was developed by Goran Enhorning and is described in detail (Enhorning, 1977). The pressure across the bubble is measured by a pressure transducer, and the surface tension is calculated by the Law of Young and Laplace. (Taken from Yu et al, 1983, with permission from the author.) B. The captive bubble apparatus (taken from Schürch et al, 1989, with permission from the author). C. Bubbles examined in the captive bubble apparatus as described by Schürch et al (Schürch et al, 1989). The phenomenon of clicking is demonstrated in these bubbles. a. A bubble before clicking, surface area=0.31 cm^2, γ=1.4 mN/m; b. The same bubble following the click, surface area=0.24 cm^2, γ=7.4 mN/m. (Taken from Schürch et al, 1989, with permission from the author.)

A



B



a

b

C

bubble model (Schürch et al, 1989; described in detail in chapter 5). This model is somewhat similar to the pulsating bubble in that a bubble is formed and cycled in aqueous suspension. However, this bubble is not in contact with the atmosphere and is thus held "captive" in the suspension (see Figure 1.1B). In this model, surface tension is calculated arithmetically from a fourth order polynomial which relates maximum height and diameter of the bubble (Malcolm & Elliott, 1980). With this instrument measurements can be made under conditions where the risk of leakage is completely eliminated, and the phenomenon known as "clicking" is observed (Figure 1.1C).

1.5 Physiological Significance of Surfactant

Surfactant is required in the alveolus to stabilize this structure during expiration. According to the Law of Young and Laplace (above), at a fixed surface tension the pressure across a bubble is inversely related to the radius. Thus, in the absence of surface active material, the pressure across the alveolus would rise upon expiration leading to alveolar collapse, much the way small soap bubbles coalesce into larger ones. Pulmonary surfactant decreases the surface tension upon compression minimizing the pressure difference thereby preventing collapse.

Surfactant also plays a role in the resorption of fetal lung liquid in the first few hours after birth. The movement of fluid across the alveolar epithelium

depends on a number of parameters including the surface tension across the air-liquid interface (Guyton et al, 1984). The transition from the fluid-filled to the aerated alveolus is likely to be only possible if the pressure gradient for resorption is greater than the retractive force at the interface, which is dependent upon the surface tension and the radius of the alveolus. In the surfactant deficient state, where the surface tension is high, fluid-filled alveoli are often observed (Guyton et al, 1984).

Surfactant is required to maintain the integrity of the alveolar epithelium. Disruption of the surfactant system, as is observed in neonatal and adult respiratory distress syndromes, results in leakage of blood components into the alveolar space. This may be the result of high transpulmonary pressures combined with physical disruption of the epithelium (Guyton et al, 1984; Robertson & Lachmann, 1988).

The host defense system of the lung is affected by surfactant. The surfactant filled hypophase provides the environment in which alveolar macrophages and monocytes reside. It has been demonstrated that surfactant components can stimulate phagocytosis and granule release by macrophages (Tenner et al, 1989; van Iwaarden et al, 1990; discussed in more detail below).

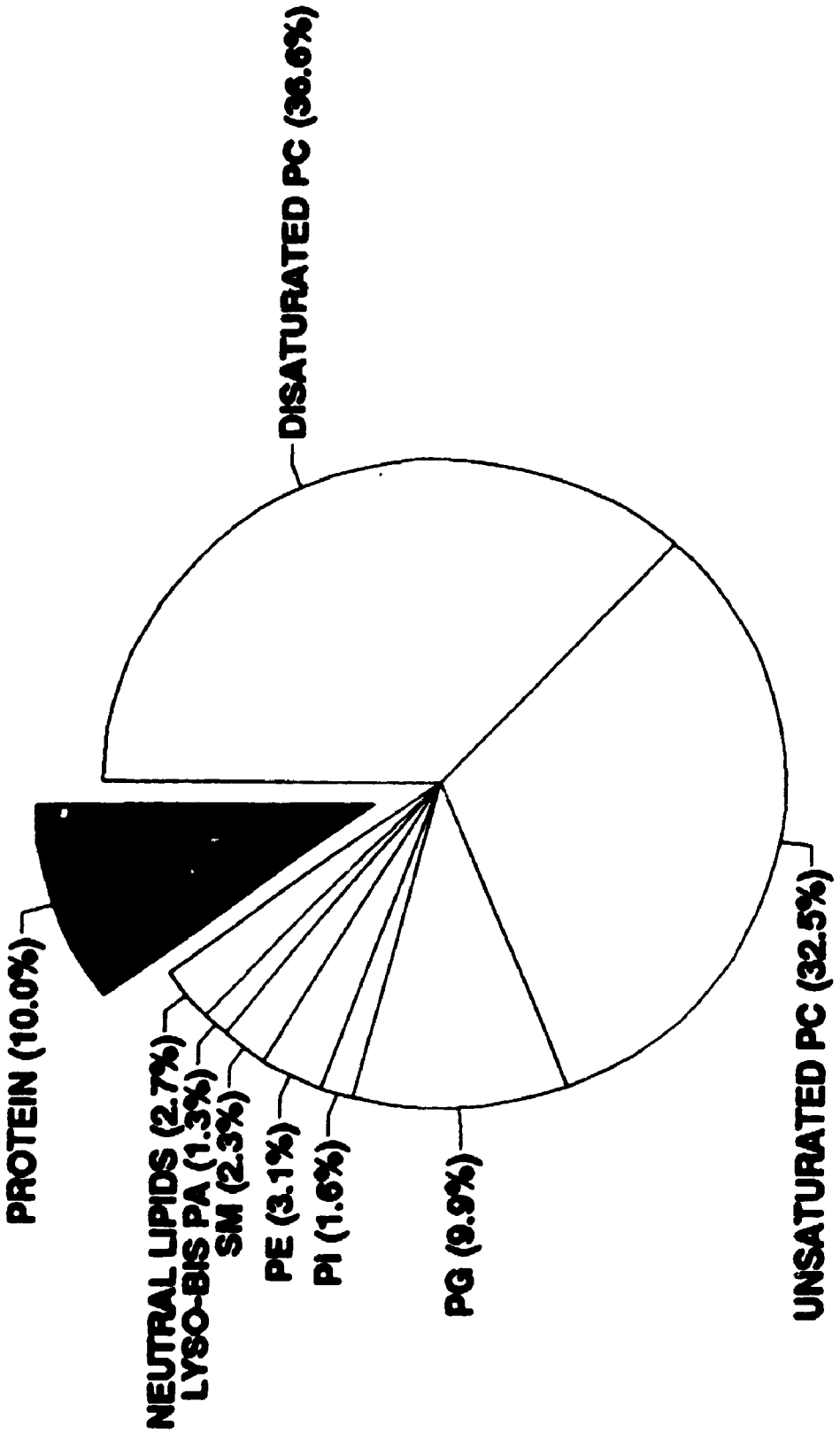
1.6 Composition of Pulmonary Surfactant

Pulmonary surfactant is composed of approximately 90% lipids and 10%

protein. The composition is highly conserved among species (Shelley et al, 1984; Possmayer, 1984). Figure 1.2 is a schematic depiction of the composition of bovine pulmonary surfactant obtained by centrifugation of lavage material (Yu et al, 1983). The protein fraction contains the surfactant-associated proteins (discussed below) and a small amount of contaminating blood protein (depending on the isolation procedure). The lipid fraction is ~97% phospholipid and ~3% neutral lipid. The neutral lipid is mostly cholesterol (~90%), with the remainder being diacylglycerol. The amount of neutral lipid varies between the species and depends on the purification protocol (for a review see Possmayer, 1984). The phospholipid fraction is predominantly phosphatidylcholine (PC), representing 80%, and phosphatidylglycerol (PG) representing 11%, of the total phospholipid. The remainder includes small amounts of phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and lyso-*bis*-phosphatidic acid.

Over half of the phosphatidylcholine is disaturated (containing saturated fatty acids at both the *sn* 1 and 2 positions). The bulk of the remaining PC is monoenoic. Other surfactant lipids do not show this preponderance of disaturated species.

Figure 1.2 The composition of bovine pulmonary surfactant. The percentage by weight of the surfactant components is represented. Data obtained from Yu et al, 1983.


DISATURATED PC (36.6%)
PROTEIN (10.0%)
NEUTRAL LIPIDS (2.7%)
LYSO-BIS PA (1.3%)
SM (2.3%)
PE (3.1%)
PI (1.6%)
PG (9.9%)
UNSATURATED PC (32.5%)

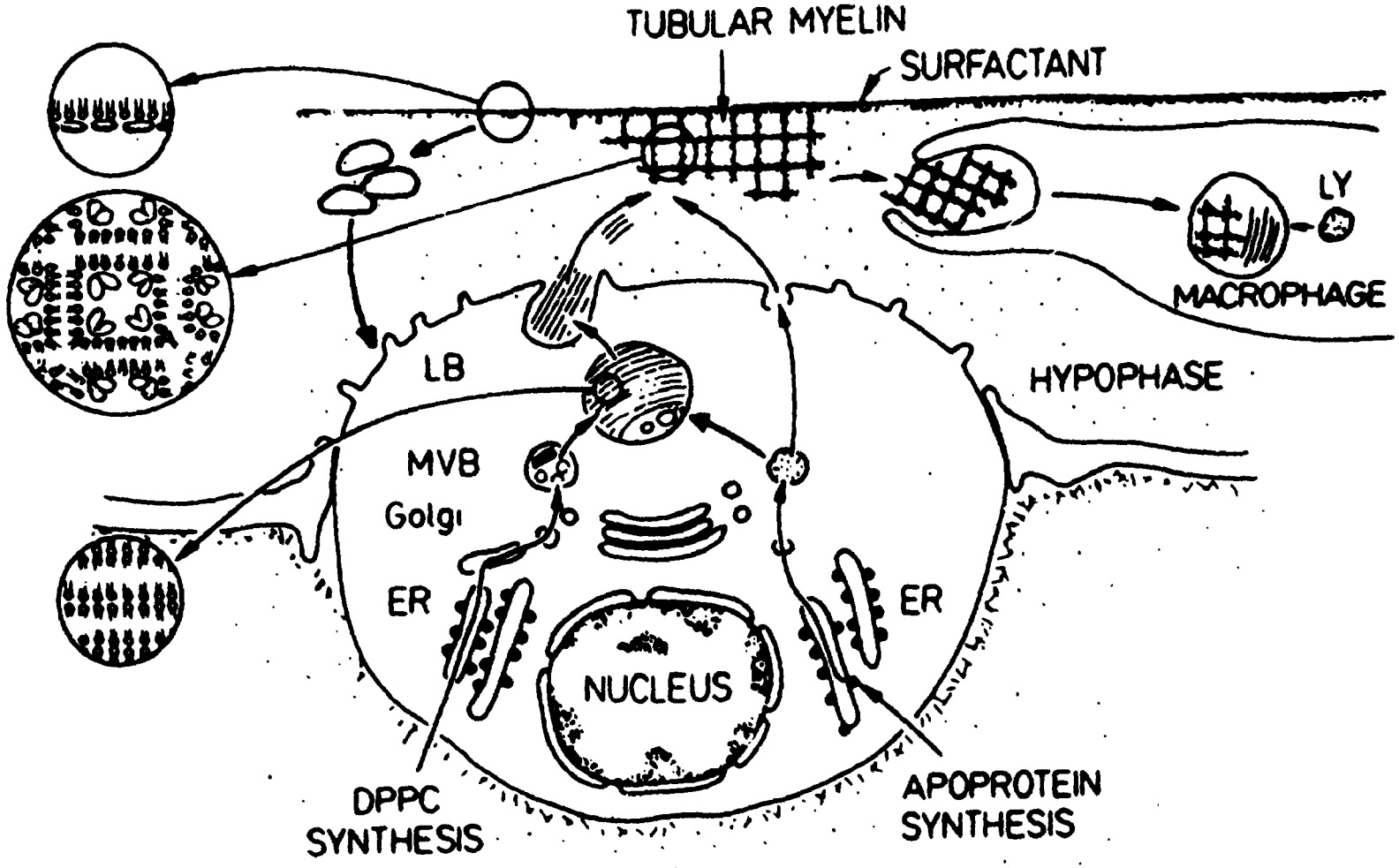
1.7 Metabolism of Surfactant

1.7.1 Subcellular Location of Surfactant Synthesis

It is now generally accepted that surfactant components are synthesized and secreted from the alveolar type II epithelial cell. Chevalier and Collet administered radioactively labelled choline to mice and determined the sequence in which the organelles of the type II cell became labelled (Chevalier & Collet, 1972). Shortly after injection, most of the radioactivity was localized in the endoplasmic reticulum. The label then followed a course through the Golgi complex, the lamellar bodies, and finally appeared in lattice-like structures in the alveolar space. Labelled leucine incorporated into protein followed a similar path in type II cells, however it appeared to move between the Golgi complex and the lamellar bodies via a multi-vesicular system which was not apparent with the labelled phospholipid. Figure 1.3 schematizes our present understanding of the synthesis and secretion of surfactant components by alveolar type II cells.

The lamellar body is the intracellular storage form of surfactant which appears to be similar to a multi-lamellar vesicle. This organelle can be secreted from the cell by exocytosis by fusion of the limiting membrane with the plasma membrane. In the alveolar space this lamellar structure can be transformed into the lattice-like tubular myelin. It is generally accepted that tubular myelin may represent the immediate precursor of the surface monolayer film (Goerke, 1974).

Figure 1.3 A schematic representation of the intracellular and extracellular forms and processing of pulmonary surfactant. ER= endoplasmic reticulum; MVB= multivesicular body; LB= lamellar body. (Modified from Weibel, 1984.)


TUBULAR MYELIN
SURFACTANT
LY
MACROPHAGE
LB
HYPOPHASE
MVB
Golgi
ER
ER
NUCLEUS
DPPC
SYNTHESIS
APOPROTEIN
SYNTHESIS

Material derived from the surface film may form small vesicular structures in the alveolar space. These vesicles are probably destined for re-uptake into the type II cell (Jacobs et al, 1983). Wright and Clements calculated that in the rat, more than 50% of the extracellular DPPC was required to form a pure monolayer covering the entire alveolar surface, this calculation suggests that surfactant concentrations may not be in vast excess (Wright & Clements, 1987).

1.7.2 Biochemistry of Phospholipid Synthesis

Formation of PC in type II cells appears to occur via the Kennedy pathway (Kennedy, 1962). Phosphatidic acid is produced by two successive acyl transfer steps to glycerol-3-phosphate or dihydroxyacetone phosphate. Phosphatidic acid is dephosphorylated by phosphatidate phosphohydrolase to diacylglycerol. Phosphatidylcholine is formed by the transfer of phosphorylcholine from CDP-choline to diacylglycerol by the action of cholinephosphotransferase. Phosphatidic acid occurs at an important branch point in the synthesis pathway because it is also a substrate for phosphatidate cytidyltransferase which forms CDP-diacylglycerol, a precursor of the acidic phospholipids, PG and PI.

The high proportion of disaturated PC found in surfactant suggests the presence of an acyltransferase with specificity for saturated fatty acids and/or a remodelling pathway (Rooney, 1985). Two remodelling mechanisms have been proposed; 1) deacylation at the 2-position of PC followed by reacylation by

palmitoyl-CoA by lysophosphatidylcholine acyltransferase, or 2) deacylation followed transacylation between two molecules of 1-palmitoyl-lysophosphatidylcholine. Although evidence exists for both mechanisms, the deacylation-reacylation mechanism is favoured (Batenburg, 1984). Another pathway of acyl-specific PC synthesis involving the acylation of glycero-3-phosphocholine has been proposed (Infante, 1984). However, this pathway cannot be demonstrated in the lung (Veldhuizen et al, 1989) and it appears as though radioactive intermediates, on which the existence of the pathway was based, were falsely identified (Veldhuizen et al, 1991).

1.7.3 Secretion of Lamellar Bodies

PC synthesized in the endoplasmic reticulum of type II cells is transported to the lamellar body (Chevalier & Collet, 1972). Magoon et al identified two populations of lamellar bodies, with low and high phospholipid/protein ratios (dense and light respectively)(Magoon et al, 1983). Labelling studies indicated that small, dense lamellar bodies became mature, light lamellar bodies. The proteins associated with surfactant can also be stored in the lamellar body prior to secretion. Recent observations that the SP-A content of lamellar bodies is much less than in the alveolar subphase, suggest that this protein may also be secreted independently of the lamellar body (Farrell et al, 1990; Oosterlink-Dijksthuis et al, 1991; Randell et al, 1991). Furthermore, some lung tumour cells and Clara

cells, which don't have lamellar bodies, secrete the surfactant-associated proteins (O'Reilly et al, 1989; Auten et al, 1990).

Secretion of lamellar bodies into the alveolar space *in vivo* is stimulated by adenosine, acetylcholine, β-adrenergic mediators and prostaglandins (Oyarzún & Clements, 1977 & 1978; Wright & Dobbs, 1991). The effects of β-adrenergic agonists are mediated by an increase in cAMP concentration in type II cells (Dobbs & Mason, 1979). The tumour promoter, TPA (12-O-tetra-decanoylphorbol-13-acetate), also stimulates secretion of lamellar bodies suggesting a role for protein kinase C in the regulation of surfactant release (Dobbs & Mason, 1979). SP-A can inhibit the secretion of lamellar bodies from type II cells *in vitro* (Dobbs et al, 1987).

1.7.4 Surfactant Catabolism and Recycling

Surfactant PC released into the alveolar space has a turnover time of 3-10 hours (Jacobs et al, 1982). It has been suggested that PC and other surfactant components can be reutilized by re-uptake into type II cells, re-packaging into lamellar bodies, and re-secretion into the alveolar space. It was demonstrated that labelled phospholipid from instilled surfactant appeared in the lamellar bodies (Hallman et al, 1981). Pulse-chase experiments demonstrated that labelled PC remains in the lamellar bodies and alveolar space far longer than predicted by turnover times. Re-uptake of phospholipids by type II cells may be controlled in

part by SP-A which stimulates the process (Wright et al, 1987). Evidence for the presence of a receptor for SP-A on the type II cell has been demonstrated, however, the nature of this receptor remains unclear (Wright et al, 1990; Voelker et al, 1990).

The catabolism of surfactant can occur via a number of pathways including endocytosis and degradation by alveolar macrophages (Naimark, 1973), enzymatic degradation in the alveolar space, and lysosomal degradation in the type II cell.

1.7.5 Hormonal Control of Surfactant Synthesis

A large number of hormones control the maturation of the lung and surfactant synthesis (Ballard, 1989). Glucocorticoids are the most studied of the hormones affecting the surfactant system. Administration of glucocorticoids to the fetus causes an acceleration of lung maturation which includes a stimulation of phospholipid synthesis and varying effects on the surfactant-associated proteins, as well as the overall maturation of the lung. Interestingly, the effects of glucocorticoids may be mediated by the fibroblast which produces a factor called Fibroblast Pneumocyte Factor (FPF) upon stimulation with glucocorticoids (Smith, 1978). Other hormones such as thyroid hormones, insulin, cAMP and TGF-β have also been implicated in lung maturation (Ballard, 1989).

1.8 The Roles of Surfactant Lipids

The major surfactant component, DPPC, is thought to be the material which is responsible for the achievement of very low surface tensions upon compression of the surface film. The high transition temperature of this phospholipid, 41°C, means that under physiological conditions a pure DPPC film would be in the gel phase. The tight packing and limited mobility in this phase suggest that compressed films of surfactant which are highly enriched in DPPC may be very rigid or even solid (Bangham, 1987).

Pure DPPC however has very poor adsorption characteristics. Thus, it is essential to have more fluid lipids present in the surfactant mixture to allow rapid adsorption of surfactant onto the expanding interface upon inspiration. This is accomplished in surfactant by the presence of unsaturated PC, PG, and/or cholesterol. Indeed dry mixtures of DPPC and unsaturated PG appear to have adequate adsorptive properties when assayed *in vitro* (Bangham et al, 1979).

However, in order for these mixtures to be good surfactants, the unsaturated lipid must be removed from the surface film to prevent collapse of the film upon compression. This has been demonstrated by mechanical compression with simple lipid mixtures and pulmonary surfactant (Bangham et al, 1979; Chung et al, 1990; Egberts et al, 1989). This process is referred to as "squeeze-out" or monolayer purification (Goerke & Clements, 1986).

The presence of relatively large amounts of anionic phospholipids, in particular PG, is characteristic of pulmonary surfactant. The precise role of this class of phospholipids remains to be determined. Suzuki et al have demonstrated

that PG is required for the formation of tubular myelin *in vitro* (Suzuki et al, 1989). The interaction of this lipid with the surfactant-associated proteins is discussed below. The roles of other surfactant lipids are unknown.

1.9 The Structure and Function of Surwctant-associated Proteins

1.9.1 SP-A

The proteins which are isolated specifically with pulmonary surfactant have been given the designation surfactant-associated protein, or SP- (Possmayer, 1988). The first protein to be discovered and the best characterized is SP-A. This protein is a large hydrophilic oligomer with a monomeric molecular weight of 26-36 kDa depending on the species and extent of glycosylation (Weaver & Whitsett, 1991). The oligomeric molecular weight of SP-A is approximately 650 kDa (King et al, 1989). It has recently been demonstrated by rotary shadowing electron microscopy that native SP-A oligomers are 18'mers made up of 6 trimers (Voss et al, 1988) structurally analogous to the complement component C1q.

The N-terminus of the protein consists of a short 7-10 amino acid sequence containing an invariant cysteine residue which forms an interchain disulphide bond important for the oligomerization of the protein. This is followed by a 72 residue collagen-like domain consisting of 23 or 24 repeats of the Gly-X-Y triplet, where X and Y are often proline or hydroxyproline. Results of circular dichroism

experiments suggest that this region forms a triple helix with the collagenous parts of two other SP-A monomers (King et al, 1989). The collagen-like rod is interrupted in the 13th repeat by the insertion of Pro-Cys-Pro-Pro which results in a kink. The collagenous region is followed by a 148 residue globular domain. This region contains four invariant cysteines which form intrachain disulphide bonds. This domain is also the site of N-linked glycosylation.

SP-A has a high degree of structural and sequence homology with the mannose-binding protein (Drickamer & McCreary, 1987). It has been suggested that these proteins belong to a family of calcium-dependent animal lectins which also includes the asialoglycoprotein receptor (Drickamer et al, 1986).

SP-A is highly modified post-translationally. The molecule is glycosylated, acetylated, sialylated, sulphated, and proline hydroxlated (Hawgood, 1989). It was suggested that SP-A was a substrate for vitamin K-dependent carboxylation of glutamic acid (Rannels et al, 1987), however this has been subsequently challenged (Wallin et al, 1988).

SP-A is a phospholipid-binding protein. SP-A is isolated associated with surfactant lipids, and is particularly enriched in the tubular myelin fraction, where the ratio of phospholipid to SP-A is 3-5:1 (weight to weight)(Hawgood & Shiffer, 1991). This association is not dependent upon calcium (Benson et al, 1984). The association of SP-A with phospholipids is favoured at temperatures below the gel to liquid-crystalline phase transition and this interaction broadens the transition isotherm (King et al, 1986). More recent observations, made with different

techniques, also point to a specific interaction between SP-A and DPPC (Kuroki & Akino, 1991). These data suggest that a hydrophobic region of SP-A interacts specifically with DPPC.

The list of functions of SP-A appears to be growing exponentially. It has been demonstrated that SP-A enhances the adsorption of surfactant lipids onto the air-liquid interface (Hawgood et al, 1987). This function requires the presence of SP-B and calcium. SP-A causes calcium-dependent aggregation of surfactant lipids (King, 1984; Haagsman et al, 1991). It has also been demonstrated that SP-A is required for the formation of tubular myelin *in vitro* (Suzuki et al, 1989). The presence of tubular myelin in fractions isolated from surfactant has been correlated with an increased rate of adsorption (Benson et al, 1984) suggesting that these ordered structures may play an important role in surface tension reduction.

SP-A is a calcium-dependent carbohydrate binding protein (Haagsman et al, 1987; Haagsman et al, 1990). The precise significance of this activity is not clear, however it may participate in the interactions of the molecule with cellular receptors. SP-A is an autocrine factor, which inhibits the secretion of surfactant from type II cells (Dobbs et al, 1987), and stimulates the uptake of phospholipids into these cells (Wright et al, 1987). Thus, it seems likely that SP-A regulates the level of surfactant in the alveolar space. SP-A also interacts with alveolar macrophages stimulating phagocytosis and killing of bacteria and viruses (Tenner et al, 1989; van Iwaarden et al, 1990).

1.9.2 SP-B

SP-B is a small hydrophobic protein of 79 amino acids (Curstedt et al, 1988; Olafson et al, 1987). SP-B forms disulphide dependent dimers which are the predominant form isolated from lavage. The mature polypeptide is derived from a 40 kDa precursor protein by proteolytic processing (Weaver & Whitsett, 1991). SP-B contains 7 cysteine residues, forming 3 intrachain disulphide bonds and one interchain bridge essential for oligomerization. Amino acid sequence analysis suggests that the polypeptide forms a number of amphipathic helices.

The addition of SP-B to synthetic lipid mixtures mimicking surfactant leads to an acceleration of adsorption of lipids to the interfacial film (Hawgood et al, 1987; Yu & Possmayer, 1988; Pison et al, 1990). SP-B is also required for the formation of tubular myelin *in vitro* (Suzuki et al, 1989). Indirect evidence of the importance of SP-B in surfactant came from the observation that injection of mice with antibodies to SP-B resulted in respiratory failure (Suzuki et al, 1988). A series of elegant experiments led to the conclusion that SP-B specifically promotes the removal of PG from the monolayer. It was demonstrated that simple lipid mixtures containing DPPC and egg PG supplemented with SP-B reduce the surface tension to less than 1 mN/m in the first pulsation on the pulsating bubble surfactometer. This effect was only observed if the sample was allowed to adsorb and held at equilibrium briefly prior to cycling (Yu & Possmayer, 1990). Since these results are not observed when unsaturated PC is included in the mixture or

with LES, this suggests that PG may have a specific interaction with SP-B. Results of fluorescence anisotropy experiments also demonstrate that SP-B interacts selectively with PG in model membranes (Baatz et al, 1990).

1.9.3 SP-C

SP-C is the smallest and most hydrophobic of the surfactant proteins. The mature polypeptide is 33-35 amino acids long, and like SP-B is derived from a larger precursor of 21 kDa (Weaver & Whitsett, 1991). This molecule contains a very hydrophobic region at the carboxy terminus of 23 hydrophobic residues which includes a tract of 6 valine residues. It has been proposed that this region forms a hydrophobic, potentially transmembrane, alpha helix. An interesting structural feature of SP-C is the presence of 2 palmitoyl groups attached to the cysteine residues in the N-terminal region of the molecule (Curstedt et al, 1990) possibly providing a membrane anchor. Canine SP-C contains a single palmitoyl group (Johansson et al, 1991)

The role of SP-C in the surface activity of surfactant is less obvious than that SP-B or SP-A. It enhances the adsorption of surfactant lipids (Yu & Possmayer, 1988), but is apparently unable to cause monolayer purification. Recently, it has been reported that some preparations of SP-C can impart excellent surface activity to lipid mixtures, however this may depend upon the isolation and reconstitution protocols (Seeger et al, 1991; Venkitaraman et al,

1991). Recent studies also indicate that recombinant SP-C may impart some resistance to blood protein inhibition (Seeger et al, 1991; Venkitaraman et al, 1991). Clearly, mixtures which contain both SP-B and SP-C have excellent surface activity when assayed at high enough concentrations (Yu & Possmayer, 1988).

1.9.4 SP-D

A fourth protein thought to be associated with surfactant has recently been identified (Persson et al, 1988, Persson et al, 1989). SP-D is a 43 kDa hydrophilic protein with some similarity to SP-A. It has a collagenase-sensitive domain, hydroxyproline, N-linked carbohydrate, and has calcium-dependent lectin activity (Persson et al, 1990). The role of SP-D in surfactant is not known. It was recently reported that this protein has considerable homology with bovine conglutinin (Crouch, oral communication to the American Lung Association Meeting, Anaheim, 1991), and may modulate the lung host defense system.

1.10 The Role of Calcium in Surfactant

Many of the properties of surfactant are enhanced by the addition of calcium. The calcium concentration in the alveolar hypophase has been estimated at 1.5-3 mM (Nielson, 1988). The addition of calcium to organic extracts of

surfactant, which contain all of the lipids and SP-B and SP-C, but not SP-A, enhances the adsorption and surface activity of these preparations (Weber & Possmayer, 1984; Kobayshi & Robertson, 1983; Chung et al, 1989). The formation of tubular myelin depends upon the presence of calcium (Benson et al, 1984; Efrati et al, 1987; Suzuki et al, 1989). SP-A binds calcium with high affinity (Haagsman et al, 1990) and this interaction is required for the aggregation of lipids by SP-A (King, 1984; Haagsman et al, 1991). Other functions of SP-A require calcium (described in results chapters), and it appears as though SP-A may reduce the calcium requirement of surfactant to physiological calcium concentrations (Chung et al, 1989).

1.11 Inhibition of Surfactant by Blood Components

In 1985 Seeger et al reported that protein leakage into the alveolar space caused severe inhibition of pulmonary surfactant activity (Seeger et al, 1985a). In this comprehensive study it was demonstrated that protein leakage into isolated perfused lungs (induced by high pressure perfusion with protein containing solutions) caused an alteration of the pressure-volume characteristics of these lungs, and that the extent of damage depended upon the particular protein perfused. The studies were confirmed *in vitro* by measurements with a Wilhelmy plate. The authors ranked the potency of the inhibitors with fibrin and fibrinogen being the most potent, albumin having intermediate strength, and elastin and the

immunoglobulins being the weakest inhibitors.

Holm et al reported that alveolar protein levels increased in animals injured by exposure to 100% O_2 (Holm et al, 1985b). The rise in alveolar protein content correlated with decreased lung compliance and pulmonary function and with decreased surface activity of the lavage material when assayed *in vitro* with the pulsating bubble surfactometer. The interactions between natural and extracted surfactants with albumin have been characterized in some detail, and it was demonstrated that this inhibition could be abolished by increasing the surfactant concentration (Holm et al, 1985a).

It is now clear that a large number of blood components interfere with the surface tension reducing properties of surfactant. A protein isolated from the airways of premature lambs is a potent inhibitor of surfactant (Ikegami et al, 1984). Hemoglobin and cell membrane lipids have also been demonstrated to inhibit surfactant both *in vitro* and in excised lungs (Holm & Notter, 1987). The inhibition of surfactant by lysophospholipids has been reported (Keicher et al, 1990) and is discussed in the results chapters.

As mentioned above, the inhibitory potential of these proteins is inversely proportional to the surfactant concentration, such that at high concentrations inhibition is not observed (Holm et al, 1985a; Ikegami et al, 1984). A potential mechanism of inhibition by protein which has been proposed suggests that the protein competes with surfactant lipid for the interface (Holm et al, 1988). This model is based on albumin inhibition measured with a Wilhelmy plate.

Differences may exist between proteins with respect to inhibitory mechanism as is suggested by their varying inhibitory potential.

Recent research has addressed the roles of the surfactant-associated proteins in inactivation by blood proteins. Results with SP-A are presented in Chapter 2. Synthetic lipid mixtures which have good surface activity in the absence of inhibitory protein were very sensitive to the addition of albumin (Holm et al, 1990). Lipid mixtures supplemented with SP-B and SP-C are considerably more resistant to inhibition (Venkitaraman et al, 1991). Interestingly, mixtures containing only SP-C were more resistant than mixtures containing only SP-B. Mixtures of recombinant SP-C with lipids are more sensitive to inhibition than natural extracts, but are more resistant than the lipids alone (Seeger et al, 1991).

1.12 Surfactant Abnormalities in Disease States

The discovery in 1959 that the development of neonatal respiratory distress syndrome (NRDS) was related to a deficiency of pulmonary surfactant was the first conclusive demonstration that abnormalities of the surfactant system may be involved in lung disease (Avery & Mead, 1959). In the case of NRDS, the primary defect appears to be delivery of the infant prior to the completion of lung maturation. The surfactant deficient state leads to poor gas exchange, alveolar collapse, edema and the formation of hyaline membranes.

More recently, surfactant abnormalities in adult respiratory distress

(ARDS) have been examined (Holm & Matalon, 1989; Seeger et al, 1990). The inhibition or inactivation of the surfactant system in ARDS is a secondary effect which appears to be the result of leakage of blood proteins into the alveolar space and the contamination of surfactant with non-surfactant lipids (Jobe, 1989). After examination of the bronchoalveolar lavage of a number of patients with ARDS, Seeger et al have concluded that the extent of leakage of blood proteins into the lung is a major factor in the progression and severity of respiratory distress (Seeger et al, 1990).

Changes in the lipid composition of surfactant have been documented. Von Wichert et al reported a six-fold increase in the lyso-PC content of bronchoalveolar lavage from rats with septic shock, an experimental model of ARDS (von Wichert et al, 1981). This increase was apparently due to the action of phospholipase A_2, which was five-fold elevated in the lavage of septic animals versus controls. Hallman et al reported increases in lyso-PC, sphingomyelin and phosphatidylserine in the bronchoalveolar lavage of ARDS patients (Hallman et al, 1982). Increased levels of glycolipid have also been detected in ARDS patients (Rauvala & Hallman, 1984). These changes appear to be the result of contamination by cell membrane lipids. The effects of these changes on surfactant activity have not been examined in depth.

1.13 Surfactant Replacement Therapy

Since the discovery of Avery and Mead in 1959 relating surfactant insufficiency and respiratory distress (Avery & Mead, 1959), replacement therapy has become an attractive way of treating the affected infants. Clinical trials employing a number of different preparations have yielded excellent results (Robertson & Lachmann, 1988; Jobe & Ikegami, 1987). Preparations currently under investigation include totally artificial preparations such as ALEC (Morley et al, 1981) or Exosurf (Durand et al, 1985); natural surfactant prepared from amniotic fluid (Merritt et al, 1986); modified natural surfactant such as Lipid Extract Surfactant (Enhorning et al, 1985), Infasurf (Kwang et al, 1985), Survanta (Horbar et al, 1991), and Curosurf (Collaborative European Multicenter Study Group, 1988). The efficacy of different preparations is currently under investigation, however it appears as though those derived from natural surfactant are superior to artificial mixtures by a number of different criteria (Cummings et al, 1991). Compositional changes to natural surfactant extracts which have beneficial effects on the *in vitro* surface activity of these preparations are discussed in Chapters 2 and 3.

CHAPTER 2 - THE EFFECT OF SP-A ON BLOOD PROTEIN INHIBITION OF LIPID EXTRACT SURFACTANT

2.1 Chapter Summary

Although a monolayer of dipalmitoylphosphatidylcholine, the major component of pulmonary surfactant, is thought to be responsible for the reduction of the surface tension at the air-liquid interface of the alveolus, the participation of unsaturated and anionic phospholipids and the three surfactant-associated proteins is suggested in the generation and maintenance of this surface active monolayer. We have examined the effects of surfactant-associated protein A (SP-A) purified from bovine lavage material on the surface activity of Lipid Extract Surfactant (LES), an organic extract of pulmonary surfactant containing all of the phospholipids and SP-B and SP-C, but lacking SP-A. Measurements of the surface tension during dynamic compression were made on a pulsating bubble surfactometer. Addition of SP-A to LES reduces the number of pulsations required to attain surface tensions near zero at minimum bubble radius. This increase in surface activity is dependent upon the presence of Ca^{++} in the assay mixture. Maximal enhancement is observed at or below 1% of the lipid concentration (w/w). The addition of two blood proteins, fibrinogen and albumin, at physiological concentrations to LES causes severe inhibition of surface activity. Addition of SP-A in the presence of Ca^{++} completely

counteracts the inhibition by fibrinogen. The amount of SP-A required for full reversal of this inhibition was less than 0.5% of the lipid concentration. Complete reversal of inhibition by albumin was also observed, even though there was a ~5000-fold molar excess of inhibitor. Addition of lysophosphatidylcholine also inhibits LES, however SP-A has no effect on this inhibition.

2.2 Introduction

The type II epithelial cell produces and secretes pulmonary surfactant which serves to reduce the surface tension across the air-liquid interface of the alveolus, and hence facilitate breathing. This substance, composed of approximately 90% lipids and 10% protein, reduces surface tension during breathing by generating a monolayer at the interface enriched in the saturated phospholipid dipalmitoylphosphatidylcholine (DPPC) (Goerke, 1974, King, 1984, Notter et al, 1984, Possmayer et al, 1985, Van Golde et al, 1988). DPPC constitutes only ~40% of the surfactant, the remainder is mainly unsaturated phosphatidylcholines, anionic phospholipids such as phosphatidylglycerol (PG) and phosphatidylinositol (PI), and the three surfactant-associated proteins (SP-) A, SP-B and SP-C (Possmayer, 1988). It has recently been demonstrated both *in vitro* and *in vivo* that many of the surface tension reducing properties of surfactant lipids are potentiated by interactions with the small, hydrophobic proteins SP-B and SP-C which make up only ~1% of surfactant (Hawgood et al, 1987, Revak et al, 1988, Suzuki et al 1986, Takahashi & Fujiwara, 1986, Yu et al., 1987, 1988).

SP-A is an abundant protein representing 5-10% of bovine surfactant (Yu et al., 1983). The structure elucidation of this highly conserved protein has revealed some very interesting features (Hawgood, 1989). The primary translation product is a ~28 kDa protein that is post-translationally modified to

generate a glycoprotein with a molecular weight of ~36 kDa that is acetylated, sulfated, and proline hydroxylated. The protein can be divided into three functional regions: the N-terminal 7 amino acids form a short tail containing a cysteine which participates in an interchain disulphide bridge, a collagen-like stretch consisting of 23 repeats of the gly-X-Y triplet, and a glycosylated carboxy terminal region which has considerable homology with several Ca^{++} dependent lectins. The oligomeric organization of SP-A has been elucidated (Voss et al., 1988), and appears to form an octadecameric structure very similar to the complement component C1q.

The physiological role of SP-A has remained somewhat elusive and is the subject of current research. The protein has the capacity to bind certain sugars (Haagsman et al., 1987), including mannose, in a Ca^{++} dependent manner. Other *in vitro* studies have demonstrated that this protein is capable of aggregating lipid vesicles, a process that is greatly enhanced by the inclusion of anionic phospholipids and Ca^{++} (King, 1984, Wright & Clements, 1987). When added to type II epithelial cells in culture, SP-A stimulates the uptake of surfactant lipids from the medium and inhibits secretion of stored surfactant (Dobbs et al., 1987, Wright et al., 1987), suggesting a paracrine regulation of alveolar surfactant levels. More recently immunological investigations, spurred by the structural similarity between complement component C1q and SP-A, have suggested an immune modulatory role for SP-A in the prevention of pulmonary infection (Tenner et al., 1989).

Previous investigations have demonstrated the deleterious effects of blood components on the activity of various surfactants both *in vitro* and *in vivo* (Fuchimukai et al., 1987, Holm et al, 1985a,b, Holm & Notter, 1987, Ikegami et al, 1984, Kobayashi et al., 1989, Seeger et al., 1985). Leakage of blood components into the alveolar space as a result of injury or increased vascular pressure has been implicated in the pathology of the neonatal and adult respiratory distress syndromes (NRDS and ARDS respectively) (Burkhardt & Van Golde, 1989, Jobe & Ikegami, 1987, Robertson and Lachman, 1988). The hyaline membranes associated with NRDS are composed largely of fibrin clots arising from the transudation of serum proteins. Examination of the interactions between surfactant components and blood components *in vitro* could lead to a better understanding of the basic mechanisms involved in RDS.

In this investigation we have examined the effects of three blood components on Lipid Extract Surfactant (LES). We have observed that SP-A can prevent inhibition by the proteins fibrinogen and albumin, even in the presence of a vast molar excess of inhibitor. We have also observed that the surface activity is compromised by lysophosphatidylcholine. This inhibition is unaffected by the addition of SP-A. We conclude that SP-A has a specific function in preventing protein inhibition *in vitro*.

2.3 Materials and Methods

2.3.1 Reagents.

All chemicals were reagent grade or better. Bovine serum albumin, lysophosphatidylcholine, and fibrinogen were obtained from Sigma. Fibrinogen was also obtained from Kabivitrum, Helena Labs. Immobilized D-mannose was purchased from Pierce. The silver stain reagents were purchased as a kit from Bio-Rad.

2.3.2 Assays.

Phospholipid concentrations were determined using the phosphorus assay of Rouser (Rouser et al., 1960). Protein purity was assayed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (Laemmli, 1970), followed by staining with Coomassie R-250 or silver stain. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Fibrinogen concentrations were also determined using the Bio-Rad assay (Bradford, 1976) and the BCA assay (Smith et al., 1984) from Pierce. All protein concentration assays used bovine serum albumin as a standard. A large discrepancy was observed between the concentrations determined for fibrinogen by the three different protein assays. Fibrinogen concentrations reported in this paper refer to those obtained with the Bio-Rad Method. Values obtained with the Bio-Rad method are ~70% of those obtained by Lowry, values obtained with the

BCA assay are ~45% of the Lowry values.

2.3.3 Surfactant Preparation.

Pulmonary surfactant was prepared from bovine lavage material by a modification of the method described elsewhere (Yu et al, 1983, Weaver & Possmayer, 1984). Briefly, Lipid Extract Surfactant (LES) is obtained by organic extraction of lavage material. Neutral lipids are removed by acetone solubilization. The material is dried, made up in chloroform/methanol 9:1 and stored at -20°C. This preparation contains all of the phospholipids of natural surfactant and the two small hydrophobic proteins SP-B and SP-C, however it has been completely stripped of SP-A. Natural Surfactant is prepared from lavage material by a series of washes and gradient centrifugations. The resulting material is lyophilized and stored at -20°C.

2.3.4 Purification of SP-A.

SP-A is purified from bovine natural surfactant by slight modifications to two previously published protocols (Haagsman et al, 1987, Ross et al, 1986). Briefly, an aqueous preparation of natural surfactant is delipidated by washing in butan-1-ol, ether:ethanol 3:1, and pure ether. The solvent is completely removed and the precipitated protein is solubilized in 5 mM HEPES, 0.1 mM Na_2EDTA pH 7.4, and centrifuged at 30 000 g to remove any insoluble material. The solubilized protein is then supplemented vith $CaCl_2$ to a concentration of 1 mM

and affinity purified on a column of immobilized D-mannose. Bound protein is eluted with 2 mM EDTA in 5 mM HEPES, pH 7.4. A Coomassie stained gel of the purified protein is shown in Figure 2.1. Note that under non-reducing conditions the band with an apparent molecular weight of 68 kDa is distinct from albumin.

2.3.5 Fibrinogen Purification.

Fibrinogen from Kabivitrum was purified as previously described (O'Brodovich et al, 1990). The fibrinogen solution containing Trasylol (American Diagnostica), a protease inhibitor, is passed over a lysine-Sepharose matrix (Pharmacia) to remove contaminating plasminogen. The preparation is then repeatedly precipitated in $(NH_4)_2SO_4$ (144g/L final concentration) to concentrate the fibrinogen and remove factor XIII. The pellet is redissolved in 5 mM sodium citrate, pH 7 5, dialysed against 0.1 M NaCl 50 mM Tris pH 7.4, and stored at -70°C.

2.3.6 Pulsating Bubble Surfactometer.

Surface tension was measured during dynamic compression using a pulsating bubble surfactometer as described (Enhorning, 1977). Briefly, a bubble of ambient air drawn through a small tube is formed in a chamber containing a surfactant suspension. This bubble is pulsated at a rate of 20 cycles per minute between fixed radii (0.4-0.55 mm) resulting in a 50% change in surface area.

The pressure across the bubble is measured by a pressure transducer and the surface tension can be calculated from the Law of Laplace: $\Delta P=2\gamma/r$. All surfactant samples assayed on the bubble surfactometer were made up in solutions containing 0.9% NaCl, 1.5 mM $CaCl_2$. Those sample assayed in the absence of Ca^{++} had 1.5 mM EDTA added to the saline buffer in place of $CaCl_2$.

Sample chambers are commercially available, however, these chambers leak on the old model used for these studies. Sample chambers were made by pulling a length of teflon capillary tubing through a narrow stainless steel tube. The inner tube fits very snugly in the outer. The ends of the inner tube are cut close to the outer tube. The whole assembly is then inserted into the end of a clear piece of nylon tubing, approximatley 1.5 cm long. The sample chamber is washed extensively before and between runs by aspirating a soap solution, followed by water, followed by 95% ethanol. The chambers are thoroughly dried before using.

Surface tension values reported are the means of three different batches of surfactant $\pm$ the standard error of the mean. Each batch is assayed 3 times, and a mean and S.E.M. are calculated. The final mean is calculated from these three means. The variability reported is the between batch variability, which is large with respect to the within batch variability. Error bars are always included on graphs except when the they fall within the symbol.

2.4 Results

Lipid Extract Surfactant reduces the surface tension at maximum (R_{max}) and minimum (R_{min}) bubble radii in the pulsating bubble surfactometer. The number of cycles required to obtain near zero surface tensions at R_{min} is inversely related to the phospholipid concentration (see Figure 2.2). Little change in the surface tension at R_{max} is observed except at very low surfactant concentrations when the initial surface tension is high. However, at the concentrations used in this study, usually 1-5 mg/mL surfactant phospholipid, the surface tension at R_{min} after 20 pulsations can be used as an indication of the rate at which surface tension decreases during dynamic compression. Table 2.1 shows the effect of addition of SP-A at a fixed concentration of 1 mg/mL to LES at 5 and 2.5 mg/mL. In the presence of Ca^{++} the addition of SP-A lowers the surface tension to near zero within 20 pulsations. In the absence of Ca^{++} a higher surface tension is observed and the addition of SP-A actually inhibits the surface activity. When a concentration range of 1-10 mg/mL surfactant was assayed in this fashion, the most pronounced effect of SP-A was observed at the lower surfactant concentrations (data not shown).

The amount of SP-A required for maximal enhancement of surface activity was determined. Figure 2.3 shows a concentration curve for SP-A, where the SP-A concentration is expressed as a percentage of the lipid concentration, 2.5 mg/mL, (w/w). Maximal enhancement of activity is observed by 0.5-1.0% SP-

A, with no further enhancement observed as the value is raised to 10%. Similar results were observed when the phospholipid concentration was 5 mg/mL (data not shown). Bovine serum albumin (BSA) is a major contaminant of crude SP-A (Figure 2.1). Therefore the effect of this protein over the same concentration was examined. BSA had only a small inhibitory effect on LES. This observation suggests that the stimulatory effect of SP-A cannot necessarily be duplicated by other proteins and may be specific.

It has been previously demonstrated that the addition of various blood components to surfactant preparations impairs their ability to reduce surface tension. These studies have shown that fibrinogen, and in particular the fibrin monomer, is a potent inhibitor of surfactant (Seeger et al, 1985, Keough et al, 1987, Fuchimukai et al, 1987). Addition of fibrinogen to LES results in high surface tensions. Figure 2.4 shows the effect of addition of increasing concentrations of fibrinogen to LES at 2.5 mg/mL. Addition of >0.1 mg/mL fibrinogen results in surface tensions of >50 mN/m at R_{max}, and ~ 40 mN/m at R_{min}, which are similar to the surface tensions of solutions containing 3 mg/mL fibrinogen in the absence of surfactant.

The effect of SP-A on the inhibition of LES by fibrinogen was determined. Inhibition of surface activity was abolished when SP-A was added prior to challenge with fibrinogen (Figure 2.5). In this experiment LES was markedly inhibited by the addition of 3 mg/mL of pure fibrinogen (open circles). However, when 5% SP-A was included in the LES preparation the surface tension

profiles in the presence and absence of fibrinogen were indistinguishable (compare filled and open triangles). In other experiments it was observed that natural surfactant, which contains SP-A, behaved similarly to LES supplemented with SP-A (data not shown). This indicates that the effect of SP-A is not an artefact of the protein isolation procedure.

The inhibition of surfactants by albumin is much weaker than by fibrinogen (Seeger et al, 1985). When low concentrations of BSA (up to 5 mg/mL) were added to LES no significant inhibition could be detected. When the concentration of albumin was increased to 50 mg/mL (and the surfactant phospholipid concentration was decreased to 1 mg/mL) a marked inhibition of surface activity, similar to that seen with fibrinogen, was observed (Table 2.2). It should be noted that this approximates the physiological concentration of albumin in serum. The addition of SP-A at 10% of the lipid concentration (=0.1 mg/mL SP-A) completely prevented the inhibition.

The amount of SP-A required for the reversal of fibrinogen inhibition was determined by adding varying amounts of SP-A to fibrinogen inhibited surfactant. The results, shown in Figure 2.6, demonstrate that only 0.5% by weight or less of the surfactant preparation need be SP-A for complete reversal of the fibrinogen inhibition.

The dependence of the reversal of fibrinogen inhibition on Ca^{++} was determined by repeating the experiments after incubating the samples at 37°C in the presence of 1.5 mM Na_2EDTA instead of $CaCl_2$. Table 2.3 shows that in the

absence of Ca^{++} SP-A has no effect on fibrinogen inhibition.

In order to obtain some indication of the manner in which SP-A reverses the inhibition by serum proteins, the effect of a potential non-protein inhibitor was examined. Table 2.4 shows that the addition of lysophosphatidylcholine (lyso-PC), which is also present in serum, to LES at 10% of the surfactant phospholipid concentration (w/w) markedly retards the reduction of surface tension. The addition of SP-A at 5% of the lipid concentration has no effect on the inhibition by lyso-PC (Table 2.4), an indication that the mechanism of inhibition by lyso-PC is distinct from that of fibrinogen and albumin. These results demonstrate that the addition of SP-A can counteract the inhibition of surface activity by blood proteins but not by lyso-PC.

Table 2.1 Effect of SP-A and Calcium on the Biophysical Activity of Lipid Extract Surfactant (LES)

Sample Description	Surface Tension after 20 pulsations (mN/m)	
	R_{min}	R_{max}
5 mg/mL LES in 1.5 mM $CaCl_2$	2.8 ± 1.5[a]	26.2 ± 0.4
+ 1 mg/mL SP-A	0.2 ± 0.3	24.7 ± 0.7
5 mg/mL LES in 1.5 mM EDTA	10.1 ± 0.0	28.3 ± 1.2
+ 1 mg/mL SP-A	15.9 ± 2.0	37.9 ± 6.4
2.5 mg/mL LES in 1.5 mM $CaCl_2$	7.8 ± 2.8	26.5 ± 0.3
+ 1 mg/mL SP-A	1.2 ± 0.4	26.2 ± 0.4
2.5 mg/mL LES in 1.5 mM EDTA	14.8 ± 0.4	33.7 ± 0.9
+ 1 mg/mL SP-A	28.0 ± 3.7	46.3 ± 2.9

[a] Values are the means ± S.E.M. of 3 separate LES samples each assayed 3 times.

Table 2.2 Effect of SP-A on the Inhibition of LES by Albumin.

Sample Description	Surface Tension after 20 pulsations (mN/m)	
	R_{min}	R_{max}
1 mg/mL LES in 1.5 mM $CaCl_2$	14.9 ± 0.3[a]	33.7 ± 1.0
+ 10% SP-A (0.1 mg/mL)	7.6 ± 1.5	27.0 ± 0.7
+ 50 mg/mL BSA	40.1 ± 2.3	49.4 ± 0.5
+ 10% SP-A	6.5 ± 1.2	27.3 ± 0.6

[a] Values are the means ± S.E.M. of 3 separate LES samples each assayed 3 times.

Table 2.3 Effect of Calcium on the Prevention of Fibrinogen Inhibition by SP-A.

Sample Description	Surface Tension after 20 Pulsations (mN/m)	
	R_{min}	R_{max}
2 mg/mL LES in 1.5 mM EDTA	16.7 ± 0.2[a]	29.7 ± 1.0
+ 5% SP-A (0.1 mg/mL)	19.5 ± 0.3	52.6 ± 0.9
+ 1 mg/mL fibrinogen	40.6 ± 0.3	53.0 ± 0.1
+ 5% SP-A	40.4 ± 0.7	53.3 ± 0.7

[a] Values are the means ± S.E.M. of 3 surfactometer assays of one LES sample.

Table 2.4 The Inhibition of LES by Lysophosphatidylcholine and the Effect of SP-A.

Sample Description	Surface Tension after 20 Pulsations (mN/m)	
	R_{min}	R_{max}
5 mg/mL LES in 1.5 mM $CaCl_2$	4.5 ± 0.3[a]	26.6 ± 0.3
+ 5% SP-A (0.25 mg/mL)	3.8 ± 0.7	26.2 ± 0.7
+ 10% lyso-PC (0.5 mg/mL)	15.0 ± 2.8	36.7 ± 2.7
5% SP-A	15.0 ± 4.0	35.2 ± 5.1

[a] Values are the means ± S.E.M. of 3 separate LES samples each assayed 3 times.

Figure 2.1 SDS-Polyacrylamide gel of SP-A stained with Coomassie R-250. *Lane A*, SP-A non-reduced; *B*, BSA non-reduced; *C and D*, SP-A and BSA non-reduced; *E*, natural surfactant reduced; *F*, SP-A reduced; *G*, molecular weight wandard. Note the presence of albumin in natural surfactant. The "dimer" of SP-A observed under non-reducing conditions does not comigrate with albumin.

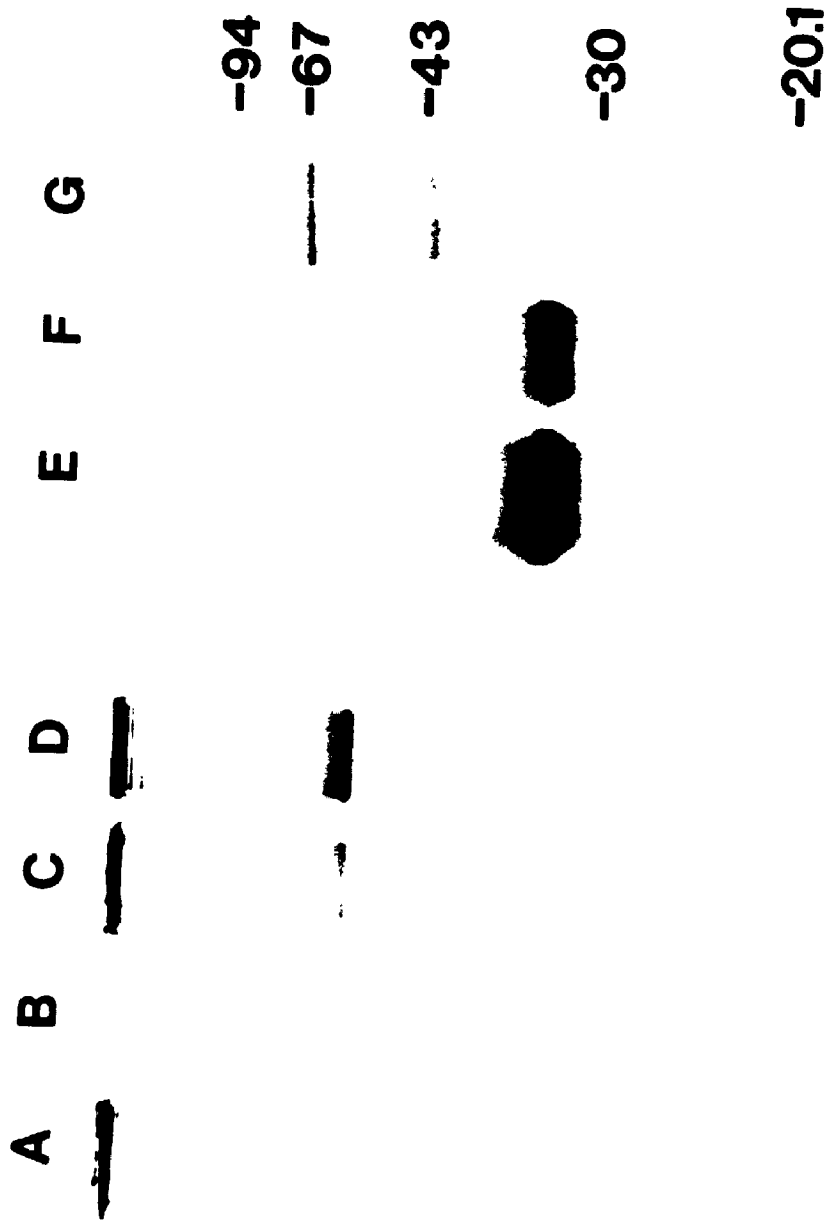

A
B
C
D
E
F
G
-94
-67
-43
-30
-20.1

Figure 2.2 Effect of phospholipid concentration on the surface tension profile of LES. Surface tension (mN/m) is plotted vs. number of pulsations for four different phospholipid concentrations of LES. ●○ 10 mg/mL; ▲△ 5 mg/mL; ■□ 2.5 mg/mL; ♦◇ 1 mg/mL. Solid symbols represent surface tension at minimum bubble radius, open symbols represent surface tension at maximum bubble radius. Values are the means ± S.E.M. of 3 separate LES samples each assayed 3 times.

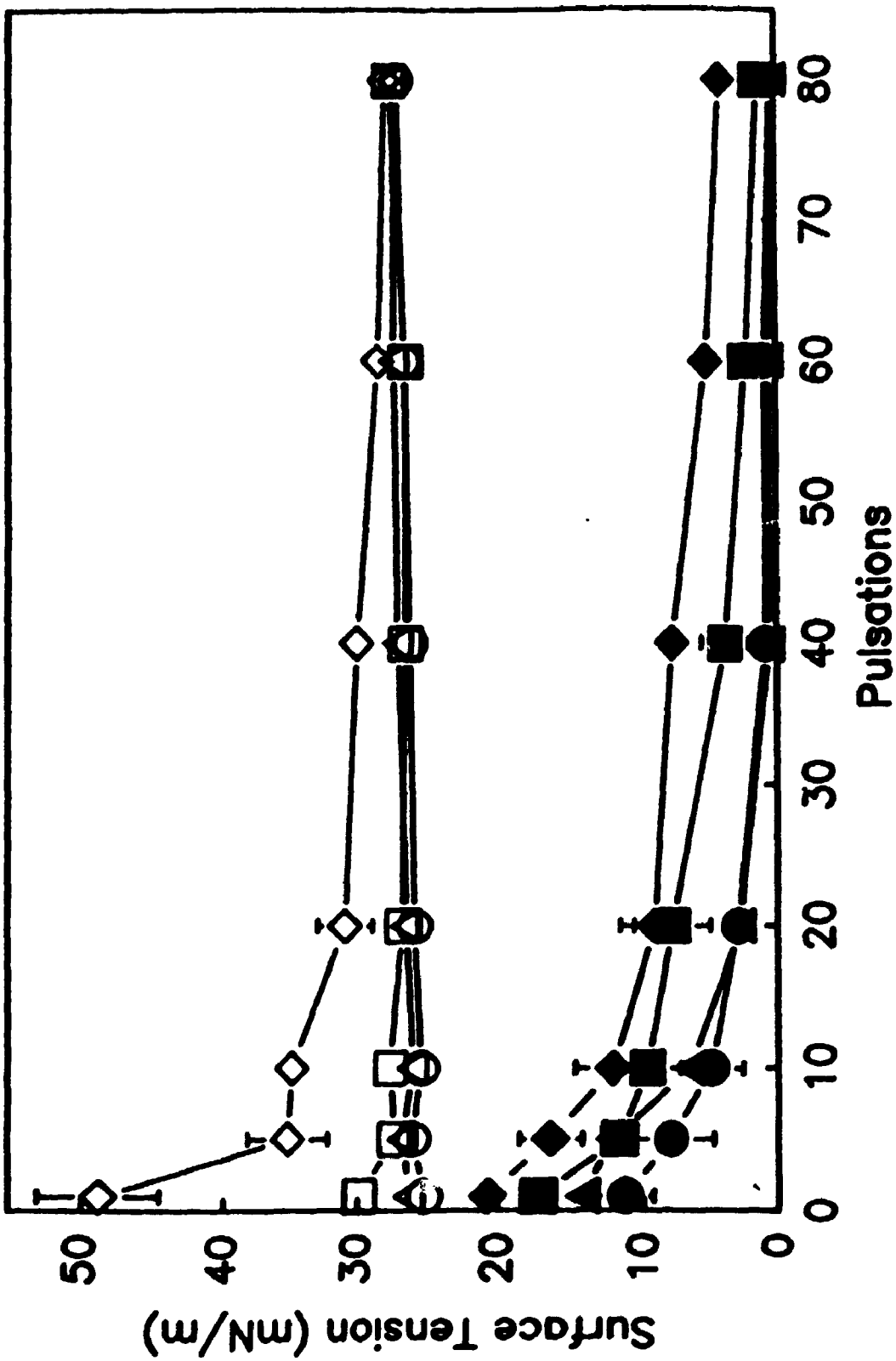

Surface Tension (mN/m)
50
40
30
20
10
0
Pulsations
0
10
20
30
40
50
60
70
80

Figure 2.3 Concentration curve for the addition of SP-A to LES. Surface tension after 20 pulsations at minimum bubble radius (mN/m) is plotted vs. protein concentration expressed as a percentage (w/w) of the lipid concentration of 2.5 mg/mL. ● represent addition of SP-A; ○ represent addition of BSA as a control. Values are means ± S.E.M. of 3 separate LES samples each assayed 3 times.

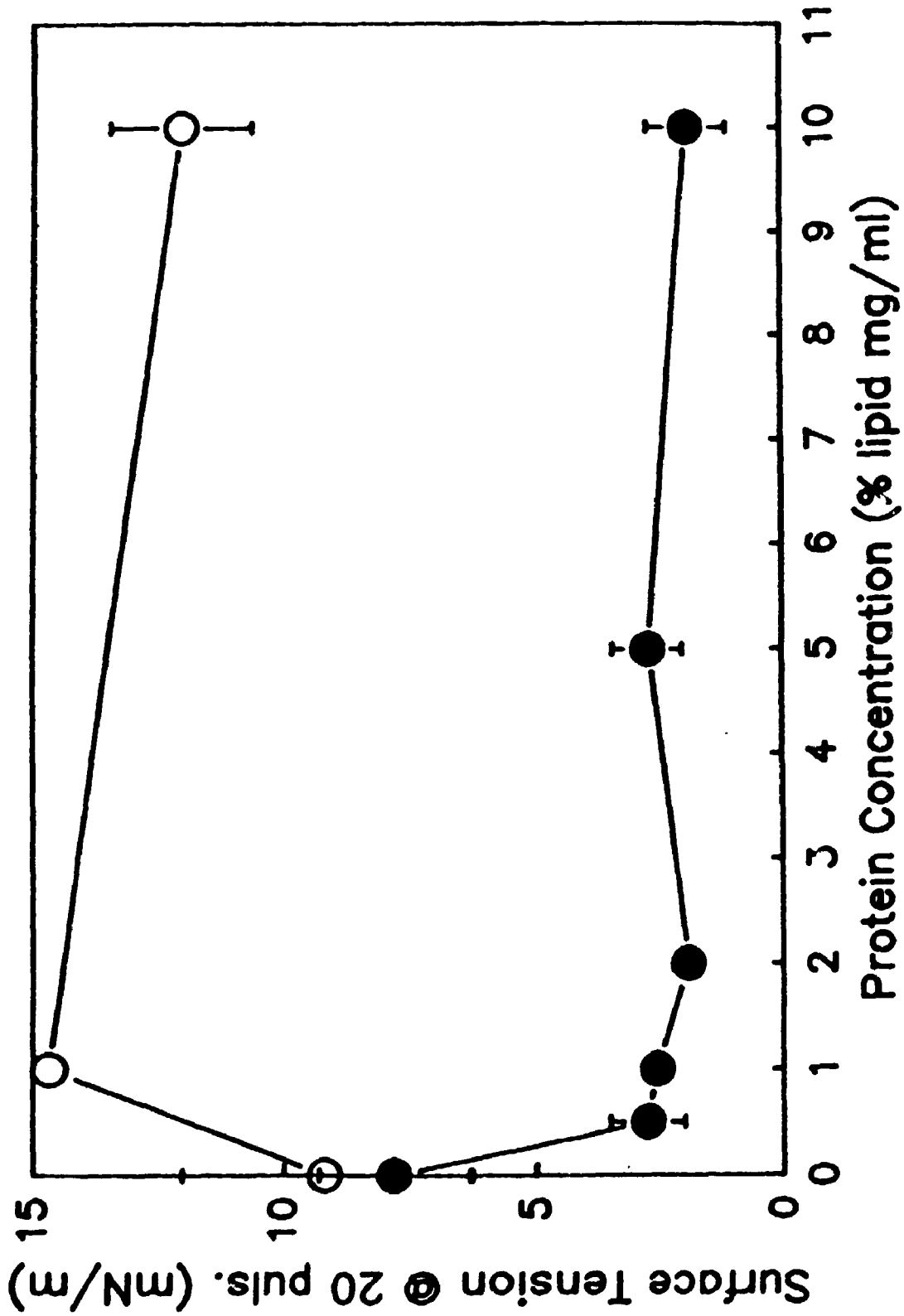

Surface Tension @ 20 puls. (mN/m)
15
10
5
0
0
1
2
3
4
5
6
7
8
9
10
11
Protein Concentration (% lipid mg/ml)

Figure 2.4 The effect of addition of fibrinogen to LES. Surface tension after 20 pulsations (mN/m) is plotted vs. the concentration of fibrinogen added (mg/mL, as determined by the method of Bradford). The LES concentration is 2.5 mg/mL. The solid symbols represent values at minimum bubble radius, and the open symbols represent maximum bubble radius. Values are means $\pm$ S.E.M. of 3 separate LES samples each assayed 3 times.

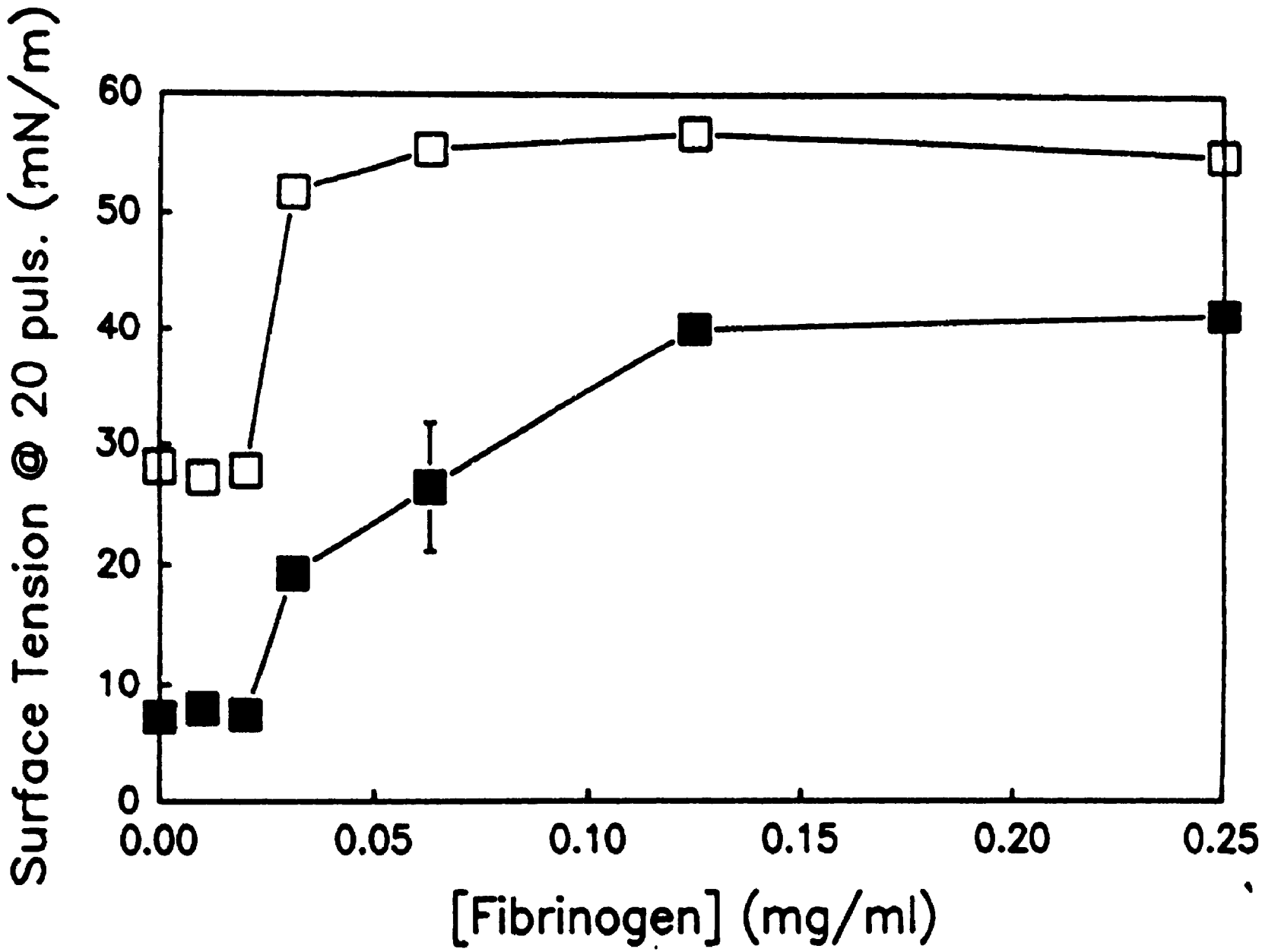

Surface Tension @ 20 puls. (mN/m)
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
0.20
0.25
[Fibrinogen] (mg/ml)

Figure 2.5 The effect of SP-A on the inhibition of LES by fibrinogen. Surface tension at minimum bubble radius (mN/m) is plotted vs. number of pulsations. ○ represents LES; ● LES + 3 mg/mL fibrinogen; △ LES + 5% SP-A; ▲ LES + 5% SP-A + 3 mg/mL fibrinogen. LES is 5 mg/mL. Values are the means ± S.E.M. of 3 separate LES samples each assayed 3 times.

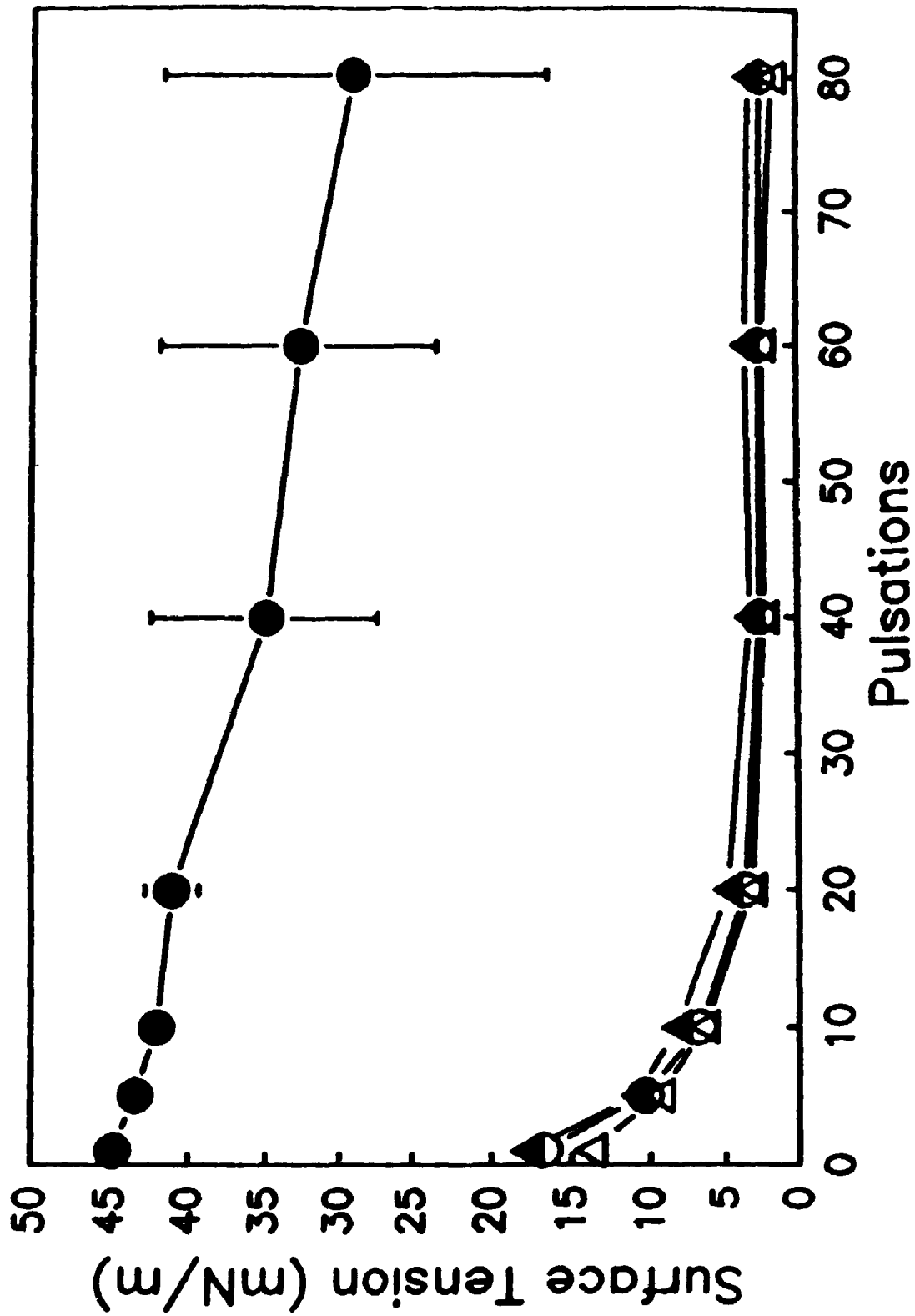
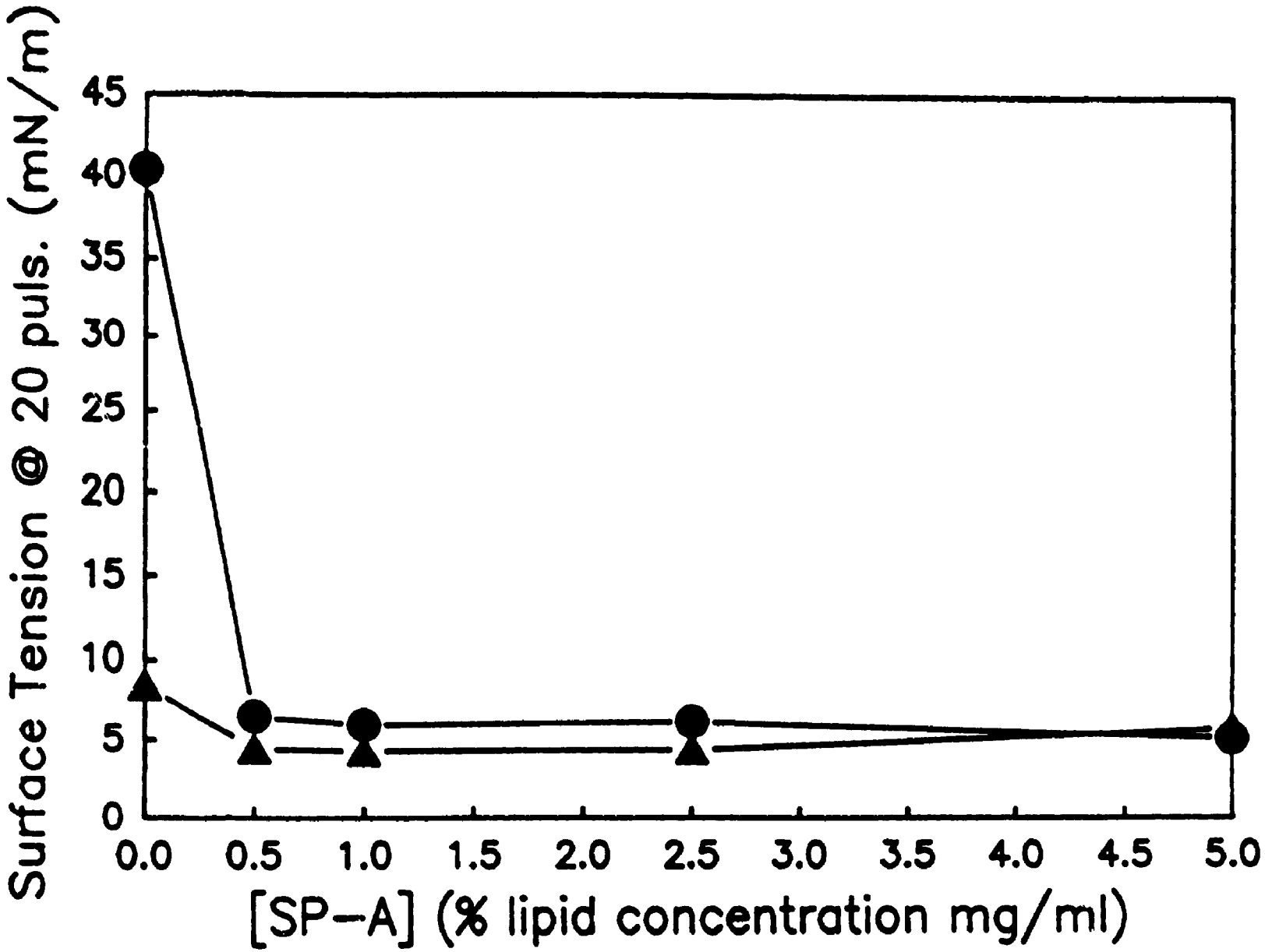

Surface Tension (mN/m)
50
45
40
35
30
25
20
15
10
5
0
Pulsations
0
10
20
30
40
50
60
70
80

Figure 2.6 Concentration curve for the effect of SP-A on the inhibition of LES by fibrinogen. Surface tension at minimum bubble radius after 20 pulsations is plotted vs the concentration of SP-A added (expressed as a percentage of the lipid concentration). ▲ represent controls, with LES and buffer; ● represent LES + 1 mg/mL fibrinogen. LES is 2 mg/mL. Values are means ± S.E.M. of 3 separate LES samples each assayed 3 times.


Surface Tension @ 20 puls. (mN/m)
45
40
35
30
25
20
15
10
5
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
[SP-A] (% lipid concentration mg/ml)

2.5 Discussion

Edema has long been implicated in the etiology of both the neonatal and adult forms of respiratory distress syndrome (Burkhardt & van Golde, 1989, Robertson & Lachmann 1988). Seeger et al (1983, 1984, 1985a, 1985b) clearly demonstrated the deleterious effects of different blood proteins on surface activity both *in vitro* and *in situ*. This study revealed that the development of edema in perfused lungs was correlated with alterations in pressure-volume characteristics and decreases in the surface activity of lavaged materials. Those perfusates that contained blood proteins, in particular fibrin and fibrinogen, caused greater inhibition of surface activity. The present investigation examines the role of SP-A in mitigating surfactant inhibition by fibrinogen and albumin.

The data presented here confirm earlier observations on the enhancement of surfactant activity by the addition of SP-A to reconstituted and organic solvent extracted surfactants (Hawgood et al, 1987, Chung et al, 1989). However, since the effect is most pronounced at low surfactant lipid concentrations, the significance of this role of SP-A in the lung is unclear. SP-A may serve to stabilize the surface tension during periods of reduced alveolar surfactant or during the establishment of the air-liquid interface upon initiation of breathing at birth. It was observed that the amount of SP-A required for maximal enhancement of activity is approximately 1/5 to 1/10 of the amount found in natural surfactant. This suggests that other functions of SP-A may account for the

in vivo concentrations which are higher than required for optimal biophysical activity.

The mechanism by which SP-A enhances the surface activity of LES may be related to the formation of tubular myelin. It has been suggested that tubular myelin may be the source of the surface active monolayer *in vivo* and under appropriate conditions *in vitro* (Goerke, 1974, Wright & Clements, 1987). Addition of SP-A to preparations containing the surfactant lipids DPPC and PG and SP-B in the presence of Ca^{++} leads to the formation of tubular myelin as observed by electron microscopy (Suzuki et al, 1989). Conditions producing an increase in the content of tubular myelin correlate with more rapid adsorption and spreading of the lipids onto the monolayer (Benson et al, 1984). Thus, the action of SP-A in the present experiments may be to change the structure of LES from a predominantly lamellar structure to tubular myelin thereby altering the spreading and monolayer purifying abilities of the surfactant. SP-A may also accelerate the squeeze out of lipids other than DPPC.

Physiological serum concentrations of fibrinogen and albumin inhibited the biophysical activity of LES. During these investigations a considerable variation in the extent of inhibition by different fibrinogen preparations was observed. Solutions of purified Kabivitrum fibrinogen are very potent. Crude preparations purchased from Sigma have only moderate inhibitory action. It was also noted that with concentrated preparations (>10 mg/mL), a loss of inhibitory activity sometimes occurred which was correlated with the appearance of a white

precipitate, presumably a fibrous clot, in the solution. These observations suggest that the fibrinogen present in the edema fluid in the lung may be a more potent inhibitor than the purified preparations.

Previous investigations with the Wilhelmy plate have revealed that whereas a preformed surfactant film is barely affected by injection of albumin into the subphase, administration of albumin prior to or with surfactant injection results in a significant inhibition (Holm et al, 1988). These observations suggest that the inhibitory proteins compete with the surfactant lipids for the interface and thereby retard the adsorption and/or spreading of surfactant phospholipids. However, since the induction of edema *in vivo* alters pressure-volume parameters, it is clear that serum proteins can affect a preformed monolayer within the alveolus. The amounts of edema fluid introduced into the alveolar space *in vivo* can greatly exceed the alveolar subphase volume. Under these conditions, edema fluid would dilute the endogenous surfactant as well as inhibit its activity. *In vitro* studies conducted with the Wilhelmy plate and the pulsating bubble surfactometer use fixed hypophase volumes. Nevertheless, the reported measurements are congruous with the competitive model, since the initial high surface tensions observed with the inhibitors tend to fall during repeated pulsation, suggesting that the inhibitory protein may be gradually removed from the interface during dynamic compression.

Clearly, the nature of the inhibitory protein affects its potential to impair surfactant action. It is likely that the protein's structure (including the extent of

denaturation in solution) and charge distribution influence its ability to occupy the interface such that one protein is a more effective inhibitor than another. In addition some proteins may interact directly with surfactant lipids preventing monolayer formation and purification. Our observations and those of others have determined that the polymerization or cleavage state of fibrinogen affects the inhibitory ability of this protein (O'Brodovich et al, 1990). Thus, the protein's conformation could have significant effects on its interaction with surfactant.

The precise mechanism by which SP-A prevents inhibition of surfactant by blood proteins is unknown. It is known that SP-A lowers the calcium level required for surface tension reduction by LES (Chung et al, 1989). SP-A has been shown to act synergistically with SP-B in surface tension reduction (Wright & Clements, 1987). SP-A does not enhance adsorption of mixtures containing only SP-C, a hydrophobic protein which is not required for the formation of tubular myelin (Pison et al, 1990). By the establishment of tubular myelin, SP-A could shift the equilibrium in favour of lipid monolayer formation. In the presence of SP-A normal surface tensions are observed even with the first pulsation indicating that compression of the bubble is not required for countering the inhibition. In the experiment in which SP-A prevented the inhibition by albumin, the concentration of inhibitor was 500 times (w/w) higher than that of SP-A. When the native molecular weight of SP-A ($\sim$650 000 Da) is considered, this represents a 5000 fold molar excess of inhibitor over SP-A. In the fibrinogen experiments, a 200 fold molar excess of inhibitor over SP-A was estimated.

Since SP-A effectively prevented inhibition in these instances, a stoichiometric model which relies on sequestration of the inhibitor by SP-A seems unlikely. It is possible however, that SP-A may prevent undesirable associations between inhibitor proteins and surfactant lipids, behaving as a kind of surfactant chaperone.

To the author's knowledge, inhibition of surfactant activity by high levels of lyso-PC has not been reported previously. Lyso-PC is present in the serum and could be produced in abnormal amounts by increased levels of alveolar phospholipase A_2 in certain types of ARDS (Hallman et al, 1982, Von Wichert et al, 1981). Lyso-PC appears to inhibit LES by a different mechanism than the blood proteins. This inhibition is insensitive to the presence of SP-A. It is likely that the detergent-like properties of lyso-PC enable it to disrupt the monolayer spreading and purifying machinery of the preparation both in the presence and absence of SP-A.

In this investigation the effects of addition of SP-A to LES on the ability of the surfactant to reduce surface tension in the presence and absence of inhibitory proteins were determined. SP-A enhances the surface activity of LES in a Ca^{++} dependent fashion. SP-A was also shown to have a protective function towards the inhibition of LES by fibrinogen and albumin. This process was also dependent on the presence of Ca^{++} and required only a small amount of SP-A. These results suggest that SP-A may play an important role in the prevention of surfactant inactivation by blood proteins which leak into the alveolus as a result of

increased permeability or high pressures. Since it is known that disruption of surface activity in the lung results in high pressures across the alveolus, the ability of a surfactant to resist the initial effects of edema, the inhibition by blood proteins, would enable it to more effectively maintain homeostasis and prevent the development of pulmonary disease. LES is currently being used in clinical trials for the treatment and prevention of NRDS (Jobe & Ikegami, 1987, Robertson & Lachman, 1988). Its use has been proposed for the treatment of ARDS as well. Consideration should be made for the possible inclusion of SP-A in this situation, where surfactant inhibition by blood proteins is well documented, for the prevention of surfactant inhibition by blood proteins.

CHAPTER 3 - THE ROLE OF PALMITIC ACID IN PULMONARY SURFACTANT

3.1 Chapter Summary

The surface activity of two surfactant preparations, Lipid Extract Surfactant (LES) and Survanta, was examined during adsorption and dynamic compression using a pulsating bubble surfactometer. At low surfactant phospholipid concentrations (1-2.5 mg/mL), Survanta reduces surface tension at minimum bubble radius faster than LES: however with continued pulsation LES obtains a lower surface tension. Addition of surfactant-associated protein A (SP-A) to LES significantly reduces the time required to reduce surface tension. Survanta is completely unresponsive to the addition of SP-A in that no further reduction of surface tension is observed. Addition of various blood components has been previously shown to inactivate surfactants *in vitro*. Addition of fibrinogen to Survanta causes an increase in surface tension when measured in the absence of calcium. When assayed in the presence of calcium, inhibition by fibrinogen is not observed. Albumin and alpha-globulin strongly inhibit Survanta at physiological serum concentrations both in the presence and absence of calcium. The surface activity of Survanta is also inhibited by lysophosphatidylcholine (lyso-PC). The role of palmitic acid in the surface activity of pulmonary surfactant was examined by adding palmitic acid to LES.

At low phospholipid concentrations addition of palmitic acid (10% w/w of the surfactant phospholipid) greatly enhances the surface activity. Maximal enhancement of surface activity and adsorption was observed at or above 7.5% added palmitic acid (w/w of surfactant lipid). LES supplemented with palmitic acid is more resistant to inhibition by fibrinogen, albumin, alpha-globulin and lyso-PC than LES alone, however the counteraction of blood protein inhibition is not as pronounced as that observed with SP-A.

3.2 Introduction

Pulmonary surfactant is a substance produced by the type II epithelial cell that reduces the surface tension at the air-liquid interface of the alveolus. Natural surfactant is a complex mixture of approximately 90% lipids and 10% proteins. The major component of the lipid fraction is phosphatidylcholine, and over half of this is the disaturated species dipalmitoylphosphatidylcholine (DPPC). It is generally accepted that a monolayer of phospholipid highly enriched in DPPC found at the interface is responsible for surface tension reduction (Goerke & Clements, 1986; Goerke, 1974; King, 1984; Notter & Finkelstein, 1984; Possmayer et al, 1984; van Golde et al, 1988). Other surfactant components include unsaturated phosphatidylcholine, anionic phospholipids such as phosphatidylglycerol and phosphatidylinositol, and the three surfactant-associated proteins SP-A, SP-B, and SP-C (Possmayer, 1988).

Surfactant deficiency and inactivation have been demonstrated in a number of pulmonary diseases. In 1959 Avery and Mead reported that a deficiency of surfactant at birth, often caused by premature delivery, was responsible for the development of neonatal respiratory distress syndrome (NRDS) (Avery & Mead, 1959). It was later realized that inactivation of the surfactant system was also involved in the development of the adult form of respiratory distress (ARDS) (Burkhardt & van Golde, 1989; Jobe & Ikegami, 1987; Robertson & Lachmann, 1988). In light of these findings, surfactant replacement therapy has been

investigated by a number of groups (Jobe & Ikegami, 1987; Enhorning, 1989). Several different preparations with differing compositions are currently been used in clinical trials around the world. Surfactant preparations used in these trials fall into two main categories:those made up solely of synthetic components and those derived from natural surfactant by organic extraction with or without subsequent modification by the addition of exogenous lipids (Jobe & Ikegami, 1987). By modifying naturally derived surfactants, investigators have sought to develop preparations with enhanced surface activity for more efficient treatment of respiratory distress. The degree to which these modifications alter the surfactant's properties has not been fully investigated.

The properties of DPPC which make it an ideal surfactant for the conditions found in the lung, namely its high gel to liquid crystalline transition temperature and its ability to be tightly packed into a gel upon compression, decrease its ability to quickly adsorb onto the monolayer. The major biophysical function of the remaining surfactant components appears to be the efficient generation and maintenance of this DPPC monolayer. For instance, 30-40% of natural surfactant consists of unsaturated species of phosphatidylcholine which is itself a poor surfactant, but when mixed with DPPC generates a more fluid mixture which can adsorb to the monolayer much more easily than pure DPPC (Goerke & Clements, 1986). Furthermore, it appears that the surfactant-associated proteins greatly enhance the adsorption of surfactant lipids to the interface (Goerke & Clements, 1986; Possmayer, 1988; Hawgood, 1989; Yu &

Possmayer, 1988; Yu & Possmayer, 1990). It has also been suggested that one of the key functions of these proteins is to remove unsaturated phospholipids from the monolayer hence enriching it in DPPC (Yu & Possmayer, 1990). The role of the anionic phospholipids, which make up about 10% of surfactant, may be to mediate the interactions of the proteins with the lipids (Yu & Possmayer, 1990; Shiffer et al, 1988; Suzuki et al, 1989).

Many of the preparations used clinically are derived from natural surfactant by organic extraction (Jobe & Ikegami, 1987; Robertson & Lachmann, 1988). This process removes aqueous soluble components such as SP-A and contaminating blood proteins, but leaves the lipids and the small, hydrophobic proteins SP-B and SP-C intact. Supplementation of such preparations with synthetic lipid components has led to the development of surfactants with good *in vitro* and *in vivo* surface tension reducing activity (Holm et al, 1990; Tanaka, et al, 1986). Addition of purified SP-A to organically extracted surfactants has also been demonstrated to enhance surface activity (Yu & Possmayer, 1990; Hawgood et al, 1987; Chung et al, 1989; Cockshutt et al, 1990). Furthermore, it has been demonstrated that the addition of SP-A to Lipid Extract Surfactant, an organically extracted preparation, imparts resistance to inhibition by blood proteins such as fibrinogen and albumin (Cockshutt et al, 1990).

In the present investigation we have combined the approaches of adding lipid and protein components to two surfactant preparations derived by organic extraction, Lipid Extract Surfactant (LES) and Survanta. These preparations

differ from each other in that LES has been acetone precipitated to remove neutral lipids such as fatty acids and triglycerides, whereas Survanta has been supplemented with palmitic acid, DPPC and triglycerides. We have demonstrated that Survanta is unresponsive to addition of SP-A, where LES is greatly enhanced. Palmitic acid added to LES enhances the surface activity at low phospholipid concentrations and partially counteracts inhibition by blood proteins, but this effect is not as pronounced as that observed with SP-A. These developments may lead to the design of surfactants which will be more suitable for the treatment of respiratory distress.

3.3 Materials and Methods

3.3.1 Reagents.

All chemicals were reagent grade or better. Survanta (or Surfactant TA) was a gift from Ross Labs, a division of Abbott Medical Supplies, Columbus, Ohio. Palmitic acid, bovine serum albumin, lysophosphatidylcholine, and fibrinogen were obtained from Sigma. Fibrinogen was also obtained from Kabivitrum, Helena Labs. Immobilized D-mannose was purchased from Pierce. The silver stain reagents were purchased as a kit from Bio-Rad.

3.3.2 Assays.

Phospholipid concentrations were determined using the phosphorus assay of Rouser (Rouser et al, 1960). Protein purity was assayed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (Leammli, 1970), followed by staining with Coomassie R-250 or silver stain. Protein concentrations were determined by the method of Lowry (Lowry et al, 1951) modified by the addition of SDS (1.8 mM SDS final concentration). Fibrinogen solution concentrations were also determined using the Bio-Rad assay (Bradford, 1976) and the BCA assay (Smith et al, 1985) from Pierce. All protein concentration assays used bovine serum albumin as a standard. A large discrepancy was observed between the concentrations determined for fibrinogen by the three different protein assays. Fibrinogen concentrations reported in this

paper refer to those obtained with the Bio-Rad method, which are ~70% of those obtained by Lowry assay and ~156% of the values obtained with the BCA assay.

3.3.3 Surfactant Preparation.

Pulmonary surfactant was prepared from bovine lavage material by a modification of the method described elsewhere (Yu et al, 1983; Weber & Possmayer, 1984). Briefly, Lipid Extract Surfactant (LES) is obtained by organic extraction of lavage material. Neutral lipids are removed by acetone solubilization. The material is dried, made up in chloroform/methanol 9:1 and stored at -20°C. This preparation contains all of the phospholipids of natural surfactant and the two small hydrophobic proteins SP-B and SP-C, however it has been completely stripped of SP-A.

3.3.4 Purification of SP-A.

SP-A is purified from bovine natural surfactant as previously described (Cockshutt et al, 1990, chapter 2). Briefly, an aqueous preparation of natural surfactant is delipidated by washing in butan-1-ol, ether:ethanol 3:1, and pure ether. The solvent is completely removed and the precipitated protein is solubilized in 5 mM HEPES, 0.1 mM Na_2EDTA pH 7.4, and centrifuged at 30 000 g for 20 minutes to remove any insoluble material. The solubilized protein is then supplemented with $CaCl_2$ to a concentration of 1 mM and affinity purified on

a column of immobilized D-mannose. Bound protein is eluted with 2 mM EDTA in 5 mM HEPES, pH 7.4.

3.3.5 Fibrinogen Purification.

Fibrinogen from Kabivitrum was purified as previously described (O'Brodovich et al, 1990). The fibrinogen solution containing Trasylol (American Diagnostica), a protease inhibitor, is passed over a lysine-Sepharose matrix (Pharmacia) to remove contaminating plasminogen. The preparation is then repeatedly precipitated in $(NH_4)_2SO_4$ (144g/L final concentration) to concentrate the fibrinogen and remove factor XIII. The pellet is redissolved in 5 mM sodium citrate, pH 7.5, dialysed against 0.1 M NaCl 50 mM Tris pH 7.4, and stored at -70°C.

3.3.6 Pulsating Bubble Surfactometer.

Surface tension was measured during dynamic compression using a pulsating bubble surfactometer as described (Enhorning, 1977). Briefly, a bubble of ambient air drawn through a small tube is formed in a chamber containing a surfactant suspension. This bubble is pulsated at a rate of 20 cycles per minute at 37°C between fixed radii (R_{min}=0.4 to R_{max}=0.55 mm) resulting in a 50% change in surface area. The pressure across the bubble is measured by a pressure transducer and the surface tension can be calculated from the Law of Laplace: $\Delta P = 2\gamma/r$; where gamma is the surface tension, ΔP is the pressure across the

bubble, and r is the radius of the bubble. All surfactant samples assayed on the bubble machine were made up in 0.9% NaCl, 1.5 mM $CaCl_2$. Those samples assayed in the absence of Ca^{++} had 1.5 mM EDTA added to the saline buffer in place of $CaCl_2$. When SP-A is mixed with the surfactant, the surfactant is made up at 2 times the final concentration, mixed with 2X SP-A in saline-calcium, and incubated at 37°C similarly to other preparations. The pH of the resulting suspensions ranged between 6.1 and 7.5. The biophysical activity of LES remains unchanged over this range as previously demonstrated (Metcalfe et al, 1980).

3.4 Results

Survanta quickly reduces surface tension at minimum bubble radius (R_{min}) during dynamic compression (see Table 3.1). Relative to LES, Survanta reduces the surface tension within fewer pulsations, but only to values of ~7 mN/m. Surface tension at maximum bubble radius (R_{max}) remains high (40-44 mN/m) relative to LES (25-27 mN/m). The surface activity of Survanta is not as dependent on phospholipid concentration as LES, and except for a relatively small increase in the surface tension at R_{max}, the activities at 1 and 2.5 mg/mL are identical. Addition of SP-A to Survanta at concentrations sufficient for maximal enhancement of LES does not cause a significant enhancement of surface activity. Enhancement of the surface activity of LES by SP-A is included in Table 3.1 for comparison.

Survanta has been previously demonstrated to be inhibited by the addition of blood proteins (Fuchimukai et al, 1987). Fibrinogen has been shown to be a particularily potent inhibitor of this and other surfactants. Figure 3.1 shows a concentration curve for the addition of pure human fibrinogen to Survanta at 1 mg/mL. When assayed in the presence of calcium little or no inhibition is observed, and a white precipitate is formed. However, when calcium is not added to the assay mixture inhibition is maximal at or below 2 mg/mL fibrinogen. Full inhibition results in surface tensions at R_{min} of ~28 mN/m. This value is considerably lower than the surface tension observed with pure fibrinogen (~43

mN/m) or LES fully inhibited by fibrinogen. It should also be noted that the amount of fibrinogen required to maximally inhibit Survanta is much greater than the ~0.1 mg/mL required for LES (Cockshutt et al, 1990, chapter 2).

Addition of albumin to Survanta at serum concentrations (50 mg/mL) leads to high surface tensions at R_{min}; those at R_{max} remain high as well as can be seen in Table 3.2. This inhibition is observed in either the presence or absence of calcium. In contrast to LES, SP-A does not affect the inhibiton of Survanta by albumin. Since the action of SP-A has been previously demonstrated to be absolutely dependent upon the presence of calcium, similar experiments on the effect of SP-A on fibrinogen inhibition of Survanta cannot be performed.

The inhibition of surfactant by another substance, lysophosphatidylcholine (lyso-PC), has been demonstrated (Cockshutt et al, 1990). The effect of added lyso-PC on Survanta is shown in Figure 3.2. Addition of 10% lyso-PC causes a large increase in the surface tension at R_{min}, but only a small increase at R_{max}. When the lyso-PC concentration is raised to 20% the surface tension at R_{min} is further increased, whereas that at R_{max} is decreased. This shows that like LES Survanta can be strongly inhibited by lyso-PC.

To investigate the cause of the differences in activity between Survanta and LES with regard to surface activity, the enhancement of activity by SP-A and the sensitivity to inhibitors, experiments were performed adding palmitic acid to LES to remove one of the differences in composition between these two surfactants. The effect of addition of palmitic acid to LES at 10% (w/w) of the surfactant

lipid concentration is shown in Figure 3.3. At this concentration palmitic acid enhances the surface activity of LES by decreasing the number of pulsations required to reach near zero surface tension at R_{min}. The effect of palmitic acid appears to be as good as or better than that of SP-A. When both SP-A and palmitic acid are present, the activity of the preparation resembles most closely the activity with palmitic acid. The surface tension at R_{max} was reduced by the addition of SP-A from 37.9 $\pm$ 1.6 mN/m to 25.7 $\pm$ 0.9 mN/m, palmitic acid reduced the value to 30.2 $\pm$ 1.2 mN/m, and the two together resulted in a surface tension of 27.4 $\pm$ 0.4 mN/m. These results show that SP-A has a more potent effect on R_{max} than palmitic acid while palmitic acid has a more rapid effect on R_{min}.

A concentration curve for the enhancement of activity of LES by addition of palmitic acid is shown in Figure 3.4. Surface tension after 30 seconds of adsorption without pulsation is plotted as well as values obtained after 10 seconds of adsorption followed by 20 pulsations. At least 7.5% (w/w of the lipid concentration) must be added to obtain maximal enhancement of surface activity observed in the twentieth pulsation. A small amount of enhancement is observed between 1-2% palmitic acid, however, between 3-6% palmitic acid surface activity is indistinguishable from control values. Above 6.5% palmitic acid enhancement is observed. This observation has been made in repeated trials and is as yet not understood. Approximately 8% added palmitic acid is required for a maximal effect on adsorption. This is in marked contrast to the addition of SP-A

to LES which requires as little as 0.1-0.5% of the lipid concentration for full enhancement (Cockshutt et al, 1990). This indicates that these two surfactant effectors may not be functioning via the same mechanism.

The effect of supplementation of LES with palmitic acid on the inhibition by blood components was determined. Figure 3.5 shows the effect of palmitic acid on the inhibition of LES by fibrinogen. Addition of 10% palmitic acid partially counteracts this inhibition. With repeated pulsation the surface tension at R_{min} begins to fall in the presence of palmitic acid. Reversal of fibrinogen inhibition by palmitic acid is not as pronounced as previously observed with SP-A. Furthermore, the addition of palmitic acid does not prevent the counteraction of inhibition by SP-A (data not shown).

The effect of palmitic acid on the inhibition of LES by albumin is similar to that seen with fibrinogen (see Table 3.3). The surface tensions at R_{min} and R_{max} in the presence of albumin are high. When palmitic acid is included in the surfactant preparation the surface tension is decreased to normal values at R_{max} and near normal values at R_{min} with repeated pulsation. The inhibition of Lipid Extract Surfactant and Survanta by alpha-globulin is shown in Table 3.4. The inhibition of LES by alpha-globulin can be reversed by the addition SP-A, whereas that of Survanta is unaffected by the addition of SP-A. Addition of palmitic acid causes only a small partial reversal of this inhibiton of LES.

The effects of palmitic acid on inhibition of LES by lyso-PC are shown in Table 3.5. Inhibition by lyso-PC is essentially removed by palmitic acid. This is

in marked contrast to what is observed with addition of SP-A to LES, which has no effect on the inhibition by lyso-PC.

Table 3.1 Effect of SP-A on the Biophysical Activity of Survanta and Lipid Extract Surfactant

Sample Description	Surface Tension after 20 pulsations (mN/m)	
	R_{min}	R_{max}
Survanta 2.5 mg/mL	7.8 ± 0.3*	43.7 ± 0.9
+ 5% SP-A (0.125 mg/mL)	6.9 ± 1.3	41.8 ± 2.0
LES 2.5 mg/mL	7.7 ± 1.3	27.1 ± 0.2
+ 5% SP-A (0.125 mg/mL)	2.8 ± 0.6	25.9 ± 0.5
Survanta 1 mg/mL	8.1 ± 0.1	47.7 ± 0.9
+ 5% SP-A (0.05 mg/mL)	7.1 ± 0.1	48.1 ± 0.4
LES 1 mg/mL	15.0 ± 1.5	37.9 ± 1.6
+ 5% SP-A (0.05 mg/mL)	7.0 ± 1.4	25.7 ± 0.9

* Values are the means ± S.E.M. of 3 separate batches of surfactant each assayed 3 times.

Table 3.2 Inhibition of Survanta by Albumin in the Presence and Absence of Calcium, and the Effect of SP-A

Sample Description	Surface Tension after 20 pulsations (mN/m)	
	R_{min}	R_{max}
Survanta 1 mg/mL, no $CaCl_2$	8.1 ± 0.4*	49.6 ± 0.3
+ 50 mg/mL BSA	42.7 ± 0.6	51.8 ± 0.3
Survanta 1 mg/mL, 1.5 mM $CaCl_2$	7.7 ± 0.5	48.5 ± 0.4
+ 50 mg/mL BSA	42.2 ± 0.3	51.6 ± 0.4
Survanta 1 mg/mL, 1.5 mM $CaCl_2$		
+ 5% SP-A (0.05 mg/mL)	7.3 ± 0.6	48.0 ± 0.6
+50 mg/mL BSA	42.7 ± 0.3	51.8 ± 0.4

* Values are the means ± S.E.M. of 3 separate batches of Survanta each assayed 3 times.

Table 3.2 Effect of Palmitic Acid on the Inhibition of Lipid Extract Surfactant by Albumin

Sample Description	Surface Tension after 20 pulsation (mN/m)	
	R_{min}	R_{max}
LES 1 mg/mL	15.0 ± 1.5*	38.0 ± 1.6
+ 10% palmitic acid	4.6 ± 1.3	30.2 ± 0.6
+ 50 mg/mL BSA	42.7 ± 0.8	51.2 ± 0.8
+ 10% palmitic acid	10.5 ± 5.4	29.8 ± 3.3

*Values are the means ± S.E.M. of 3 separate batches of LES each assayed 3 times.

Table 3.4 Effect of Alpha-globulin on Lipid Extract Surfactant, Survanta, and Lipid Extract Surfactant Supplemented with Palmitic Acid

Sample Description	Surface Tension after 20 pulsations (mN/m)	
+ 5 mg/mL alpha globulin	R_{min}	R_{max}
LES 2 mg/mL	36.9 ± 0.7*	43.4 ± 0.7
+ 5% SP-A (0.1 mg/mL)	10.1 ± 4.8	28.8 ± 2.0
+ 10% palmitic acid	29.2 ± 0.4	41.1 ± 0.7
Survanta 2 mg/mL	25.1 ± 0.0	41.1 ± 0.1
+ 5% SP-A (0.1 mg/mL)	24.6 ± 0.8	41.2 ± 0.4

* Values are the means ± the S.E.M. of 3 separate batches of surfactant each assayed 3 times.

Table 3.5 Effect of Palmitic Acid on the Inhibition of Lipid Extract Surfactant by Lysophosphatidylcholine

Sample Description	Surface Tension after 20 pulsations (mN/m)	
	R_{min}	R_{max}
LES 2.5 mg/mL	7.0 ± 1.0*	30.2 ± 1.5
+ 10% palmitic acid	5.7 ± 0.5	26.8 ± 0.4
+ 10% lyso-PC	15.1 ± 2.3	35.3 ± 2.9
+ 10% palmitic acid	6.5 ± 0.5	30.5 ± 0.3

* Values are the means ± S.E.M. of 3 separate batches of LES each assayed 3 times.

Figure 3.1 The effect of fibrinogen on Survanta in the presence and absence of calcium. Surface tension at minimum bubble radius after 20 pulsations (mN/m) is plotted vs. final fibrinogen concentration (mg/mL, as determined by the method of Bradford). Survanta is 1 mg/mL based on the phospholipid concentration. Open symbols are for samples assayed in the presence of 1.5 m$\underline{M}$ $CaCl_2$, filled symbols represent samples assayed in the absence of calcium. Values are the means $\pm$ the S.E.M. for 3 batches of Survanta each assayed 3 times.

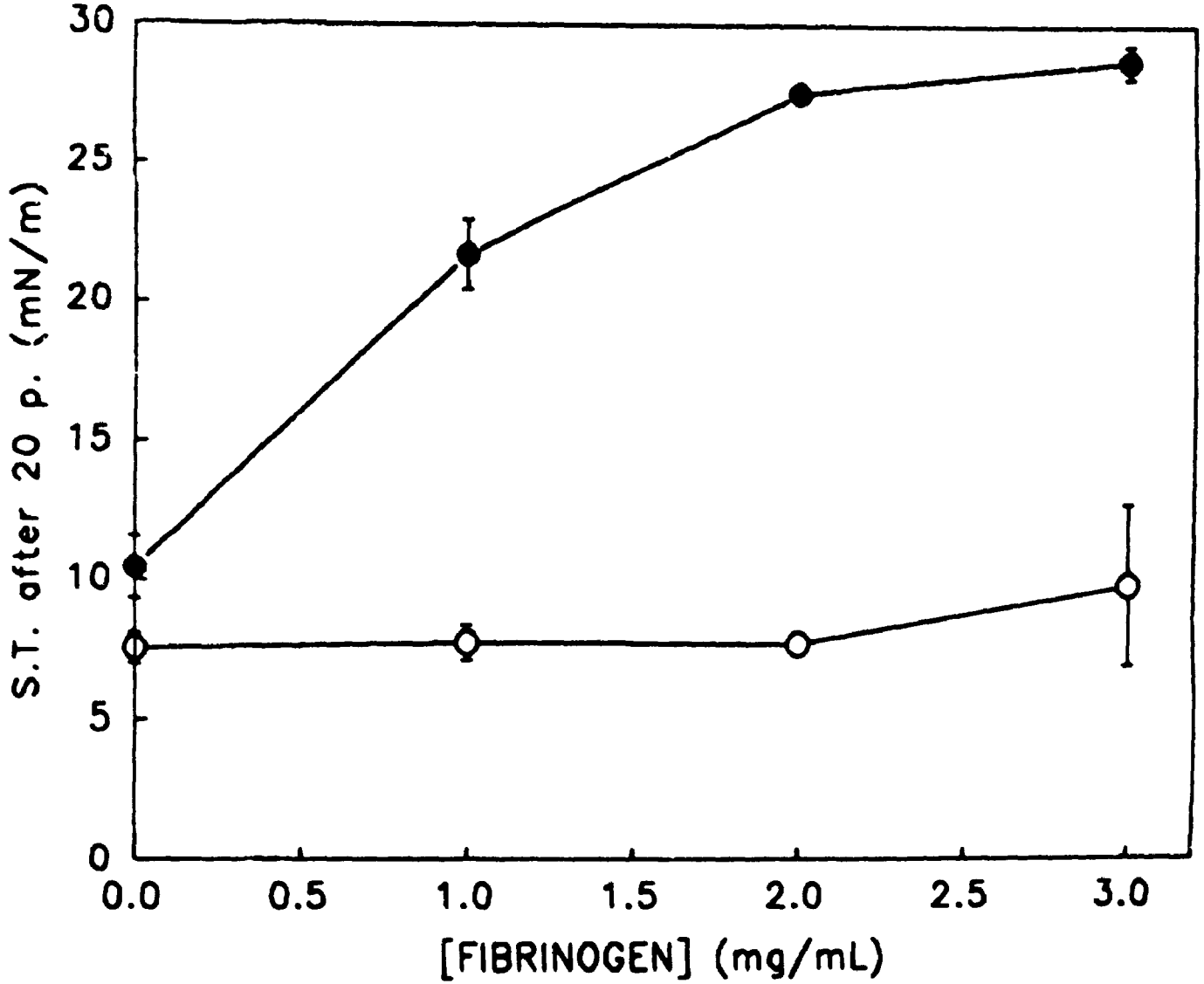

30
25
20
15
10
5
0
S.T. after 20 p. (mN/m)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[FIBRINOGEN] (mg/mL)

Figure 3.2 The effect of added lysophosphatidylcholine on Survanta. Surface tension (mN/m) is plotted vs. pulsations. Survanta is 2.5 mg/mL phospholipid. Open symbols represent surface tension at minimum bubble radius, filled symbols are at maximum bubble radius. Circles represent Survanta alone, triangles are Survanta plus 10% added lyso-PC, squares are Survanta plus 20% added lyso-PC. Values are the means $\pm$ S.E.M. of three separate batches of Survanta each assayed 3 times.

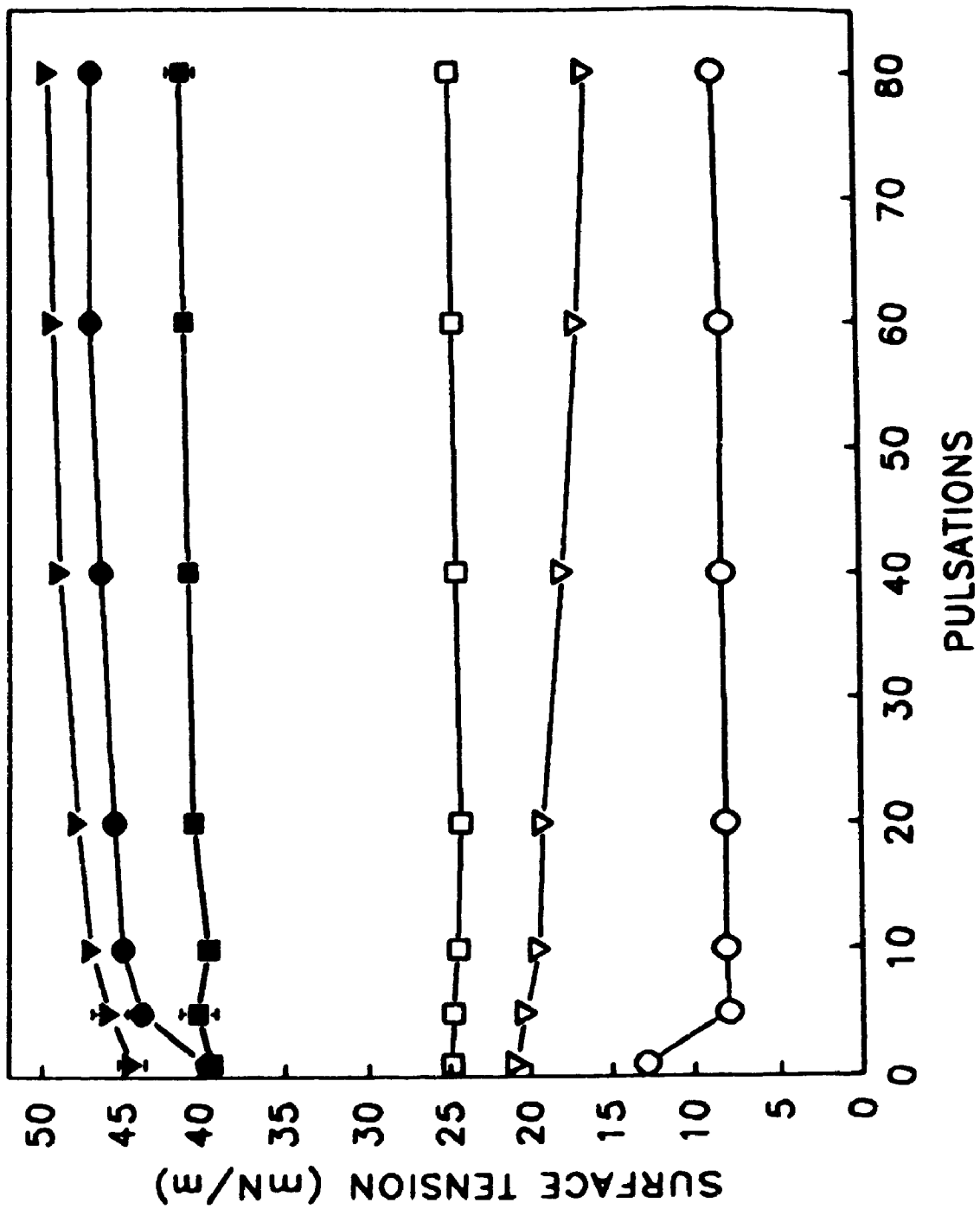

SURFACE TENSION (mN/m)
50
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
PULSATIONS

Figure 3.3 The effect of palmitic acid and SP-A on Lipid Extract Surfactant. Surface tension at minimum bubble radius (mN/m) is plotted vs. pulsations. LES is 1 mg/mL phospholipid. Open circles represent LES alone, filled circles are LES plus 5% SP-A, open triangles are LES plus 10% palmitic acid, filled triangles are LES plus 10% palmitic acid plus 5% SP-A. Values are the means $\pm$ the S.E.M. of 3 batches of LES each assayed 3 times.

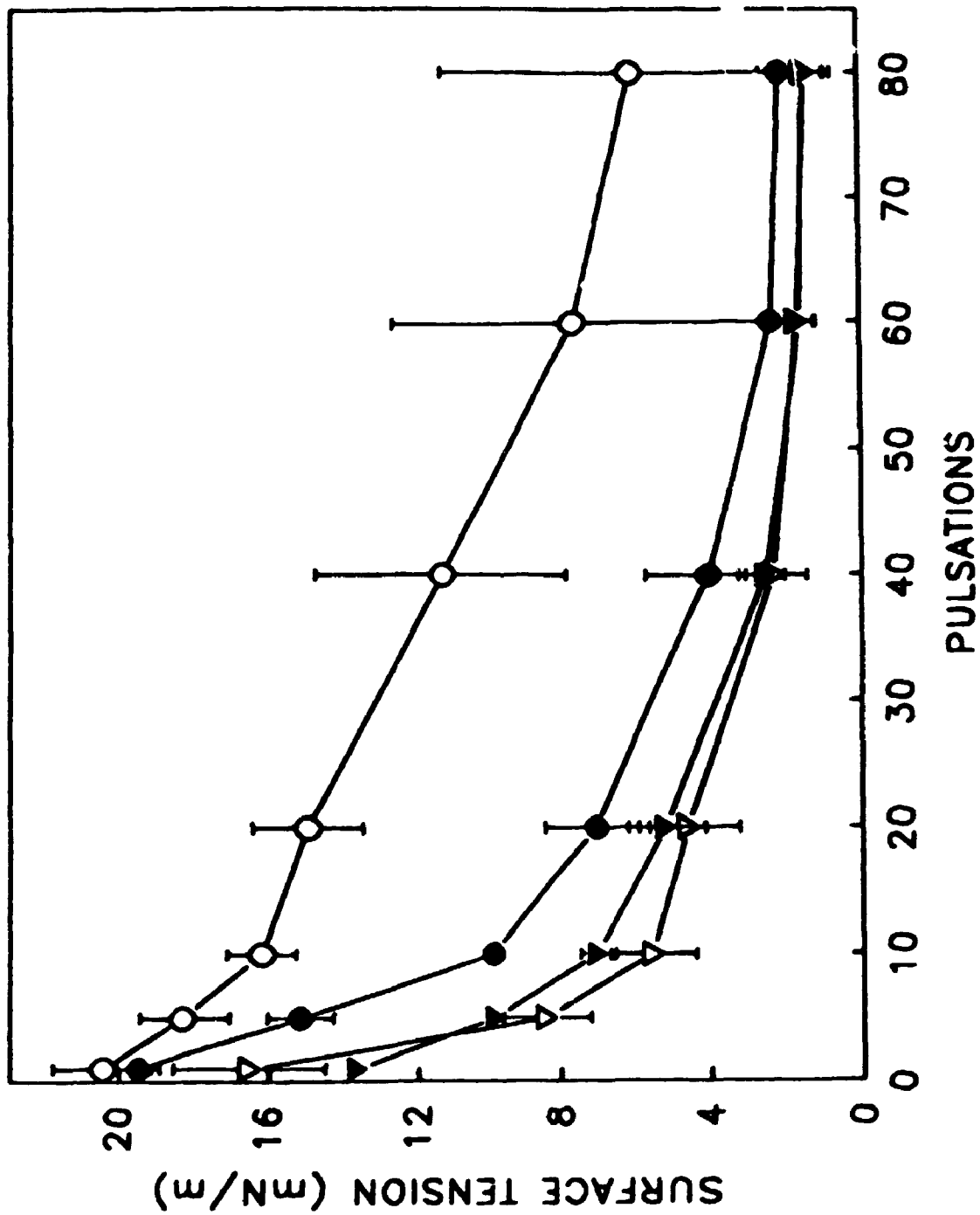

SURFACE TENSION (mN/m)
20
16
12
8
4
0
0
10
20
30
40
50
60
70
80
PULSATIONS

Figure 3.4 Concentration curves for the addition of palmitic acid to Lipid Extract Surfactant. Surface tension (mN/m) is plotted vs. the concentration of added palmitic acid expressed as a percentage of the surfactant lipid concentration (w/w). LES is 1 mg/mL based on phospholipid content. Open symbols represent surface tension at minimum bubble radius after 20 pulsations; filled symbols represent the surface tension after 30 seconds of adsorption without pulsation. Values are the means $\pm$ the S.E.M. for 3 separate batches of LES each assayed 3 times.

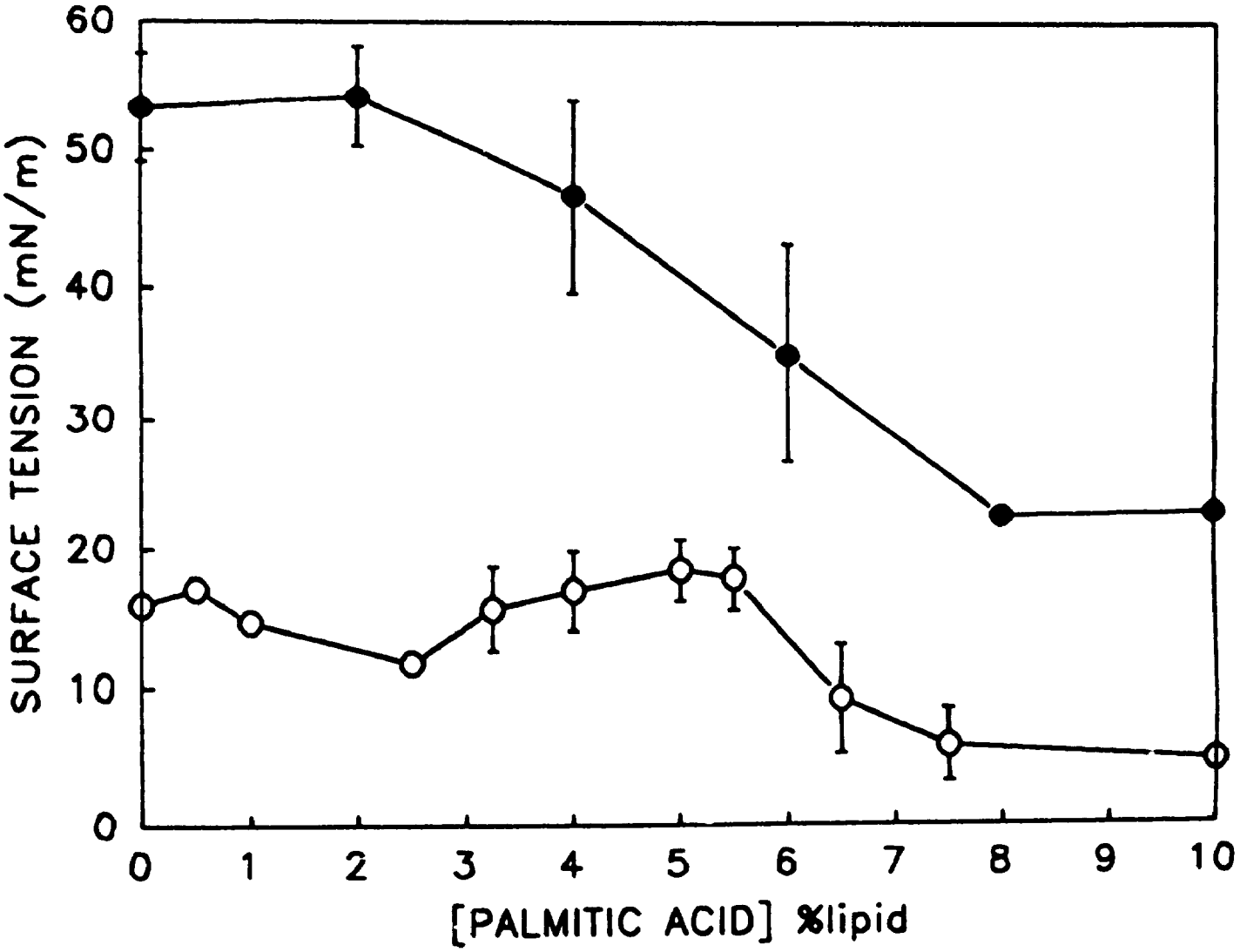
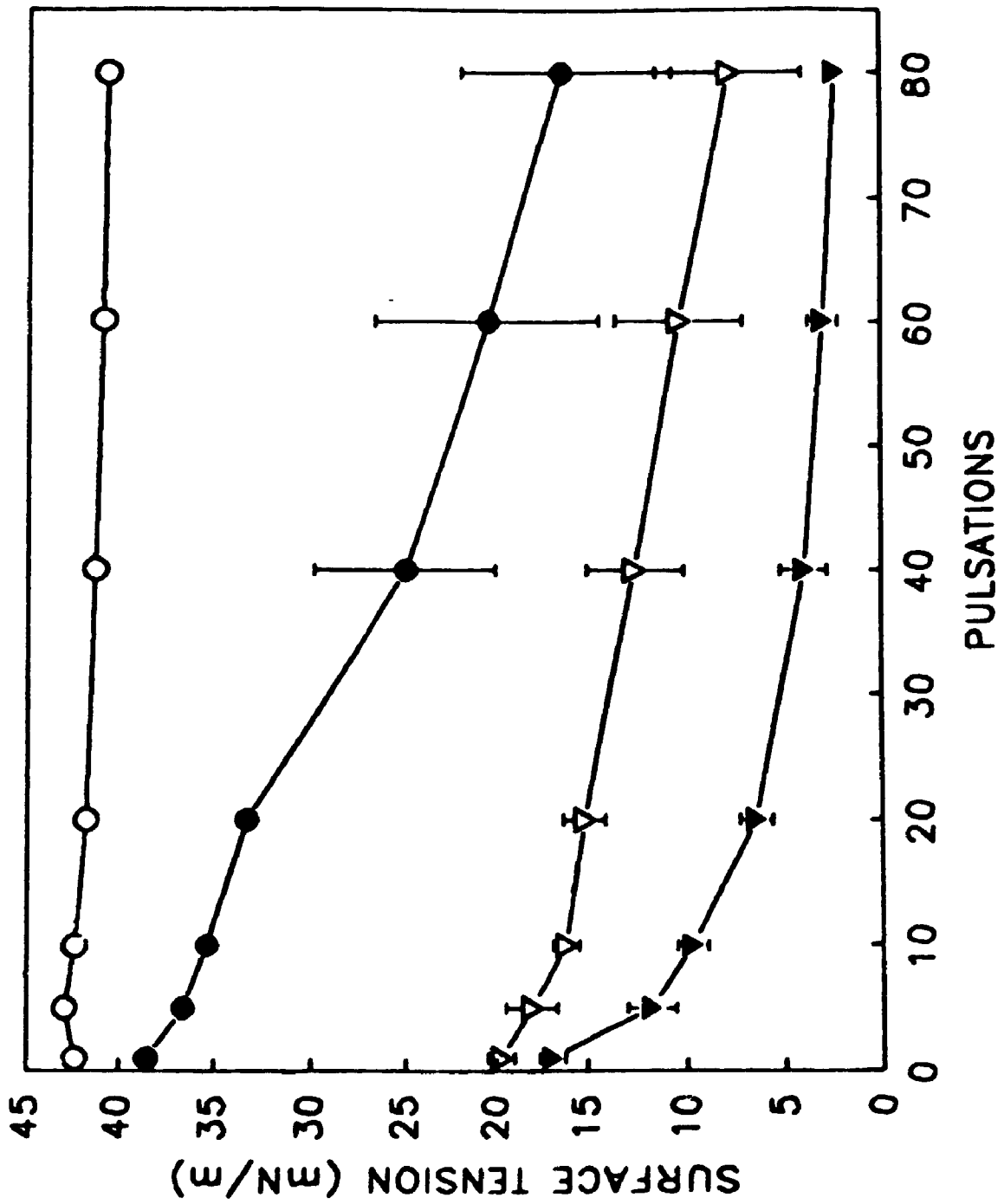

SURFACE TENSION (mN/m)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
[PALMITIC ACID] %lipid

Figure 3.5 The effect of added palmitic acid on fibrinogen inhibition of Lipid Extract Surfactant. Surface tension at minimum bubble radius (mN/m) is plotted vs. pulsations. LES is 1 mg/mL based on phospholipid concentration. Open circles represent LES with 1 mg/mL fibrinogen (as determined by the method of Bradford), filled circles are LES plus 10% palmitic acid with 1 mg/mL fibrinogen, open triangles are LES alone, filled triangles are LES plus 10% palmitic acid. Values are the means ± S.E.M. of 3 separate batches of LES each assayed 3 times.


SURFACE TENSION (mN/m)
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
PULSATIONS

3.5 Discussion

Since the discovery by Avery and Mead that surfactant deficiency causes respiratory distress in the newborn (Avery & Mead, 1959), replacement therapy has become an attractive approach to the treatment of this syndrome (Jobe & Ikegami, 1987). This discovery also incited interest in the understanding of the mechanisms of surface tension reduction with hopes that the development of improved and/or synthetic surfactants would ensue. More recently the involvement of inhibitory proteins in the development of disease has been investigated (Burkhardt & van Golde, 1989; Robertson & Lachmann, 1988; Jobe, 1989). Many products are currently undergoing clinical trials. In the present investigation two such preparations, Survanta and Lipid Extract Surfactant, were analyzed with respect to their surface activity during dynamic compression both in the presence and absence of challenge by surfactant inhibitors. Furthermore, the effect of addition of palmitic acid to LES in order to mimic in part the composition of Survanta was assayed.

Some of the surface active properties of Survanta appear to be very different from those of LES. Survanta reduces surface tension at R_{min} quickly without apparent dependence upon surfactant concentration within the range assayed. The surface activity of LES on the other hand shows a distinct inverse relationship with concentration (Chung et al, 1989; Cockshutt et al, 1990). This difference in surface activity is probably the result of the enhanced adsorptive

ability of Survanta. Presumably because of the presence of palmitic acid (8.5% by weight), Survanta adsorbs quickly to the interface even at low surfactant lipid concentrations. Experiments in this investigation demonstrate that the addition of palmitic acid to LES vastly enhances the adsorption of surfactant lipids to the interface at low surfactant concentrations. The effects of palmitic acid on the surface activity of synthetic lipid mixtures has been reported (Holm et al, 1990; Tanaka et al, 1986; Tanaka et al, 1983). In these investigations palmitic acid increased the rate of adsorption of surfactant lipids onto the monolayer and enhanced the surface activity during dynamic compression.

The surface tension of Survanta at R_{min} fell to ~ 7 mN/m within the first few pulsations, however it was never observed to decrease further, even with continued pulsation (although in the past values < 10 mN/m have been considered adequate, measurements in the lung of < 1 mN/m have been made (Schürch et al, 1976)). This suggests that this surfactant lacks the ability to efficiently enrich the monolayer in DPPC, which is thought to result in very low surface tension (0-2 mN/m) with compression. Perhaps this surfactant forms an unstable surface film which tends to collapse rather than pack upon compression resulting in higher surface tensions at R_{min}. Since the adsorption of Survanta lipids is very rapid it seems unlikely that insufficient lipid gets onto the interface following expansion to be packed upon compression. It is more likely that compression of this monolayer results in a limited amount of packing, reducing the surface tension to ~ 7 mN/m. However, compression beyond this point leads to collapse of the

monolayer rather than further packing preventing a further reduction in surface tension. This instability may be due to the presence of a relatively high proportion of non-DPPC lipids including unsaturated phospholipids and triglycerides as well as palmitate in the monolayer.

The unresponsiveness of Survanta to SP-A, even at low surfactant lipid concentrations, contrasted with the marked enhancement of LES under similar conditions, taken together with the enhancement of LES by palmitic acid indicates that the role of SP-A in Survanta is obviated by the presence of palmitic acid and that further enhancement of this nature is not possible. It has been previously demonstrated that the surface activity of LES is concentration dependent and that the effect of SP-A is most pronounced at lower surfactant concentrations (Cockshutt et al, 1990). In the current investigation it was also noted that the effect of palmitic acid was most dramatic at low surfactant lipid concentrations (data not shown). These findings suggest that a major factor in the mechanisms of enhancement by both palmitic acid and SP-A may be to increase adsorption, which is more apparent when adsorption is hampered by low bulk phase lipid concentrations.

It has been suggested that the biophysical activity of SP-A is mediated through the formation of the lipid structure tubular myelin (Goerke, 1974; Wright & Clements, 1987). It has also been shown that an increase in the content of tubular myelin can be correlated with an increase in the adsorption and spreading of lipids onto the monolayer (Benson et al, 1984). Tubular myelin may act to

store the extruded lipids and may facilitate more efficient respreading following compression. It appears that palmitic acid is acting in a more non-specific fashion which leads to the adsorption of an average mixture of bulk phase lipids. Studies of R_{min} surface tensions indicate that the rate of adsorption of LES with SP-A is similar to or even slower than that of LES with palmitic acid. However, since SP-A has a more potent effect on R_{max} it seems likely that it is not necessarily only the rate of adsorption, but also the type of adsorption that is important. One possibility is that SP-A may be able to cause selective adsorption from a pool compositionally distinct from the bulk phase lipids. This pool may be the lipids of tubular myelin.

It can be postulated that the enhanced adsorptivity of surfactant lipids which added palmitic acid imparts to LES and is observed with Survanta may lead to an alteration in monolayer configuration. This is indicated by the higher surface tensions observed at R_{max} for Survanta and for LES enhanced with palmitic acid relative to SP-A. Similarily, Holm et al (Holm et al, 1990) reported that synthetic lipid mixtures containing palmitic acid maintain higher surface tensions at R_{max} than organic extracts of natural surfactants. It is thought that the surface tension at R_{max} reflects primarily the kinetics of the adsorption and spreading of new (i.e. non-monolayer) surfactant lipid upon bubble expansion following packing and/or removal during compression. For example, if a surfactant adsorbs to the interface and spreads slowly as the monolayer is expanded, regions with low phospholipid densities will form resulting in an

increase in . rface tension far above the equilibrium value. On the other hand, if the lipids adsorb and spread quickly, equilibrium surface tensions will be achieved shortly after expansion is completed.

In an effort to mimic more closely the composition of Survanta, palmitic acid was added to LES. This addition leads to the enhancement of surface activity, especially when assayed at low phospholipid concentrations (1 mg/mL). The resulting modified surfactant reduced surface tension at R_{min} during dynamic compression faster and to lower values than either Survanta or unmodified LES. ' was also observed that addition of palmitic acid to LES vastly increased the rate of adsorption of lipids to the monolayer, such that at 8-10% palmitic acid equilibrium surface tension was achieved almost immediately. Taken together, these findings suggest that palmitic acid enhances the adsorption of surfactant lipids, and that when added to LES it produces a lipid mixture which performs excellently during both adsorption and dynamic compression.

The concentration curve for palmitic acid indicates that a relatively large amount of palmitic acid is necessary for maximal enhancement of activity. At least 7.5% palmitic acid (w/w of the lipid concentration (1 mg/ml = ~ 1.3m<u>M</u>), which corresponds to ~ 0.3 m<u>M</u>), or 1 molecule per 4-5 molecules of surfactant lipid, must be added to obtain good adsorption and low initial surface tensions. When compared with the amount of SP-A required for maximal enhancement of activity (0.5%, which is ~ 7 n<u>M</u>, based on the native molecular weight of SP-A of 650 000), which corresponds to ~ 185 000 molecules of surfactant

phospholipid per SP-A oligomer, it can be concluded that the palmitic acid must act through a distinct mechanism, whereas SP-A may be involved in forming a complex structure, namely, tubular myelin.

Interactions between palmitic acid and other lipids which have been documented include differential scanning calorimetric data (Schullery et al, 1981; Ortiz & Gomez-Fernandez, 1987). The addition of palmitic acid at mole fractions similar to those used in the present investigation increases the phase transition temperature of pure DPPC (Schullery et al, 1981). The palmitic acid in these DPPC bilayers is mostly protonated at physiological pH's having a measured pKa of ~8.5. The protonated form of the molecule has a cylindrical shape suitable for tight packing with DPPC. Furthermore, the addition of saturated fatty acid to binary mixtures suggests that the fatty acid partitions preferentially into solid-like domains (Ortiz & Gomez-Fernandez, 1987). These results suggest that when palmitic acid is added to surfactant lipids it may enhance the adsorptivity and surface activity by adsorbing onto the monolayer, preferentially to more solid areas (i.e. those enriched in DPPC), and fill gaps and bare areas upon expansion, while packing tightly with the DPPC upon compression. This would explain why relatively large amounts of palmitic acid are required for the enhancement of surface activity, and that maximal enhancement of surface activity during dynamic compression is observed at the same concentration as maximal enhancement of adsorption.

Supplementation of LES with palmitic acid at 10% of the lipid concentration imparted the surfactant with increased resistance to the inhibitors fibrinogen, albumin, alpha-globulin and lyso-PC. The counteraction of protein inhibition followed different kinetics than has been observed with SP-A (Cockshutt et al, 1990). When SP-A is used to prevent protein inhibition, adsorption and initial surface tensions are normal (i.e. indistinguishable from uninhibited) whereas the effect of palmitic acid was only seen with repeated pulsation, with adsorption impaired. This also supports the notion that adsorption mediated by SP-A is specific whereas that by palmitic acid is non-specific.

The susceptibility of Survanta to certain inhibitors is suprising considering the inhibition controlling effects of palmitic acid on LES. In particular, the inhibition by albumin, which is very strong with Survanta, is almost completely abolished by addition of palmitic acid to LES. This suggests that another component of Survanta must render this surfactant susceptible to inhibition. As well, Survanta is strongly inhibited by lyso-PC which is prevented by palmitic acid.

The effect of palmitic acid on the susceptibility of LES to inhibition by lyso-PC is intriguing. SP-A is completely ineffectual against inhibition by lyso-PC whereas addition of 10% palmitic acid almost completely reverses this phenomenon. The effects of lyso-PC may include disruption of the tubular myelin structure as well as blocking the formation of a DPPC enriched monolayer. Palmitic acid appears to be able to enhance the adsorption of

surfactant lipids to the monolayer even in the presence of lyso-PC, suggesting that the mechanism of lyso-PC inhibition may be an impedance to normal lipid adsorption which can be overcome by the addition of palmitic acid. It is also possible that the cylindrically shaped palmitic acid may pack with the conically shaped lyso-PC to form a structure less perturbing to the monolayer.

The inhibition of Survanta by fibrinogen is unusual in that it is only observed in the absence of calcium. A few explanations are possible for this observation. It is possible that Survanta contains a substance that is capable of preventing inhibition in the presence of calcium (like SP-A), however, this putative substance is ineffective against inhibition by albumin and alpha-globulin. A second possibility is that calcium is required for optimal activity of Survanta, however, when assayed in the presence and absence of calcium it behaves almost identically. A more likely explanation is that Survanta contains a substance that leads to the inactivation of fibrinogen, probably by clotting, in the presence of calcium. This is supported by the observation that a white precipitate is formed when fibrinogen is added to Survanta. As well, most clotting enzymes require calcium for their activity.

The effects of palmitic acid on the inhibition of surfactants by proteins has been previously investigated by Holm et al (Holm et al, 1990). In these investigations the susceptibility of various synthetic lipid mixtures to inhibition by albumin was compared to the inhibition of natural and organically extracted surfactants. It was observed that the synthetic lipid mixtures were strongly

inhibited by albumin regardless of whether they contained palmitic acid, whereas naturally derived mixtures were more resistant to inhibition. However, in this investigation it was not reported whether supplementation of the natural surfactants with palmitic acid or SP-A would impart increased resistance to blood protein inhibition. Taken together with the present findings, it seems likely that the presence of SP-B and/or SP-C is required for prevention of inhibition. Perhaps the enhanced adsorption imparted by the palmitic acid must be complemented by the DPPC enriching ability of the small surfactant-associated proteins for optimal activity.

These experiments demonstrate that new more potent surfactants may be developed that combine the salient features of other preparations. By the addition of palmitic acid to LES we have generated a preparation with superiour *in vitro* activity to either LES or Survanta. This hybrid preparation adsorbs more rapidly to the monolayer than LES and reduces surface tension at R_{min} lower than Survanta. This preparation is also more resistant to challenge with inhibitors such as fibrinogen, albumin, alpha-globulin and lyso-PC. In addition this preparation can be made completely insensitive to the addition of blood protein inhibitors by the subsequent addition of SP-A.

All of the data presented in the present investigation have been made in an *in vitro* model of the alveolus, the pulsating bubble surfactometer. Clearly, the situation *in vivo* would be expected to be far more complex than the observations presented here. For instance, it has been reported that labelled palmitic acid

instilled into the airspace with surfactant is very rapidly cleared from the airways (Tabor et al, 1990). These data suggest that palmitic acid may not necessarily maintain these properties when included in surfactant preparations for replacement therapy. In addition, lyso-PC has been demonstrated to cause severe injury to the type I epithelial cell when instilled into the airspaces of isolated perfused lungs (Niewoehner et al, 1987). This injury results in increased permeability and leakage of blood components into the alveolar space. These effects would compound the primary effects on the surfactant leading progressive surfactant inactivation. For these reasons it is appropriate to study the basic relationships between these surfactant components in an *in vitro* model prior to embarking upon *in vivo* studies.

An appreciation of these observations could be very important with respect to the generation and treatment of both neonatal and adult forms of respiratory distress, where leakage of blood proteins into the alveolar space and the generation of lyso-PC by phospholipase A_2 are involved in the etiology of the disease.

CHAPTER 4 - SENSITIZATION TO INHIBITION BY SERUM PROTEINS BY LYSOPHOSPHATIDYLCHOLINE

4.1 Chapter Summary

Interactions between serum protein and lysophospholipid inhibitors of pulmonary surfactant were examined *in vitro* using a pulsating bubble surfactometer. In previous studies a particular batch of Lipid Extract Surfactant (LES) was observed to be unusually sensitive to inhibition by fibrinogen. This sample was found to contain an abnormally high concentration of lysophosphatidylcholine (lyso-PC). Addition of exogenous lysophospholipid to LES at similar concentrations sensitized the surfactant to inhibition by fibrinogen. Sensitization to inhibition by lyso-PC is also observed with fetal bovine serum. Under the conditions used, inhibition by bovine serum albumin was not affected. Whereas only small amounts of lyso-PC (1 mol % added) maximally sensitize LES to inhibition by fibrinogen, co-addition of equal amounts of palmitic acid can partially offset this effect at low lyso-PC concentrations (less than 5 mol %). Lipid Extract Surfactant was digested with phospholipase A_2 to mimic the generation of endogenous lyso-PC at the expense of surfactant lipids. Digestion of 2-3% of the phosphatidylcholine to lysophosphatidylcholine vastly sensitized the surfactant to inhibition by fibrinogen. These results suggest that the degradation of surfactant phospholipids by phospholipase A_2 to lysophospholipids

could contribute to the development and progression of adult and neonatal respiratory distress syndromes.

4.2 Introduction

Pulmonary surfactant is a surface active mixture of lipids and proteins found in the terminal airways and alveoli of the lung. Surfactant is produced in the alveolar type II epithelial cell and is secreted into the aqueous hypophase where it is observed to form a number of complex lipid-based structures including lamellar bodies, tubular myelin, small vesicles, and a phospholipid monolayer which lines the air-liquid interface (Goerke, 1974; Goerke & Clements, 1986; Wright & Clements, 1987). It is a monolayer highly enriched in dipalmitoylphosphatidylcholine which is thought to be responsible for very low surface tensions upon expiration (van Golde et al, 1988; Possmayer & Yu, 1990; Notter, 1988).

In 1959 Avery and Mead reported that a deficiency of pulmonary surfactant was responsible for the development of neonatal respiratory distress syndrome (NRDS), a disease primarily affecting prematurely delivered infants (Avery & Mead, 1959). Prophylactic and therapeutic administration of surfactant preparations to these infants has greatly reduced the long term morbidity associated with this disease (Robertson & Lachmann, 1988; Jobe & Ikegami, 1987).

Some attention has been turned to the role of pulmonary surfactant and its potential inactivation in the development and progression of adult respiratory distress syndrome (ARDS)(Robertson & Lachmann, 1988; Burkhardt & van

Golde, 1989; Holm & Matalon, 1989; Seeger et al, 1990). ARDS is frequently observed following a number of diverse conditions including sepsis, shock, trauma, pneumonia, lung injury, etc. Regardless of the etiology, ARDS is characterized by decreased pulmonary function including decreased lung compliance and arterial hypoxemia. A hallmark of ARDS is the presence of drastically elevated levels of serum-derived proteins in the alveolus. It has been demonstrated that blood proteins inactivate pulmonary surfactant when assayed either *in situ* in an excised lung model (Seeger et al, 1985; Holm & Notter, 1987) or *in vitro* using the pulsating bubble surfactometer or the Wilhelmy plate (Seeger et al, 1985; Fuchimukai et al, 1987; Holm et al, 1985; Keough et al, 1987; Cockshutt et al, 1990). Although the exact mechanism of this inhibition of surface activity is not known, an investigation of the inhibition of surfactant by albumin suggested that inhibition by this protein is a result of competition of the albumin with surfactant lipid for the air-water interface (Holm et al, 1988). Considering the varying inhibitory potency of different blood proteins assayed (Seeger et al, 1985; Fuchimukai et al, 1987; Cockshutt et al, 1990), it seems probable that not all proteins inactivate surfactant via a common mechanism. Specific lipid-protein or protein-protein interactions could alter the inhibitory potential of the protein.

The leakage of blood components into the alveolar space can be effectuated by a number of factors. In the case of NRDS it has been suggested that high transpulmonary pressures resulting from surfactant insufficiency lead to

transudation of serum components into the alveolus (Notter, 1988; Burlkhardt & van Golde, 1989; Guyton et al, 1984; Jobe, 1989). Another clinically important cause of leakage is increased permeability of the alveolar endothelium and epithelium resulting from injury or chemical damage (Seeger et al, 1985b; Holm et al, 1985b).

Evidence for changes in the amount and composition of pulmonary surfactant in the ARDS lung is conflicting (Holm & Matalon, 1989: Seeger et al, 1990). Some investigations report little to no change in surfactant levels and composition, whereas others report elevations in surfactant lipids and/or changes in the lipid composition (Seeger et al, 1990; Hallman et al, 1982; von Wichert et al, 1981). Compositional changes that have been documented include the observation of increased relative levels of cellular lipids (sphingomyelin, phosphatidylethanolamine, phosphatidylserine, glycolipid...) and an increase in lysophospholipids (predominantly lyso-PC) (Hallman et al, 1982; von Wichert et al, 1981; Rauvala & Hallman, 1984). Increased levels of cellular lipids are indicative of cellular breakdown, whereas the presence of lysophospholipids indicates the action of phospholipases on the surfactant and/or cell membranes. The presence of phospholipase A_2 in the alveolus would accompany the invasion of neutrophils drawn into the area by the chemotactic activity of chemical irritants or bacterial infection, or the activation of alveolar macrophages. Thus, the observation of lysophospholipids in ARDS is not surprising. Von Wichert et al reported in 1981 that lyso-PC levels were elevated six-fold in an ARDS model,

rat lung peritonitis (von Wichert et al, 1981). The authors also reported a five-fold elevation of lung phospholipase A_2. A two-fold increase in PLA_2 activity in patients suffering from ARDS has also been demonstrated (Hallman et al, 1982).

In the present investigation the interactions between surfactant degradation by phospholipase A_2 (and the generation of lysophospholipid) and the inhibition of surfactant by serum components were examined. It was observed that the presence of endogenous, exogenous or endogenously generated lysophospholipid in the surfactant leads to increased sensitization to inhibition by some blood proteins. It is therefore suggested that the degradation of surfactant lipids by phospholipase may be important in the development and progression of respiratory distress.

4.3 Materials and Methods

4.3.1 Reagents.

All chemicals were reagent grade or better. Palmitic acid, bovine serum albumin, fibrinogen and lysophosphatidylcholine were obtained from Sigma. Fibrinogen was also obtained from Kabivitrum, Helena Labs. Pancreatic phospholipase A_2 was purchased from Boehringer Mannheim. Trasylol was purchased from American Diagnostica. Lysine-sepharose was purchased from Pharmacia. Fetal bovine serum (FBS) was obtained from Gibco.

4.3.2 Assays.

Phospholipid concentrations were determined using the phosphorus assay of Rouser (Rouser et al, 1960). Phospholipid composition of the surfactants was determined by phosphorus assay following separation of the phospholipids by thin layer chromatography using the solvent system of Touchstone (Touchstone et al, 1980). Phospholipids were visualized on thin layer chromatographs by staining for phosphorus by the method of Dittmer and Lester (Dittmer & Lester, 1964). Protein concentrations were determined by the method of Lowry (Lowry et al, 1951) modified by the addition of SDS (1.8 mM SDS final concentration). Fibrinogen solution concentrations were also determined using the Bio-Rad assay (Bradford, 1976) and the BCA assay (Smith et al, 1985) from Pierce (see Cockshutt et al, 1990, chapter 2). All protein concentration assays used bovine

serum albumin as a standard. Protein purity was assayed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (Laemmli, 1970).

4.3.3 Surfactant Preparation.

Pulmonary surfactant was prepared from bovine lavage material by a modification of the method described elsewhere (Yu et al, 1983; Weber & Possmayer, 1984). Briefly, Lipid Extract Surfactant (LES) is obtained by organic extraction of lavage material. Neutral lipids are removed by acetone solubilization. The material is dried, made up in chloroform/methanol 9:1 and stored at -20°C. This preparation contains all of the phospholipids of natural surfactant and the two small hydrophobic proteins SP-B and SP-C, however it has been completely stripped of SP-A.

4.3.4 Fibrinogen Purification.

Fibrinogen from Kabivitrum was purified as previously described (O'Brodovich et al, 1990). The fibrinogen solution containing Trasylol, a protease inhibitor, is passed over a lysine-Sepharose matrix to remove contaminating plasminogen. The preparation is then repeatedly precipitated in $(NH_4)_2SO_4$ (144g/L final concentration) to concentrate the fibrinogen and remove factor XIII. The pellet is redissolved in 5 mM sodium citrate, pH 7.5, dialysed against 0.1 M NaCl, 50 mM Tris, pH 7.4, and stored at -70°C at 2-5 mg/mL.

4.3.5 Phospholipase A_2 Digestion of Surfactant.

Lipid Extract Surfactant was dried under N_2 and resuspended in 50 mM Tris, 3 mM $CaCl_2$, pH 8.0 at a phospholipid concentration of 10 mg/mL. The suspension was repeatedly vortexed and bath sonicated in warm water. The suspension was prewarmed at 37-40°C for 10 minutes. Pancreatic phospholipase A_2 (0.14 U/mg phospholipid) was added and the mixture was shaken at 37-40°C. Samples were removed at intervals and placed in 1.11 volumes of cold chloroform:methanol 1:1 and vortexed to stop the reaction. The samples were extracted by the method of Bligh and Dyer (Bligh & Dyer, 1959). The organic phase was washed twice with 1% potassium chloride. An aliquot of the organic phase was transferred to a centrifuge tube and dried thoroughly under N_2 onto the sides of the tube. Ten mL cold acetone was added and the tube was placed at -70°C for ~2 hours. The tubes were centrifuged at -10 °C for 15 minutes at 1500 rpm. The supernatant (containing neutral lipids) was discarded. The pellet was dried under N_2 and dissolved in chloroform:methanol 9:1.

4.3.6 Pulsating Bubble Surfactometer.

Surface tension was measured during dynamic compression using a pulsating bubble surfactometer as described (Enhorning, 1977). Briefly, a bubble of ambient air drawn through a small tube is formed in a chamber containing ~20 uL of surfactant suspension. This bubble is pulsated at a rate of 20 cycles per minute at 37°C between fixed radii (R_{min}=0.4 to R_{max}=0.55 mm) resulting in

a 50% change in surface area. The pressure across the bubble is measured by a pressure transducer and the surface tension can be calculated from the Law of Laplace: $\Delta P=2\gamma/r$; where gamma is the surface tension, ΔP is the pressure across the bubble, and r is the radius of the bubble. All surfactant samples assayed on the bubble machine were made up in 0.9% NaCl, 1.5 m<u>M</u> $CaCl_2$.

4.3.7 Inhibition Assay.

Surfactant in organic solvent was dispensed into glass tubes and thoroughly dried under N_2. NaCl 0.9%, 3 mM $CaCl_2$ was added to generate 2X lipid suspensions (in most cases 4 mg/mL). The tubes were vortexed and shaken on a WristAction Shaker for 20 minutes at room temperature. Samples were incubated at 37°C for at least 2 hours. Approximately 5 minutes before assaying, the samples were mixed with equal volumes of inhibitory protein in saline solutions in eppendorf tubes. The samples were returned to the water bath before assaying (more rigorous mixing by vortexing or sonicating leads to decreased inhibitory potential of fibrinogen, presumably due to aggregation). Final concentrations of salts were 0.9 % NaCl, 1.5 mM $CaCl_2$. Fibrinogen inhibited samples also contained 25 mM Tris, pH 7.4. When lyso-PC was added as an inhibitor it was mixed with the LES in organic solvent. These samples were dried down and resuspended as above.

4.4 Results

During previous investigations of the inhibitory action of serum proteins on pulmonary surfactant (Cockshutt et al, 1990) it was noted that a particular batch of Lipid Extract Surfactant (LES) had a greater sensitivity to inhibition by crude fibrinogen than other preparations (Figure 4.1). This batch, LES #91, was discarded from those studies, but the nature of this increased sensitivity was investigated. Examination of the surfactant-associated protein composition by polyacrylamide gel electrophoresis showed no difference in this batch from normal LES (data not shown). The phospholipid composition of this batch was compared to normal LES by separation of the phospholipids by thin layer chromatography and subsequent visualization of the phospholipids by Dittmer and Lester spray (Figure 4.2a.). The phospholipid profile was quantitated by scraping the silica and measuring the phosphorus content of each section, the results of this analysis are shown in Table 4.1. The major difference between this batch and other batches is the amount of lyso-PC in LES #91, 5.5 mol % compared to 0.2-0.3% in normal samples. This is apparently at the expense of the PC which represents a smaller fraction of the total phospholipid in LES #91 than in normal LES. Another difference is the presence of a novel spot in LES #91 migrating just above the PE spot. This spot, which represents 1.3% of the phosphorus in the sample migrates at the position corresponding to lyso-PG (a spot migrating at this position is seen in some PG standard preparations, and with PG digested with

PLA_2). Thus, the anomaly in this batch appears to be the presence of abnormally high amounts of lysophospholipid. The lyso-PC and lyso-PG in this batch represent 7% and 8.5% respectively of the PC and PG pools.

To determine if lyso-PC was the component of LES #91 causing increased sensitization to fibrinogen inhibition, lyso-PC was added to normal LES at similar concentrations (5 mol %) and the fibrinogen sensitivity curves were compared (Figure 4.2b.). At low concentrations of purified fibrinogen (less than 0.2 mg/mL) LES + lyso-PC is more inhibited than normal LES. LES + lyso-PC and LES #91 behave very similarly (data not shown), with higher surface tensions being observed at both maximum and minimum bubble radius.

The inhibitory and sensitizing activities of sphingomyelin, another lipid which has been observed to increase in ARDS, was also assayed. This component neither inhibited surface activity nor sensitized the preparation to inhibition by fibrinogen even when as much as 15% (w/w) was added (data not shown).

The ability of lyso-PC to sensitize the surfactant to inhibition by other blood components was assayed. Inhibition by fetal bovine serum was slightly sensitized by addition of lyso-PC (Figure 4.3a.), although the effect was not as dramatic as that observed with fibrinogen. Interestingly, no increase in the inhibition by albumin was observed when lyso-PC was added (Figure 4.3b.) or with LES #91 (data not shown). In all experiments with inhibitory blood proteins the surface tension at minimum bubble radius at high protein concentrations was

slightly lower when lyso-PC was present. This was not observed at maximum bubble radius.

A concentration curve for the addition of lyso-PC to LES in the presence and absence of challenge by fibrinogen is shown in Figure 4.4a. A fibrinogen concentration was chosen, 0.105 mg/mL (from Figure 4.2b.), such that the sensitization effect was maximized. As little as 1 mol % added lyso-PC is sufficient to cause maximal sensitization to fibrinogen inhibition with very little inhibition of the surfactant by the lyso-PC alone. This demonstrates that at low levels of inhibitor the sensitization effect is more than simply additive.

When lyso-PC is generated from PC by the action of PLA_2 fatty acid is also released at equal molar concentrations. Since palmitic acid has been demonstrated to both enhance surface activity and partially reverse blood protein and lyso-PC inhibition in LES (Cockshutt et al, in press, chapter 3), the effect of co-addition of equal molar amounts of lyso-PC and palmitic acid was assayed (Figure 4.4b.). Low amounts of palmitic acid appeared to partially suppress the sensitization effect. However, at higher concentrations of added lipid (5 and 10 mol %) sensitization was indistinguishable from the addition of lyso-PC alone.

In the above experiments lyso-PC has been added to the LES. A situation more likely to occur *in vivo* is that lysophospholipid should arise from digestion of the surfactant phospholipid, thus at the same time depleting the intact phospholipid pool and introducing the inhibitory lysophospholipid. This was mimicked *in vitro* by the digestion of LES with phospholipase A_2 (PLA_2). The

results of this digestion are shown in Figure 4.5. As can be judged from the lyso-PC standard curve on the plate, approximately 3-4%, 6-8%, 15% and 20% lyso-PC is generated after 15, 30, 60 and 120 minutes of PLA_2 digestion respectively. It should also be noted the PG and PE are also digested in a similar manner (on this plate lyso-PG comigrates with PE). To analyze the fatty acid effect in these samples, the digested material was divided and half was precipitated with acetone to remove neutral lipids (including palmitic acid). The average recovery of phospholipid following acetone washing was 91.2%.

These samples were assayed for their sensitivity to fibrinogen inhibition relative to the zero time sample (Figures 4.6). In acetone precipitated samples (Figure 4.6a.) low levels of PLA_2 digestion (15 and 30 min.) drastically sensitize the surfactant to inhibition by fibrinogen. This is observed at minimum bubble radius at both low (0.056 mg/mL) and high (0.105 mg/mL) fibrinogen concentrations. A large effect on surface tension at maximum bubble radius is observed only at low fibrinogen concentrations. After longer periods of digestion (60 and 120 min.) the sensitization effect was significantly reduced, however in these cases the surfactant was markedly inhibited even in the absence of fibrinogen. These effects are somewhat less apparent with the non-acetone washed samples (Figure 4.6b.). Surprisingly, even the zero time sample for the non-acetone washed samples had increased sensitivity to fibrinogen inhibition relative to the untreated or acetone washed samples. The phospholipid profiles of these samples are indistinguishable from the acetone washed samples. These

observations suggest that an acetone soluble substance may be introduced to the surfactant during the digestion procedure, or that small amount of pre-existing contaminants are removed by the acetone wash. The sensitization observed with the endogenously generated lyso-PC was similar to that observed with added lyso-PC, suggesting that these experiments may indeed reflect situations occurring in the lung.

Table 4.1 The Phospholipid Composition of Three Different Batches of Lipid Extract Surfactant

Phospholipid	% of total phosphorus		
	LES #90	LES #91	LES #92
lyso-PC	0.2 ± 0.03	**5.5 ± 0.11***	0.3 ± 0.10
SM	1.4 ± 0.23	2.6 ± 0.24	1.9 ± 0.49
PC	78.9 ± 0.53	**72.6 ± 0.55***	80.2 ± 0.88
PI	1.0 ± 0.16	1.1 ± 0.18	1.0 ± 0.10
LPG(?)	0.1 ± 0.04	**1.3 ± 0.12***	0.1 ± 0.00
PE	3.0 ± 0.20	3.3 ± 0.19	2.7 ± 0.09*
PG	15.4 ± 0.32*	14.0 ± 0.20	13.8 ± 0.72

lyso-PC=lysophosphatidylcholine; SM=sphingomyelin; PC=phosphatidylcholine; PI=phosphatidylinositol; LPG(?)= migrates at the proposed position of lyso-phosphatidylglycerol; PE= phosphatidylethanolamine; PG=phosphatidylglycerol.

Data represent the mean ± S.D. of three determinations. *Denotes values different from the other 2 preparations ($p<0.05$)(by ANOVA followed by Student's t-test).

Figure 4.1 Lipid Extract Surfactant batch #91 is more sensitive to inhibition by crude fibrinogen than other batches. Surface tension at R_{min} after 20 pulsations is plotted versus the fibrinogen concentration. Circles represent LES #90, open triangles represent LES #91, filled triangles represent LES #92. All samples contain LES at 2.5 mg/mL phospholipid. Fibrinogen is crude human fibrinogen from Sigma. Values are means $\pm$ S.E.M. of 3 assays for each batch. *Denotes values significantly different from the other 2, $p < 0.05$ (by ANOVA followed by Student's t-test).

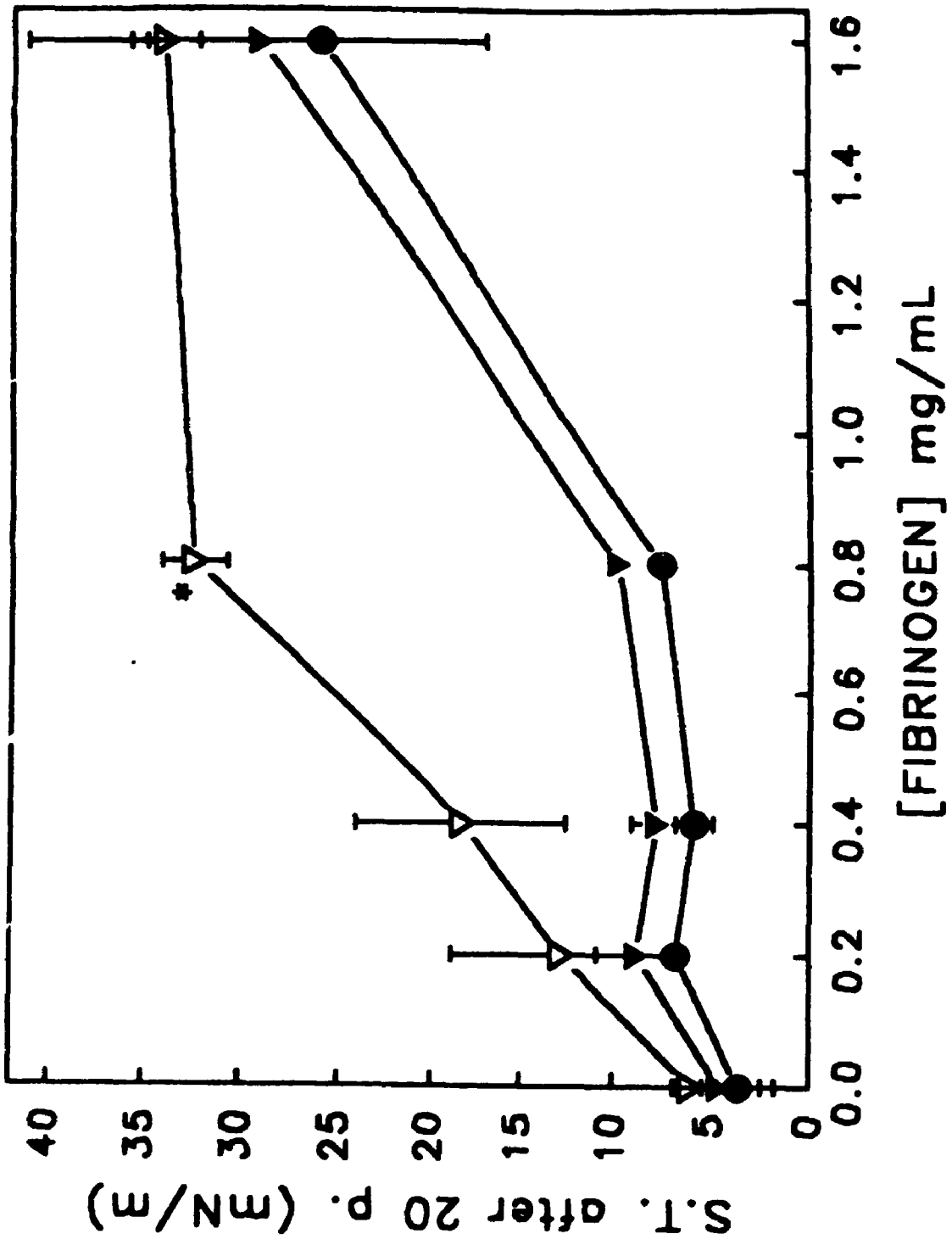
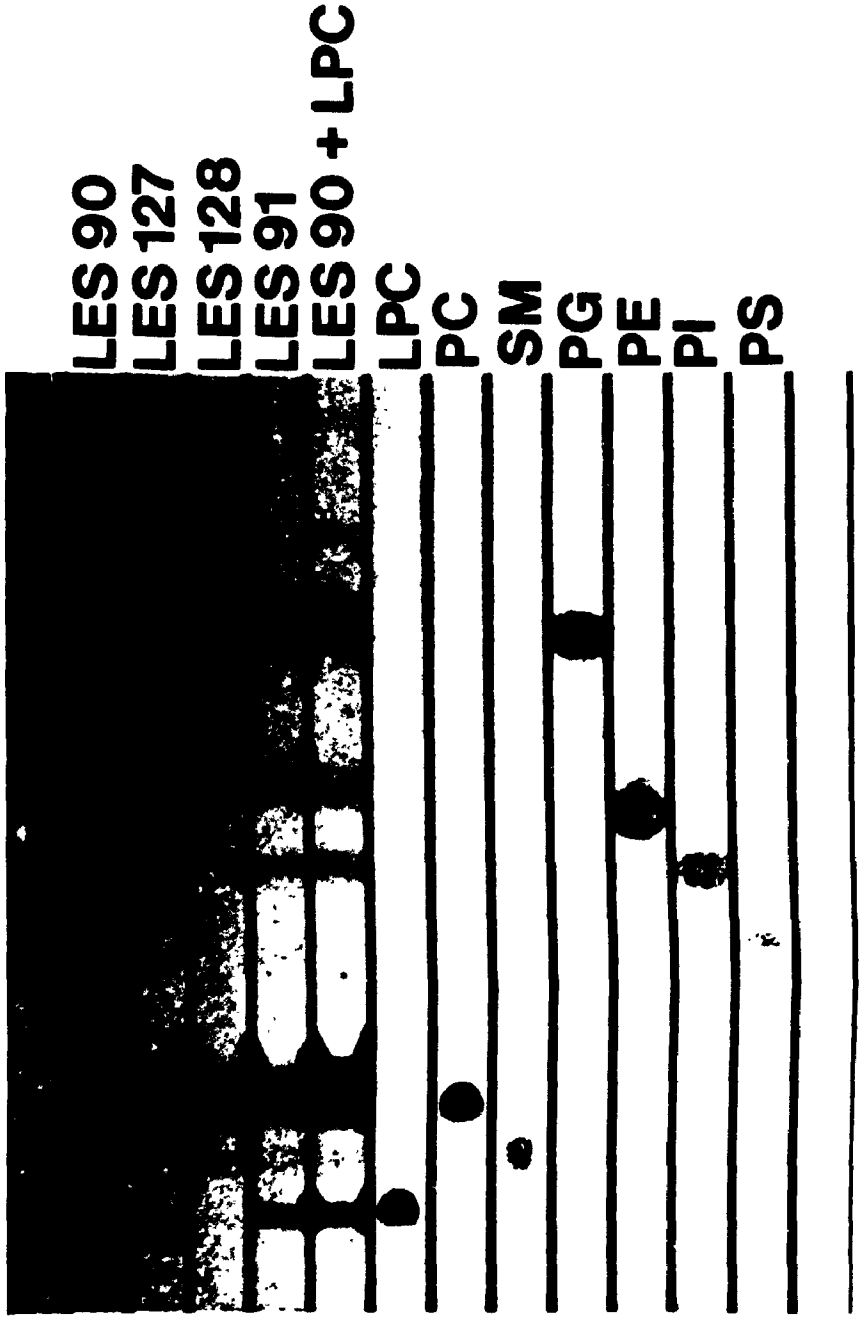

S.T. after 20 p. (mN/m)
40
35
30
25
20
15
10
5
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
[FIBRINOGEN] mg/mL
*

Figure 4.2 The effect of endogenous and exogenous lyso-phosphatidylcholine on the inhibition of Lipid Extract Surfactant by purified fibrinogen. *A.* A thin layer chromatogram of different batches of LES and standards developed in Touchstone reagent and visualized with Dittmer and Lester phosphorus spray. Numbers denote the batch designation, for definition of abbreviations see Table 4.1. *B.* (page 127) Surface tension after 20 pulsations (mN/m) is plotted versus the fibrinogen concentration (mg/mL). Fibrinogen from Kabivitrum, purified as described in the Materials and Methods, was used for this and all subsequent figures. Circles represent LES alone; triangles represent LES + 5 mol % lyso-PC. Open symbols are values at R_{min}, filled symbols are values at R_{max}. All samples contained 2 mg/mL LES. Values are the means ± S.E.M. for three different batches of surfactant each assayed three times. *Denotes LES + lyso-PC values that are significantly different ($p < 0.05$) from LES alone for each concentration of inhibitor.


LES 90
LES 127
LES 128
LES 91
LES 90 + LPC
LPC
PC
SM
PG
PE
PI
PS

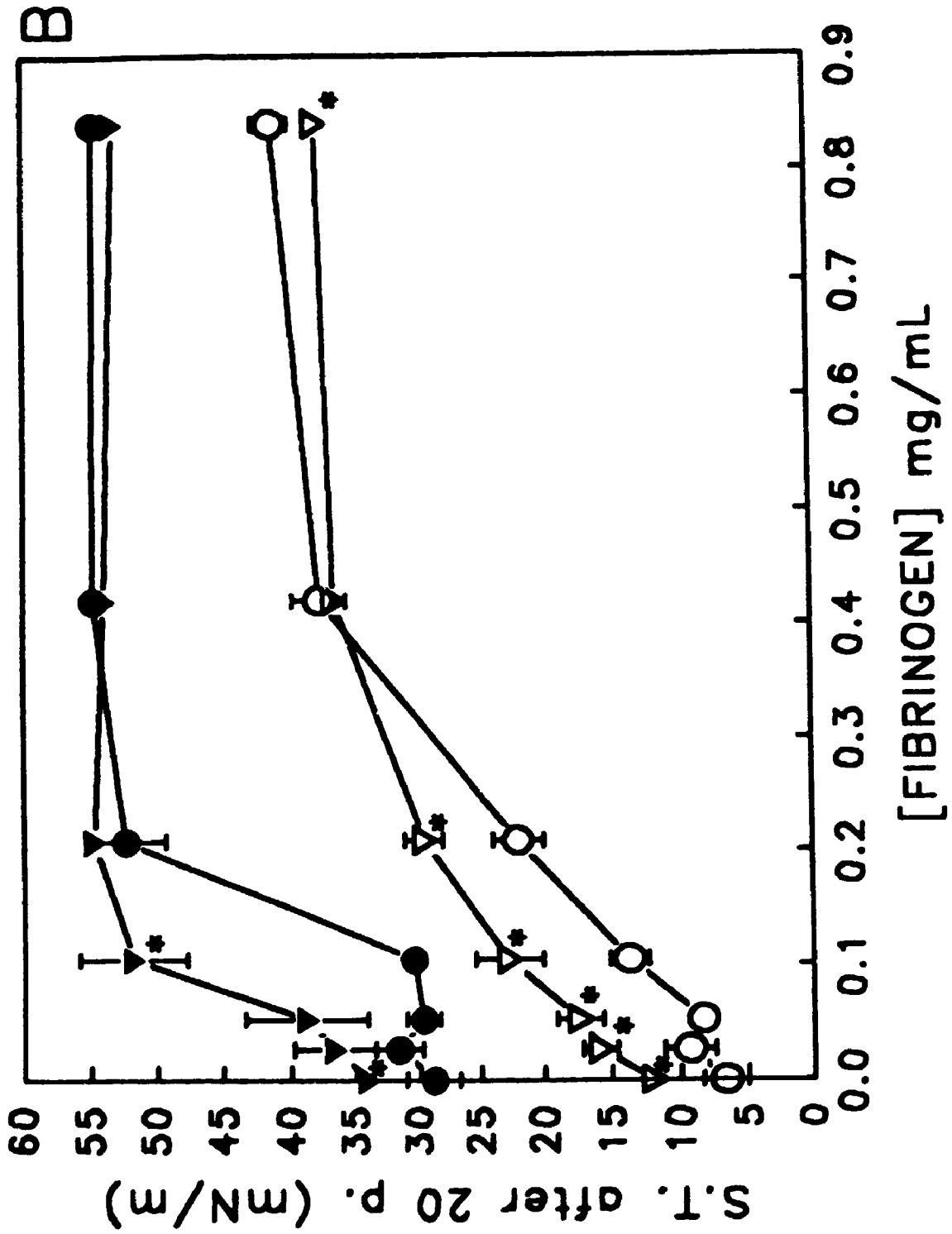

B
S.T. after 20 p. (mN/m)
[FIBRINOGEN] mg/mL
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
25
30
35
40
45
50
55
60

Figure 4.3 The effect of exogenous lyso-PC on the concentration curves for the addition of bovine serum albumin and fetal bovine serum to Lipid Extract Surfactant. *A*. Surface tension after 20 pulsations (mN/m) is plotted versus the concentration of FBS protein (mg/mL). Circles represent LES alone; triangles represent LES + 5% lyso-PC. Open symbols represent values at R_{min}; closed symbols represent values at R_{max}. *B*. Surface tension after 20 pulsations (mN/m) is plotted versus the concentration of albumin (mg/mL). Symbols are identical to above. All samples contain LES at 2 mg/mL phospholipid. Values are the means ± S.E.M. of three different batches of surfactant each assayed three times. *Denotes LES + lyso-PC values that are significantly different ($p < 0.05$) from LES alone for each concentration of inhibitor.

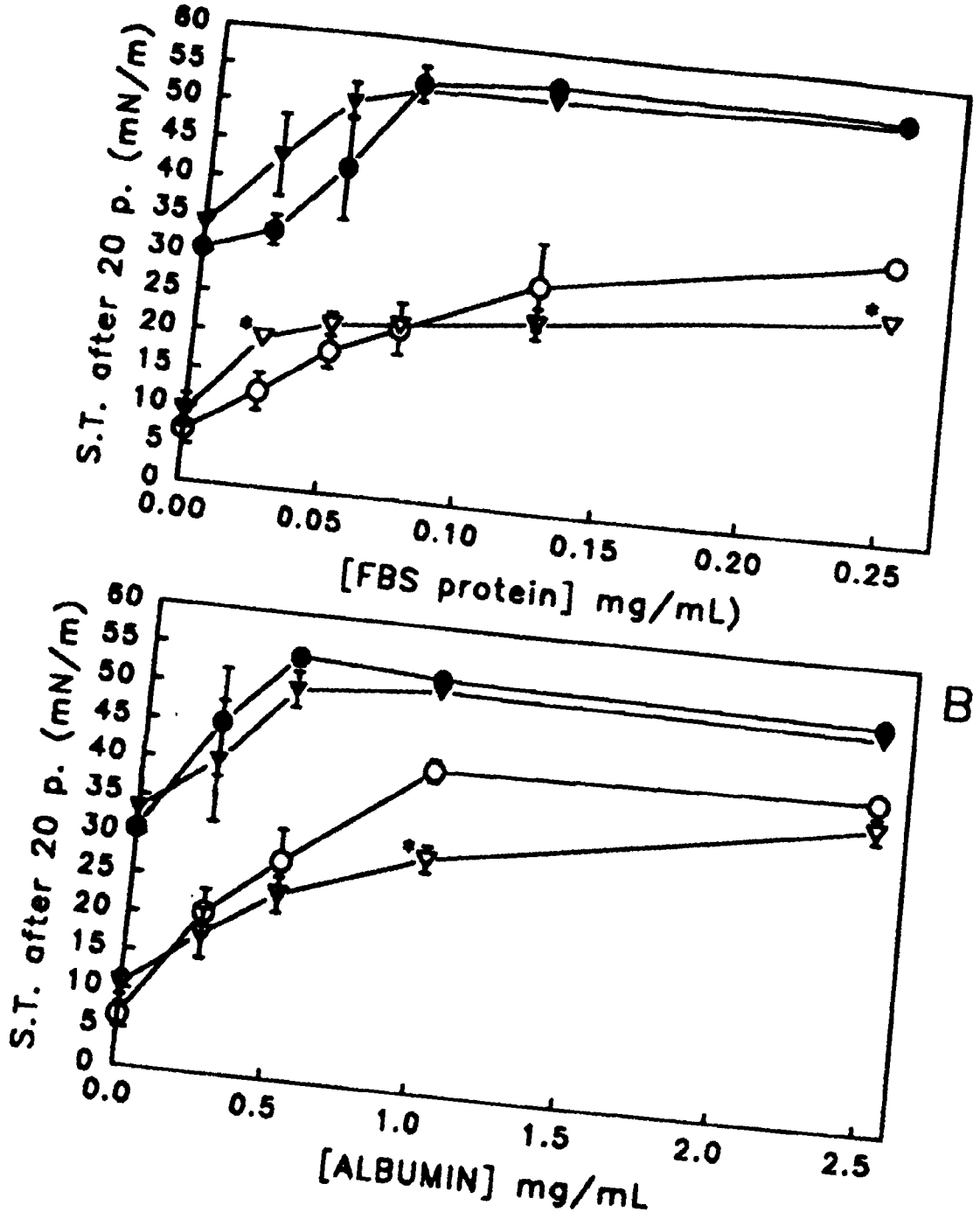

S.T. after 20 p. (mN/m)
60
55
50
45
40
35
30
25
20
15
10
5
0
0.00
0.05
0.10
0.15
0.20
0.25
[FBS protein] mg/mL)
A
S.T. after 20 p. (mN/m)
0.0
0.5
1.0
1.5
2.0
2.5
[ALBUMIN] mg/mL
B

Figure 4.4 Concentration curve for the addition of lyso-PC and the co-addition of lyso-PC and palmitic acid to LES in the presence and absence of inhibition by fibrinogen. *A*. Surface tension after 20 pulsations (mN/m) is plotted versus the concentration of added lyso-PC (mol % of LES). Circles represent the control (without fibrinogen); triangles represent samples inhibited by the addition of 0.105 mg/mL purified human fibrinogen. Open symbols are values at R_{min}; filled symbols are values at R_{max}. *B*. Surface tension after 20 pulsations (mN/m) is plotted versus the concentration of lyso-PC and palmitic acid (mol % of LES). Symbols are the same as above. All samples contain 2 mg/mL LES phospholipid. Values are the means $\pm$ S.E.M. for three batches of surfactant each assayed three times. *Denotes values significantly different ($p < 0.05$) from the corresponding 0% lyso-PC values.

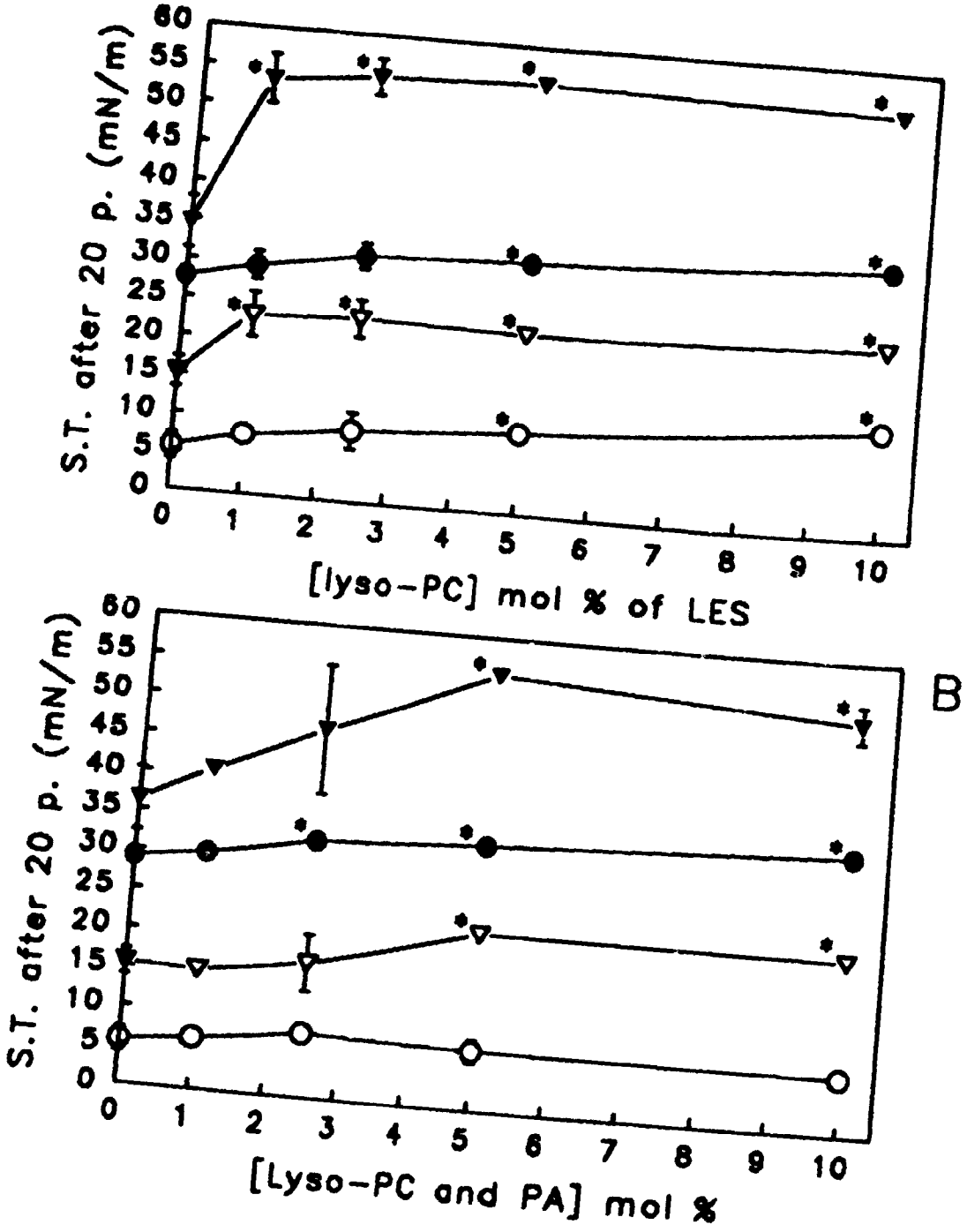

A
S.T. after 20 p. (mN/m)
60
55
50
45
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10
[lyso-PC] mol % of LES
B
S.T. after 20 p. (mN/m)
60
55
50
45
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10
[Lyso-PC and PA] mol %

Figure 4.5 Results of a time course of phospholipase A_2 digestion of Lipid Extract Surfactant. A thin layer chromatogram of samples developed in Touchstone reagent and visualized with Dittmer and Lester phosphorus spray. "Ace" indicates samples that have been acetone precipitated to remove neutral lipids; "non" indicates samples which have not been precipitated. The numbers 0, 15, 30, 60, and 120 refer to the duration of PLA_2 digestion in minutes. A standard curve of lyso-PC from 1 to 25 % by weight of the amount of surfactant phospholipid added is included. A sample of undigested LES is also included. 500 ug of surfactant phospholipid is loaded into the lanes. PG= phosphatidylglycerol; PE=phosphatidylethanolamine; PI= phosphatidylinositol; PC=phosphatidylcholine; SM=sphingomyelin; LPC=lyso-phosphatidylcholine.

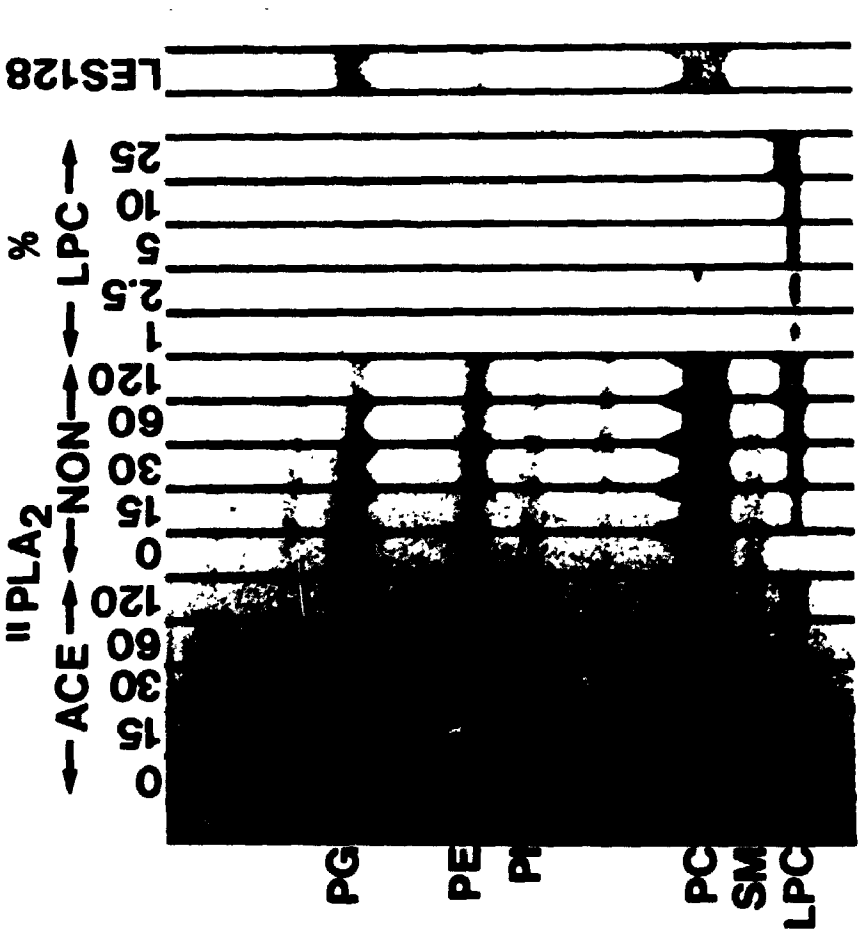
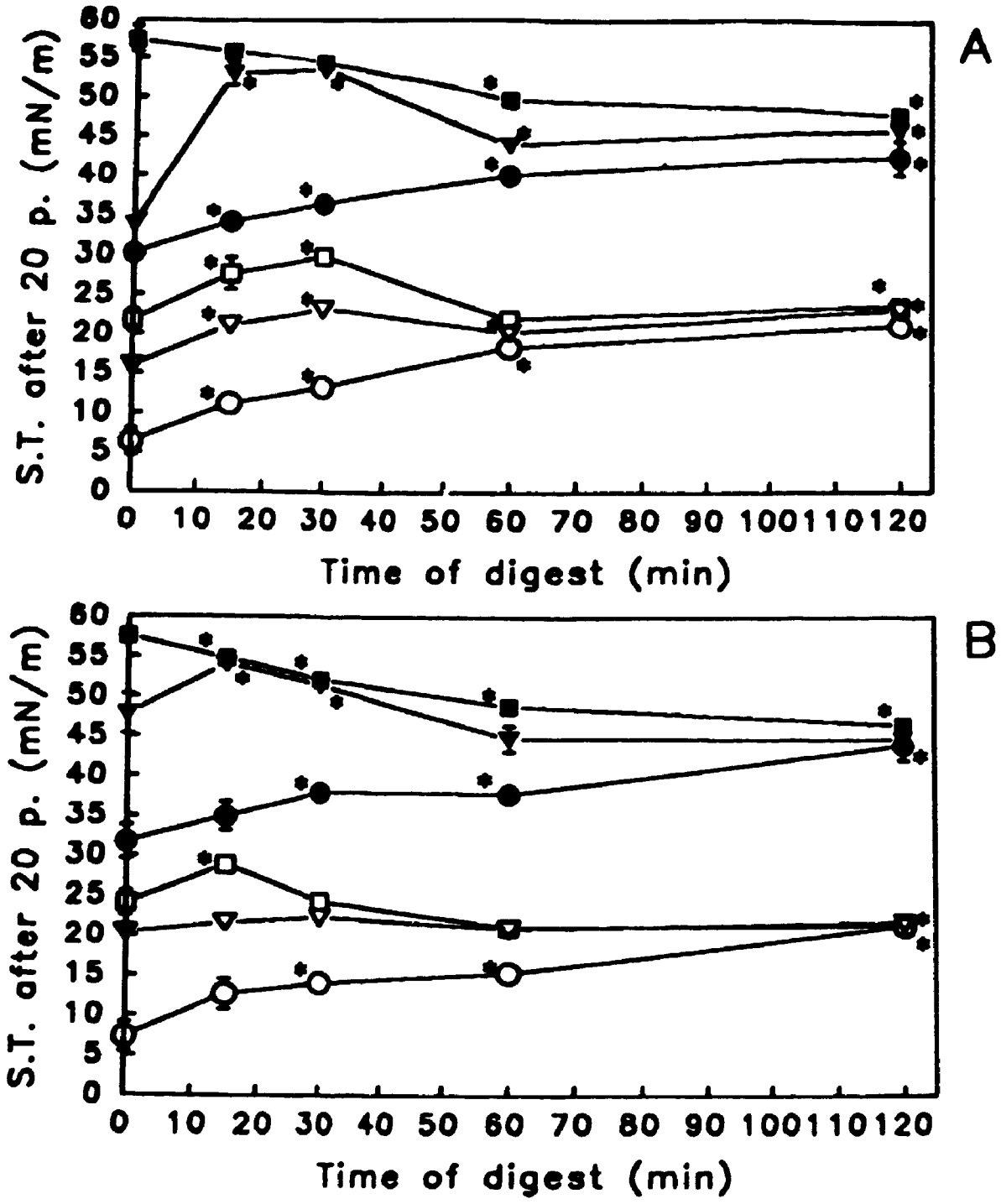

LES128
%
LPC
25
10
5
2.5
1
120
60
30
15
0
NON
120
60
30
15
0
ACE
II PLA2
PG
PE
PC
SM
LPC

Figure 4.6 The effect of phospholipase A_2 digestion of Lipid Extract Surfactant on the inhibition by fibrinogen. *A.* Surface tension after 20 pulsations (mN/m) is plotted versus the time of PLA_2 digestion (min.) for surfactant which has been acetone precipitated. Circles represent control surfactant (without addition of fibrinogen); triangles represent the addition of 0.056 mg/mL purified human fibrinogen; squares represent the addition of 0.105 mg/mL fibrinogen. Open symbols are values at R_{min}; filled symbols are values at R_{max}. *B.* Surface tension after 20 pulsations (mN/m) is plotted versus the time of PLA_2 digestion (min) for non-acetone precipitated samples. Symbols are the same as above. All surfactants were assayed at 2 mg/mL original phospholipid concentration. Values are the mean $\pm$ S.E.M. for three assays. *Denotes values significantly different ($p < 0.05$) from the corresponding 0" time value.


A
S.T. after 20 p. (mN/m)
0
5
10
15
20
25
30
35
40
45
50
55
60
0 10 20 30 40 50 60 70 80 90 100 110 120
Time of digest (min)
B
S.T. after 20 p. (mN/m)
0
5
10
15
20
25
30
35
40
45
50
55
60
0 10 20 30 40 50 60 70 80 90 100 110 120
Time of digest (min)

4.5 Discussion

It is now well established that regardless of the precipitating cause, a major complicating factor in the progression of respiratory distress is the leakage of blood components into the alveolus (Holm & Matalon, 1989; Seeger et al, 1990). One consequence of this leakage would be the inactivation of the pulmonary surfactant system resulting in high transpulmonary pressures leading to further leakage and damage (Jobe, 1989). In the present investigation we have sought to investigate the role of surfactant phospholipid degradation in the process of pulmonary surfactant inactivation leading to respiratory distress. Our studies demonstrate that the presence of endogenous lyso-PC in surfactant suspensions sensitizes the surfactant to inhibition by blood proteins, in particular to inhibition by fibrinogen. The addition of exogenous lyso-PC or the generation of lysophospholipid from surfactant lipids *in vitro* similarly sensitize the surfactant to inhibition. This effect is observed as a shifting of the inactivation concentration curve such that the surfactant is inhibited at lower inhibitory protein concentrations.

The extent of sensitization appears to depend upon the nature of the protein causing the inhibition. For example, whereas lyso-PC dramatically sensitizes LES to fibrinogen inhibition, albumin inhibition is not aggravated by lyso-PC addition. This provides evidence that the mechanism of protein inhibition may not be the same for different proteins. Blood proteins have been

demonstrated to have widely varying inhibitory potential. It has been shown that of the blood proteins examined, fibrinogen and the fibrin monomer were the most inhibitory whereas albumin is the least inhibitory (Seeger et al, 1985a; Fuchimukai et al, 1987; Cockshutt et al, 1990). Low surfactant concentrations must be used to detect albumin inhibition, as this inhibition is very sensitive to surfactant concentration. Whereas SP-A appears to possess the ability to counteract the inhibitory action of all of the blood proteins assayed (Cockshutt et al, 1990; Venkitaraman et al, 1990), the ability of palmitic acid to reverse inhibition varies from protein to protein (chapter 3). In particular, inhibition by fibrinogen and alpha-globulin was only partially reversed whereas albumin inhibition was almost completely abolished by palmitic acid addition. These differences suggest that the lipid binding properties of albumin may affect its inhibitory potential. The binding of palmitic acid to albumin may change its properties such that it can no longer effectively compete with surfactant lipids for the air-liquid interface.

It has been demonstrated that at the levels used in this investigation palmitic acid and lyso-PC have contrasting effects on the surfactant (chapter 3). Palmitic acid enhances the surface activity of LES as well as partially reversing blood protein and lyso-PC inhibition of this surfactant. Therefore, one might expect that as PLA_2 degrades PC and releases lyso-PC and fatty acid, that these two effectors would tend to cancel each other out. However, the data from both the co-addition curve and the *in vitro* generated lyso-PC experiments suggest that

this effect is only partial. Fatty acids other than palmitic acid would be released from the sn-2 position of PC including unsaturated fatty acids which do not enhance the surface activity of mixtures as palmitic acid does (Tanaka et al, 1986; Tanaka et al, 1983). It should also be noted that the half life of palmitic acid administered to the alveolus is relatively short because it is rapidly reabsorbed into the type II and possibly other cells (Tabor et al, 1990). For these reasons, palmitic acid may not remain in the hypophase following its release from PC, and could not reverse the effects of lyso-PC. Thus, the mediatory effects of the liberated fatty acid may be much weaker than those seen with the addition of pure palmitic acid.

The mechanism of lyso-PC inhibition of surfactant is not known. The inhibition of LES by lyso-PC follows different kinetics than blood protein inhibition and in contrast to protein inhibition is not reversed by the addition of SP-A (Cockshutt et al, 1990). Inhibition of surfactant activity by lyso-PC has also been demonstrated by the treatment of the surfactant with PLA_2 during measurement with a pulsating bubble surfactometer (Keicher et al, 1990). The inhibition by lyso-PC is almost entirely lost when large amounts (10% by weight) of palmitic acid are added to the surfactant (chapter 3). PC, and in particular DPPC, are cylindrically shaped which allows them to pack readily into bilayers and monolayers (Cullis & Hope, 1985). Lyso-PC has a conical shape which accounts for its detergent properties and ability to form micelles (Cullis & Hope, 1985). Addition of small amounts of lyso-PC to phospholipid mixtures slightly

enhances phospholipid adsorption presumably by increasing the fluidity and curvature of the bilayers (MacDonald et al, 1991). However, adsorption of lyso-PC onto the air-liquid interface could lead to disruption of the monolayer upon compression because the large hydrated polar head group and single acyl chain of lyso-PC would interfere with the regular packing of disaturated phosphatidylcholine. The presence of equimolar amounts of palmitate should compensate for the lyso-PC in the monolayer. Degradation of a large proportion of the phospholipid in the outer leaflet of erythrocyte plasma membranes by phospholipase A_2 does not result in membrane disruption unless the free fatty acids are removed from the membrane by incubation with albumin (Zwaal et al, 1973). Since palmitate also packs in monolayers as cylinders, the presence of moderate excesses of palmitate in surfactant preparations need not disrupt the surface monolayer. The results of the co-addition curve indicate that in the absence of challenge by fibrinogen this appears to be the case, as no inhibition by lyso-PC is observed. An alternative explanation is that lyso-PC somehow interferes with the interactions between the low molecular weight surfactant proteins SP-B and SP-C and the surfactant lipids which lead to the enrichment of dipalmitoylphosphatidylcholine in the monolayer, and hence to the reduction of surface tension to very low values. It is also possible that the SP-B/SP-C monolayer enriching function cannot remove lyso-PC from the interface, as readily as it removes unsaturated and non-PC components (Pison et al, 1990; Yu & Possmayer, 1990). The observation that SP-A cannot reverse inhibition by

lyso-PC supports these arguments since it has been shown that the SP-A effects require the presence of the low molecular weight surfactant proteins (Hawgood et al, 1987; Venkitaraman et al, 1991; Pison et al, 1990; Yu & Possmayer, 1988; Yu & Possmayer, 1990).

The mechanism of sensitization of surfactant to inhibition by blood proteins by the addition of lyso-PC is also not known. Inhibition by blood proteins is dependent on the surfactant phospholipid concentration. It appears as though lysophospholipid addition decreases the effective surfactant concentration, thus shifting the surfactant inactivation curve. If a simple competition model were occurring, where surfactant lipids and inhibitory proteins were competing for the interface, one might expect that the addition of 5 or 10% of added lyso-PC, which does increase the rate of adsorption of surfactant lipids (MacDonald et al, 1991), would essentially counteract protein inhibition. Such a surfactant would be expected to behave simply like lyso-PC inhibited surfactant. On the contrary, the addition of even small amounts of lyso-PC dramatically sensitizes the surfactant to inhibition by blood proteins. This effect is particularly apparent at R_{max} where the surface tensions are increased by $\sim$20 mN/m upon sensitization with lyso-PC. The surface tension at R_{max} reflects the ability of the surfactant lipids to adsorb and re-spread during expansion of the monolayer. This activity is obviously impaired in the samples containing lyso-PC and fibrinogen. If the model of lyso-PC preventing surfactant lipid-surfactant protein interactions is considered, this sensitization phenomenon is not surprising since several

investigations have demonstrated that synthetic lipid mixtures containing SP-B and SP-C are far more resistant to inhibition by blood proteins than the mixtures alone (Venkitaraman et al, 1991; Seeger et al, 1991). Thus, it seems probable that lyso-PC could be interfering with the lipid-protein interactions that are required for the efficient reduction of surface tension, and that this compromised surfactant is not able to deal with even small amounts of inhibitory blood proteins.

Considering the data presented in the present investigation a model of surfactant inactivation potentially leading to ARDS can be proposed. Following injury or damage to a region of the lung, local influx of neutrophils may occur. Release of PLA_2 from these activated neutrophils or from cells influenced by the neutrophil could cause degradation of surfactant and/or cellular lipids to generate lysophospholipid and free fatty acid. Should the injury or invasion of neutrophils be accompanied by the leakage of blood proteins into the alveolar space, the degraded surfactant would be more sensitive to inactivation by the blood proteins than intact surfactant. Since lyso-PC also possesses strong cytolytic activity, and has been shown to destroy lung epithelial cells (Aronson & Johns, 1977), its presence alone would jeopardize the integrity of the epithelium and promote leakage into the alveolus. Furthermore, lyso-PC may reduce the threshold for blood protein inhibition sufficiently that leakage that would normally be sub-clinical or constitutive could lead to surfactant inactivation, since even the lavage from healthy lungs contains trace amounts of serum contaminants. When such extrapolations are made from *in vitro* data to the *in vivo* situation, one must bear

in mind that the experiments were performed with a modified natural surfactant under conditions which do not necessarily reflect those found in the lung. However, it has been demonstrated with a similar preparation of surfactant that the concentrations used in the present study are at or above the threshold required for good *in vitro* and *in vivo* activity (Kobayashi et al, 1990). Other data comparing the inhibitory potential of serum *in vitro* and *in vivo* suggests that there are differences between these two systems, with greater inhibition being observed with the pulsating bubble than in a lung model (Kobayashi et al, 1991), but the *in vitro* experiments were performed at much higher surfactant concentrations than those used in the present investigation. These differences are probably the result of the presence of small amounts of endogenous SP-A in the lungs which has been shown to counteract the inhibition by blood proteins (Cockshutt et al, 1990). Taking these findings into consideration, one can postulate that although the experiments reported here may not accurately represent the complete *in vivo* situation, they are indicative of a subset of interactions which can be expected to be found in the more complex environment in the lung.

A more moderate suggestion is that lyso-PC content may be a predictor of the severity or speed of development of respiratory distress. Thus, if alveolar lyso-PC is high when leakage of blood proteins begins, this lung may progress towards complete respiratory failure more rapidly than a lung whose surfactant is not compromised.

CHAPTER 5 - SP-A ENHANCES ADSORPTION AND SURFACE SORTING

5.1 Chapter Summary

The effect of surfactant concentration and supplementation with surfactant-associated protein A (SP-A) on the surface activity of Lipid Extract Surfactant (LES) was examined using a captive bubble technique. Adsorption of LES is strongly concentration dependent over the range of 50-1000 ug/mL. Addition of SP-A (0.5-4.0% w/w) to LES at low concentrations (200 ug/mL) dramatically increases the rate of adsorption in the presence of calcium. In quasi-static cycling experiments, samples containing SP-A require less compression to achieve low surface tensions (1 mN/m) even in the first compression cycle. The calculated film compressibilities at 15 mN/m indicate that SP-A alters the surfactant monolayer such that within a small number of cycles the compressibility is indistinguishable from that of pure DPPC. Furthermore, SP-A reduces the incidence of bubble "clicking", suggesting a stabilization of the monolayer at low surface tensions. In dynamic cycling experiments, SP-A reduces the area compression requirement and eliminates the purification plateau and adsorption shoulder observed with low LES concentrations. Experiments with EDTA suggest that the SP-A mediated effects are not absolutely dependent upon but are greatly enhanced by calcium.

5.2 Introduction

Pulmonary surfactant is a complex mixture of lipids and proteins secreted from the alveolar type II epithelial cell which reduces the surface tension across the air-liquid interface of the alveolus. Despite the complex nature of the mixture, it is thought that a mono-molecular layer enriched in the saturated phospholipid dipalmitoylphosphatidylcholine (DPPC) is responsible for the reduction of surface tension to very low values upon expiration (Clements, 1977; Goerke & Clements, 1986; Notter, 1984). Other surfactant components, which include unsaturated and negatively charged phospholipids and the surfactant-associated proteins, are required to generate and maintain this monolayer highly enriched in DPPC.

Although surfactant composition varies depending on the method of isolation, the species, and the purification protocol, all surfactants contain large proportions of phosphatidylcholine, and a relatively large amount of an anionic phospholipid, either phosphatidylglycerol or phosphatidylinositol (Shelley et al, 1984; Yu et al, 1983). Preparations of natural surfactant also contain at least three surfactant-associated proteins denoted SP-A, SP-B and SP-C (Possmayer, 1988; Weaver & Whitsett, 1991). Elucidation of the roles of the various surfactant components in surface tension reduction has been the subject of intense research. These experiments have demonstrated that the low molecular weight, hydrophobic proteins SP-B and SP-C are essential for good surface activity,

regardless of the assay system used (Hawgood et al, 1987; Pison et al, 1990; Yu & Possmayer, 1988). SP-C is important for rapid adsorption (Yu & Possmayer, 1988), whereas SP-B promotes both adsorption and the removal of phosphatidylglycerol from the monolayer (Yu & Possmayer, 1988; Yu & Possmayer, 1990). SP-A has little effect on its own, but enhances adsorption in conjunction with SP-B (Hawgood et al, 1987).

Measurements of surface activity can be made in systems which incorporate the lungs of animals, either *in situ* or excised (Robertson & Lachmann, 1988). While these experiments may give an accurate picture of the physiological situation, they are often too complex to elaborate precise data on molecular mechanisms. Many researchers have turned to *in vitro* techniques to address these questions. A wealth of information has been obtained using the Langmuir-Wilhelmy surface balance and the pulsating bubble surfactometer. In these systems, an air-liquid interface is generated, either on the surface of a trough or a bubble in a suspension, and adsorption of surfactant lipids and surface tension during dynamic cycling can be measured (Enhorning, 1977; Robertson & Lachmann, 1988). While these systems mimic many of the characteristics of the lung, large area compressions are required to obtain low surface tensions. Furthermore, these monolayers are much less stable than those found in the lung, in that the surface tension will rise quickly when a compressed monolayer is held at a fixed area (Hawgood et al, 1987; Schürch et al, 1976). This is presumably

due to film collapse and/or leakage which results in a loss material from the monolayer (Goerke & Clements, 1986).

Schürch et al (Schürch et al, 1989) recently described a new *in vitro* method for the assessment of the surface activity of films adsorbed from aqueous solutions. In this captive bubble technique, a bubble of diameter up to 8 mm is analyzed in a leak-proof chamber. Surfactants examined in this manner mimic closely the *in situ* behaviour observed in the lung. In particular, the surface films are very stable, even at very low surface tensions, and relatively little compression (reduction of surface area) is required to reduce the surface tension to 1 mN/m.

It was suggested by Clements (Clements, 1977) that the stability and low surface tensions observed in the lung were due to the squeezing-out of components other than DPPC from the alveolar lining during expiration. This was supported by the finding that monolayers of pure DPPC spread from organic solvents are far more stable than those of pulmonary surfactant (Goerke & Gonzales, 1981; Hildebran et al, 1979; Notter, 1984). Experiments with pulmonary surfactant and model mixtures of surfactant containing DPPC and phosphatidylglycerol have demonstrated using a variety of techniques that non-DPPC lipids can be removed from the monolayer by mechanical compression leaving a film which is enriched in DPPC and capable of withstanding high surface pressures ((Bangham et al, 1979; Chung et al, 1990; Egberts et al, 1989). Furthermore, it was demonstrated using the pulsating bubble surfactometer that

repeated cycling was required to obtain low surface tension upon compression, suggesting that the process of repeated expansion and compression results in an alteration of monolayer composition and/or configuration (Enhorning, 1977). Quasi-static cycling with the captive bubble has also demonstrated that considerably less reduction of surface area is required to achieve surface tensions <2 mN/m when the bubble has been previously cycled (Schürch et al, 1989). All of these data suggest that monolayer purification must be taking place as a result of breathing movements. However, the mechanism and compositional requirements of this process remain largely unknown.

In the present investigation we have examined the role of surfactant-associated protein A in adsorption of surfactant lipids to the air-liquid interface and in the purification or surface sorting of that monolayer using the captive bubble technique. We have found that supplementation of Lipid Extract Surfactant, an organic extract of pulmonary surfactant which contains the lipids and small hydrophobic proteins SP-B and SP-C, with SP-A results in enhanced adsorption at low surfactant concentrations. SP-A also appears to accelerate monolayer purification, perhaps through selective adsorption of DPPC. Thus, SP-A apparently enables the surfactant to function efficiently at very low concentrations and with minimal reduction of surface area.

5.3 Materials and Methods

5.3.1 Reagents.

All chemicals were reagent grade or better. Immobilized D-mannose was obtained from Pierce. The silver stain reagents were purchased as a kit from Bio-Rad.

5.3.2 Assays.

Phospholipid concentrations were determined using the phosphorus assay of Rouser (Rouser et al, 1960). Protein concentrations were measured by the method of Lowry (Lowry et al, 1951) modified by the addition of SDS (1.8 mM final). Protein purity was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (Laemmli, 1970) followed by staining with Coomassie R-250 and/or silver stain.

5.3.3 Surfactant Preparation.

Pulmonary surfactant was prepared from bovine lavage material by a modification of the method described elsewhere (Yu et al, 1983; Weber & Possmayer, 1984). Briefly, Lipid Extract Surfactant (LES) was obtained by organic extraction of lavage material. Neutral lipids were removed by subsequent acetone solubilization. The material was dried under N_2, made up in chloroform/methanol 9:1 and stored at -20°C. This preparation contains all of the

phospholipids of natural surfactant and the two small hydrophobic proteins SP-B and SP-C, however it has been stripped of SP-A. Prior to use, the surfactant was suspended in 0.9% saline solutions containing 1.5 mM $CaCl_2$. In experiments examining the requirement for calcium, 1.5 mM Na_2EDTA was added in place of the calcium.

5.3.4 Purification of SP-A.

SP-A was purified from bovine natural surfactant as previously described (Cockshutt et al, 1990). Briefly, an aqueous suspension of natural surfactant was delipidated by washing in butan-1-ol, ether/ethanol 3:1, and pure ether. The solvent was completely removed and the precipitated protein was solubilized in 5 mM HEPES, 0.1 mM Na_2EDTA, pH 7.4 and insoluble material was removed by centrifugation at 30 000 g for 20 minutes. The solubilized protein was then supplemented with $CaCl_2$ to a concentration of 1 mM and affinity purified on a column of immobilized D-mannose. Bound protein was eluted with 2 mM EDTA in 5 mM HEPES, pH 7.4.

5.3.5 Captive Bubble Apparatus.

The captive bubble method is an *in vitro* technique developed for the accurate determination of the surface tension of a bubble in a surfactant suspension. The design and construction of the instrument are described at length by Schürch et al (Schürch et al, 1989). Dr. Samuel Schürch and his technician

Stanley Cheng performed all of the measurements on this apparatus with samples prepared by the author. The instrument consists of sample chamber constructed from an inverted glass syringe. The ceiling of the chamber is plugged with a thin, slightly concave coating of 1% agar gel to generate a completely hydrophilic surface for contact with the bubble. The temperature of the chamber is regulated and maintained at 37°C. The sample chamber is filled with the suspension to be assayed and a small bubble (1-3 mm in diameter) is introduced.

5.3.6 Calculation of Surface Tension.

The bubble is recorded continuously throughout the experiment on a video recorder (WVD 5000 camera and AG6060 recorder, Panasonic, Matsushita Canada, Mississauga, Ontario). Video images are printed by a video copier (P60U, Mitsubishi Canada, Toronto, Ontario). Surface tension is calculated from the parameters of maximum height and diameter of the bubble as measured from the video image, using the method of Malcolm and Elliott (Malcolm & Elliott, 1980). A rationale and discussion of the use of this method for surface tension calculation is given elsewhere (Schürch et al, 1989).

5.3.7 Adsorption Measurements.

To measure the adsorption of surfactant onto the monolayer a small atmospheric bubble (1-2 mm diam.) was rapidly expanded to a maximum size of 7-8 mm, resulting in an approximately 10-fold increase in bubble surface area in a

third of a second interval. The chamber contents were stirred during the adsorption period by a small magnetic bar. Expansion of the bubble represents the starting time of the adsorption measurements. The bubble shape is recorded continuously on a video recorder and surface tension is calculated as above.

5.3.8 Quasi-static Isotherms.

A small atmospheric bubble was rapidly expanded as described for adsorption measurements. Following adsorption to ~25 mN/m the film was compressed stepwise, waiting at each step until the bubble shape (i.e. surface tension) no longer changed within 20-30 seconds (this corresponds to a change in surface tension of less than 0.5 mN/m in that period). Between 10-15 steps were taken for each compression and expansion part of the cycle.

5.3.9 Dynamic Hysteresis Loops.

A bubble is formed as described above and cycled dynamically at 20 cpm. The compression results in a reduction of surface area to approximately 20% of the original area. The bubble is recorded continuously throughout the cycling process.

5.3.10 Calculation of Film Compressibility.

The compressibility of a film is a useful parameter for the interpretation of compression data. The film compressibility at a surface tension of 15 mN/m is

reported here for comparison to pure DPPC films. It is calculated from the equation, $C_{15}=(1/A)(dA/d\gamma)$, where A is the relative area from the start of compression, and γ is the surface tension. This value is obtained from the isotherm by determining the relative area at 15 mN/m, and the slope of the tangent to the curve at this surface tension. The C_{15} for a pure DPPC film is 0.005.

5.4 Results

The captive bubble technique was used to examine the concentration dependence of the adsorption of surfactant lipids onto the air-liquid interface. Figure 5.1 shows the time course of adsorption of Lipid Extract Surfactant (LES) at different concentrations following a rapid expansion of surface area. The process is strongly concentration dependent, such that samples at 50 ug/mL require more than 30 minutes to reach equilibrium, whereas samples at 800 ug/mL or greater require less than 5 seconds to reach equilibrium surface tensions of 22-25 mN/m. The initial, or first measurable, surface tension value following bubble expansion, is also an indicator of adsorption rate, since it reflects the amount of adsorption which occurs during the expansion period of ~0.3 seconds. Suspensions with very slow adsorption rates will have initial surface tension values approaching the 70 mN/m corresponding to aqueous solutions. Indeed, LES at 50 ug/mL has initial values greater than 60 mN/m.

Addition of SP-A to LES at low concentrations in the presence of calcium results in a dramatic increase in the rate of surfactant lipid adsorption. Figure 5.2 demonstrates the effect of addition of increasing amounts of SP-A to LES at 200 ug/mL (expressed as % w/w of the surfactant lipid concentration, such that 1.0% SP-A = 2 ug/mL in this case). In the absence of SP-A, adsorption to equilibrium takes ~20 seconds and initial surface tensions greater than 45 mN/m are observed (see Figure 5.1b.). With the addition of 0.5% SP-A the adsorption

period is decreased to approximately 3-5 seconds, and initial surface tensions less than 35 mN/m are observed. The addition of larger amount of SP-A, 1.0% and 4.0%, leads to adsorption to equilibrium within 1 second of bubble expansion.

Following adsorption of the surfactant to equilibrium, the bubble can be cycled in a quasi-static fashion. The isotherms generated from such an experiment with an LES concentration of 200 ug/mL are shown in Figure 5.3. Clearly, the compression and expansion parts of the cycle do not overlap, i.e. there is hysteresis. As can be noted on these isotherms, there is a "click" observed at low surface tensions which results in a spontaneous decrease in surface area and concomitant increase in surface tension (an in depth discussion of the clicking phenomenon is given elsewhere, (Schürch et al, 1989)). Sequential isotherms of this nature (Figure 5.3a. is the first cycle, b. the second, and c. the fourth) demonstrate that monolayer purification or surface sorting occurs with cycling. This process is reflected in the greater reduction of surface area that is required to obtain very low surface tensions in the first cycle than the fourth. As well, the hysteresis is much less in the fourth cycle than in the first. These differences are indicative of an increased proportion of DPPC in the cycled monolayer.

The addition of SP-A changes the appearance of these isotherms as can be seen in Figure 5.4. When 0.5% SP-A is added (5.4a.,b.,c.), less hysteresis is observed in the first cycle and compression of only 21% results in surface tensions of 1 mN/m. In this case clicking was observed only in the first cycle.

By the fourth cycle a compression of 12% results in these very low surface tensions, this is the same area change as observed with pure DPPC films. Higher concentrations of SP-A, such as 1.0% (5.4d.,e.,f.), result in further enhancement such that clicking is eliminated and 12% compression results in low surface tensions in the second cycle Increasing the SP-A concentration to 4.0% did not lead to further enhancement (data not shown). The results of the quasi-static isotherms are summarized in Table 5.1, and the calculated film compressibility at 15 mN/m is included. With the addition of SP-A, the film compressibility is indistinguishable from that of pure DPPC by the fourth cycle.

Bubbles formed in the captive bubble apparatus can also be cycled dynamically (at 20 cycles per minutes). The results of such experiments are shown in Figure 5.5, where the data from four consecutive cycles centring on the 20^{th} cycle is plotted. When LES at 200 ug/mL is cycled in this fashion (Figure 5.5a.) several interesting features are observed. Compressions of ~80% of the surface area are required to reach low surface tensions. Even after 20 cycles compressibility at 15 mN/m is 0.042 which is almost 10 times the value observed for pure DPPC. In the compression segment of the loop a large plateau just below 25 mN/m is observed. It is thought that this plateau represents monolayer purification or surface sorting rather than simply the packing of surfactant lipids. Similarly, in the expansion part of the cycle, at a surface tension of ~35 mN/m, a shoulder is present. This shoulder may represent the adsorption of surfactant lipids from a "surface reservoir" distinct from the bulk suspension.

At higher surfactant concentrations the loops have a very different shape (Figure 5.5b.). At 1000 ug/mL LES the purification plateau and the adsorption shoulder essentially disappear. Furthermore, a compression of only 32% is required to achieve low surface tensions. It should also be noted that the surface tension at maximum bubble area is ~35 mN/m compared to the ~45 mN/m observed at the lower surfactant concentration. Film compressibility at 15 mN/m is 0.013. LES at 200 ug/mL supplemented with 4.0% SP-A behaves similarly to the higher surfactant concentration (Figure 5.5c.). In this case no purification plateau or adsorption shoulder is observed. Maximum surface tensions of less than 35 mN/m are observed and compression of less than 30% is required to obtain low surface tensions. Compressibilities of these samples at 15 mN/m are 0.011. Interestingly, in these samples, and to a lesser extent with high concentrations of LES in the absence of SP-A, the shape of the expansion segment of the loop at low surface tension is less steep. The surface tension remains below 1 mN/m for a short period as the area begins to increase upon expansion. This is in marked contrast to what is observed in the quasi-static experiments (compare Figure 5.5c. to the isotherms in Figure 5.4.).

Previous experiments have demonstrated that the effects of SP-A depend on the presence of calcium for optimal activity (Benson et al, 1984; Cockshutt et al, 1990; Hawgood et al, 1987; King, 1984). Adsorption and cycling experiments were performed in the presence of EDTA to assess the role of calcium in this system. Figure 5.6 shows the effect of incubation of samples with

EDTA on adsorption time courses. LES alone adsorbs much slower in the absence of calcium. Addition of SP-A does increase the rate of adsorption, however times of 3 minutes as compared to 1 second are required to achieve equilibrium surface tension at 4.0% added SP-A. Incubation with EDTA also altered the behaviour of the samples during quasi-static and dynamic cycling. During dynamic cycling even with 4.0% SP-A a large purification plateau is observed and compression of more than 50% is required to reduce the surface tension to low values (data not shown). The results of quasi-static cycling of LES at 200 ug/mL are summarized in Table 5.2. In the presence of EDTA, LES alone does not reduce the surface tension below 18 mN/m, even with compression of 80% of the surface area. Addition of SP-A does result in the achievement of lower surface tensions, however 45-65% compressions are required, as opposed to about 30% when calcium is present and the samples are incubated similarly. Controls are included in this table to demonstrate that although the surfactant is somewhat compromised by the overnight incubation at 37°C (compare values in Table 5.1 and Table 5.2), the effect of EDTA is still readily apparent. This loss of activity is presumably due to lipid peroxidation or degradation.

Table 5.1 Summary of Quasi-static Isotherms of LES ± SP-A

SP-A (%)	Cycle #	γmax (mN/m)	γmin (mN/m)	ΔArea[‡] (%)	C_{15}[†]
0	1st	26.0[*]	2.0	28.7	0.015
	2nd	26.0	1.8	21.5	0.010
	4th	26.0	1.0	17.5	0.008
0.5	1st	25.0	1.0	20.7	0.015
	2nd	25.0	1.0	18.0	0.007
	4th	25.0	1.0	18.0	0.006
1.0	1st	25.0	1.0	21.5	0.009
	2nd	25.0	1.0	13.7	0.006
	4th	25.0	1.0	12.0	0.005

[*]Values are means, n=4.

[†]C_{15} is the film compressibility at γ of 15 mN/m$=(1/A)(dA/d\gamma)$, C_{15} for pure DPPC film = 0.005.

[‡]Total area change.

Table 5.2 The Effect of EDTA on Quasi-static cycling of LES

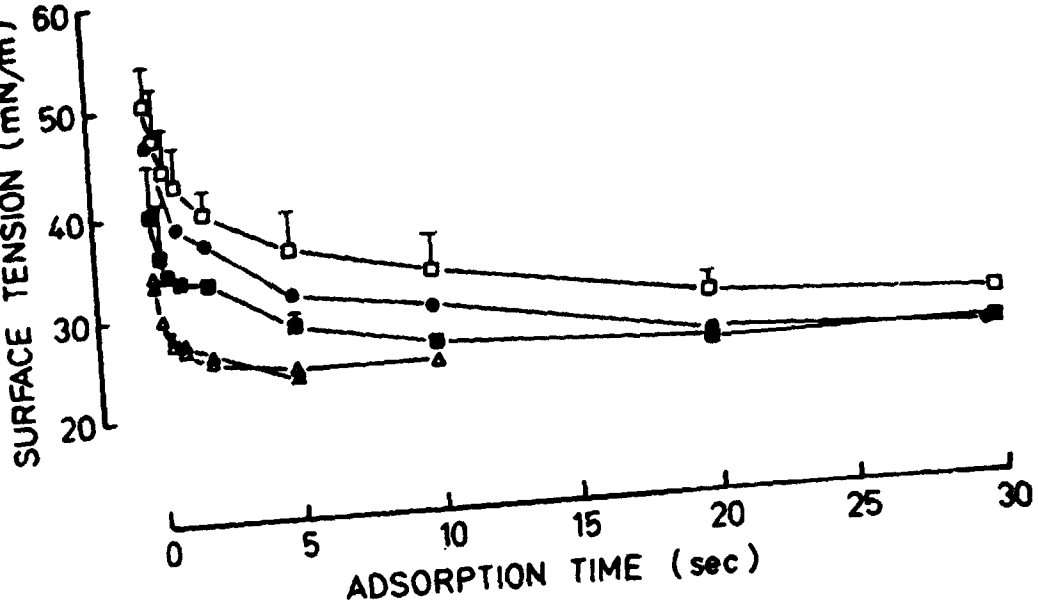
Incubation conditions[†]	SP-A %	γmax mN/m	γmin mN/m	ΔA_T[‡] %	ΔA_{25}[§] %	C_{15}
1.5 mM EDTA	0	46.7[*]	18.0	79.8	69.3	-
	1.0	32.5	3.0	67.5	62.5	.023
	4.0	25.2	2.5	44.8	44.8	.009
CONTROLS						
1.5 mM $CaCl_2$	0	26.3	3.6	43.5	40.6	.028
	1.0	24.6	1.7	32.5	32.5	.022
1.5 mM EDTA +3mM $CaCl_2$ next day	1.0	24.9	2.3	32.1	32.1	.014
1.5 mM $CaCl_2$ O/N, SP-A added next day	1.0	25.6	1.5	30.3	30.3	.013

[*]Values given are for the first quasi-static cycle

[†]All samples incubated overnight (16-18 hours) in buffers indicated.

[‡]Total area change, [§]area change from 25 mN/m to minimum surface tension.

Figure 5.1 Time courses for the adsorption of Lipid Extract Surfactant (LES) at different concentrations. A. Surface tension (mN/m) is plotted versus adsorption time (minutes) for LES at 50 ug/mL. Samples are suspended in 0.9% NaCl, 1.5 mM $CaCl_2$. B. Surface tension (mN/m) is plotted versus adsorption time (seconds) for LES 100-1000 ug/mL. □, 100 ug/mL; ●, 200 ug/mL; ■, 400 ug/mL; △, 800 ug/mL; ▲, 1000 ug/mL. Samples suspended as in part A. Values are the means ± S.D., n=4. Note different scales of axes in parts A. and B. (minutes as opposed to seconds).


SURFACE TENSION (mN/m)
60
50
40
30
20
0
5
10
15
20
25
30
ADSORPTION TIME (sec)

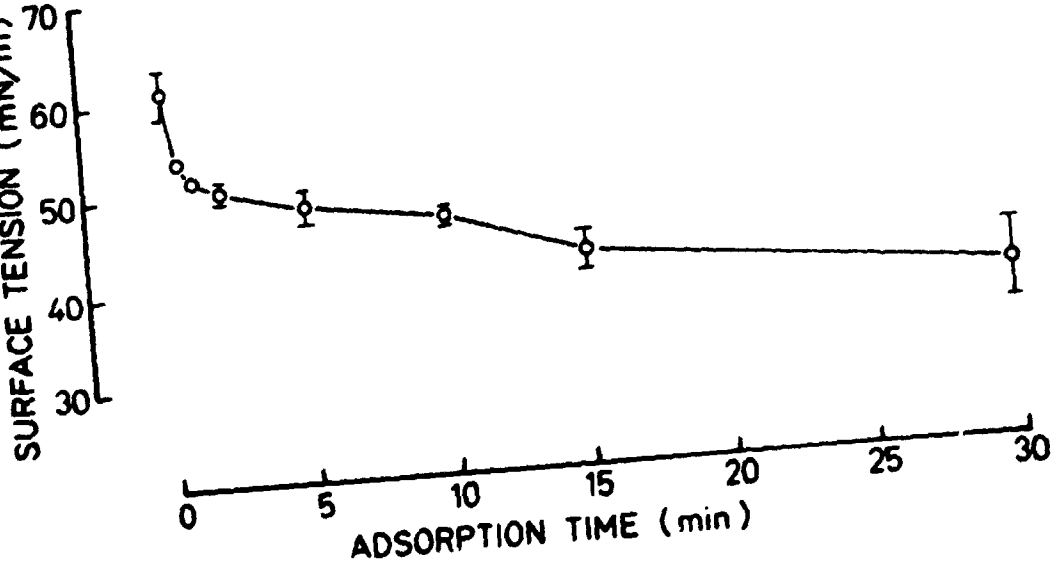

SURFACE TENSION (mN/m)
70
60
50
40
30
0
5
10
15
20
25
30
ADSORPTION TIME (min)

Figure 5.2 Time courses for the adsorption of Lipid Extract Surfactant supplemented with different amounts of SP-A. Surface tension (mN/m) is plotted versus adsorption time (seconds) for LES at 200 ug/mL. ○, 0.5% SP-A; ●, 1.0% SP-A; □, 4.0% SP-A. Samples are suspended in 0.9% NaCl, 1.5 mM $CaCl_2$. Values are the means ± S.D., n=4.

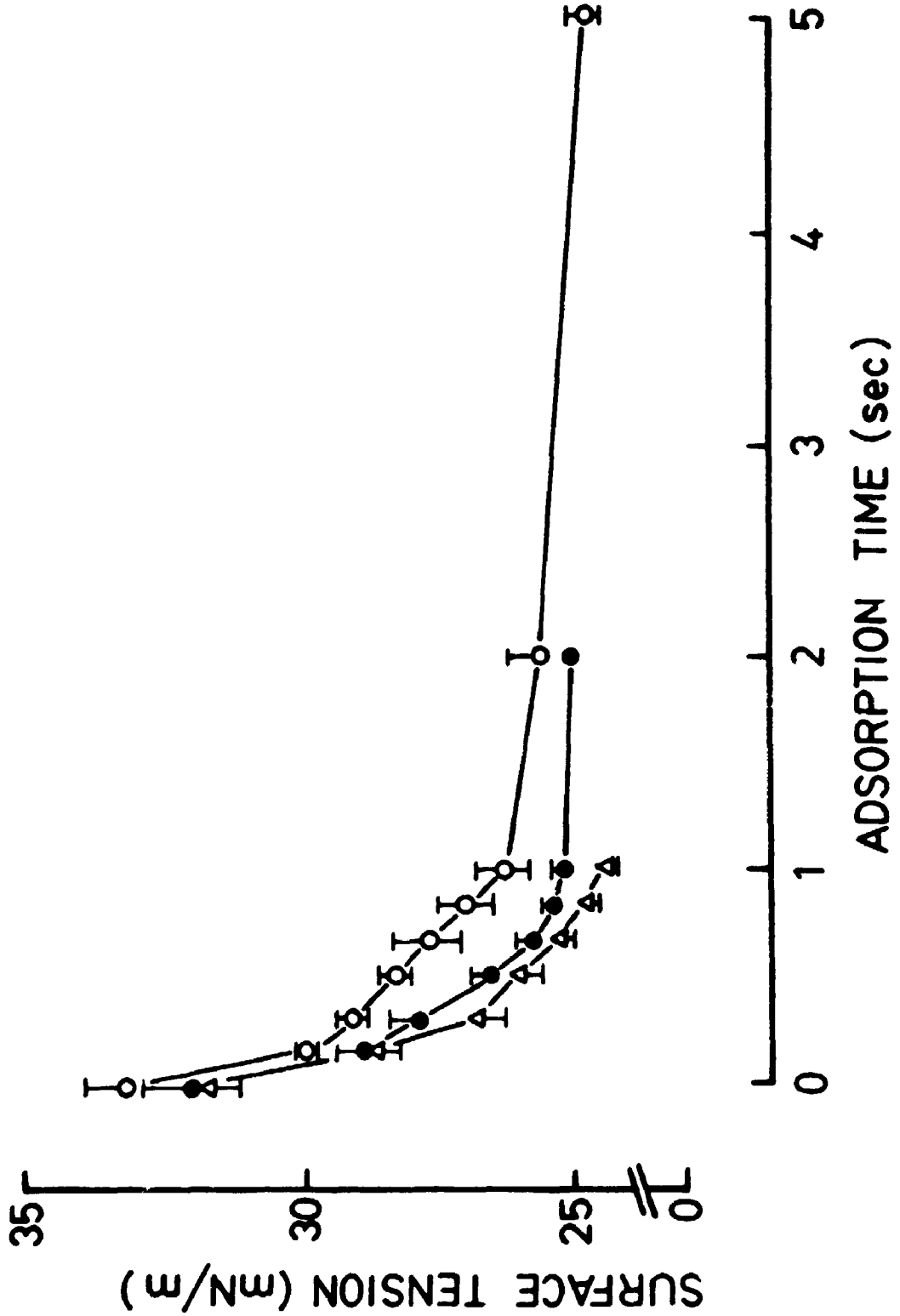

SURFACE TENSION (mN/m)
35
30
25
0
ADSORPTION TIME (sec)
0
1
2
3
4
5

Figure 5.3 Quasi-static isotherms of Lipid Extract Surfactant. Surface tension (mN/m) is plotted versus relative area for LES at 200 ug/mL. Open symbols represent measurements made during compression, filled symbols represent those made during expansion. Samples are suspended in 0.9% NaCl, 1.5 mM $CaCl_2$. Values are the means ± S.D., n=4. Part A. represents the first cycle, part B. the second and part C. the fourth quasi-static cycle. The gap between the last compression value and the first expansion value represents a bubble "click".

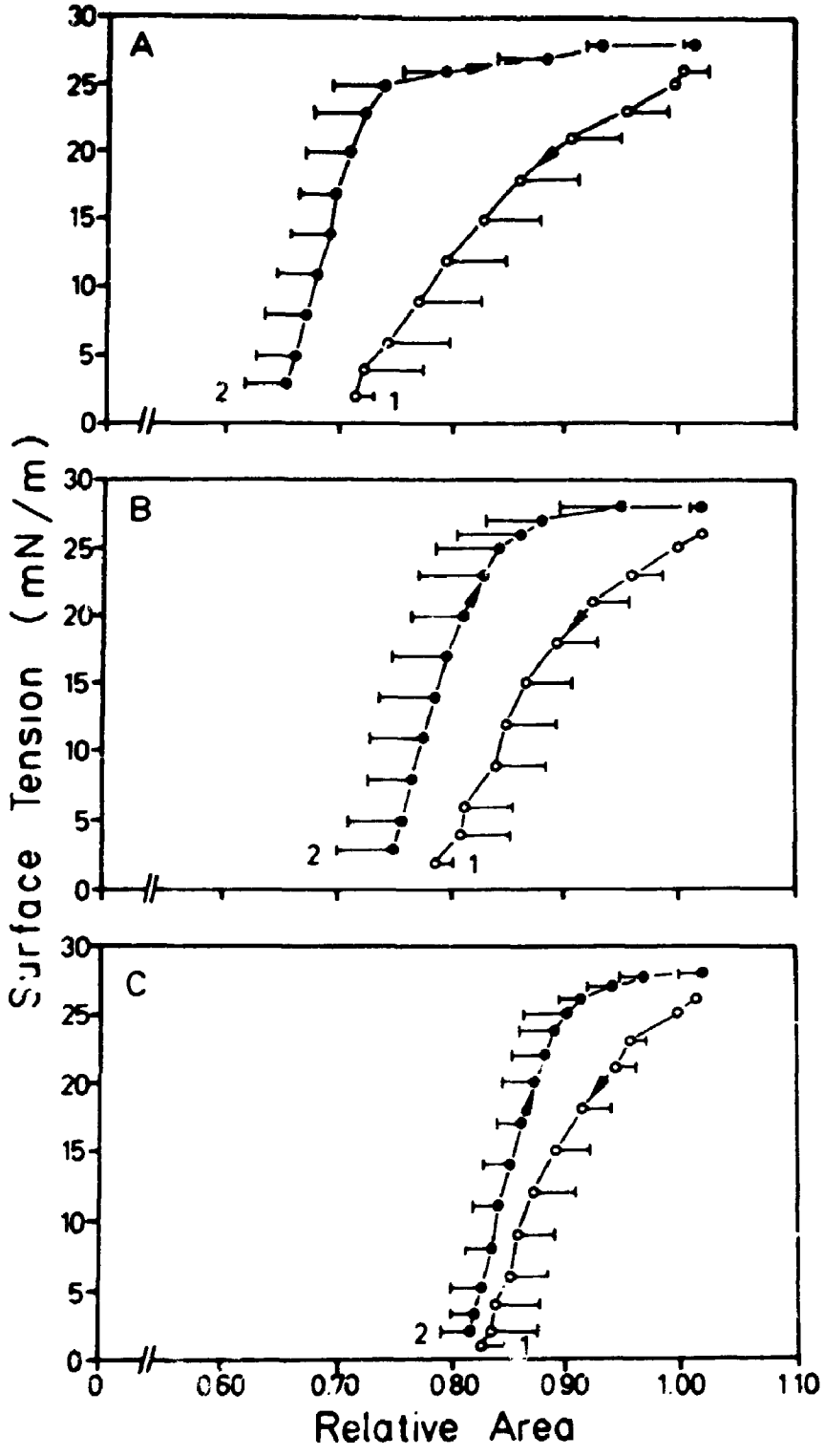
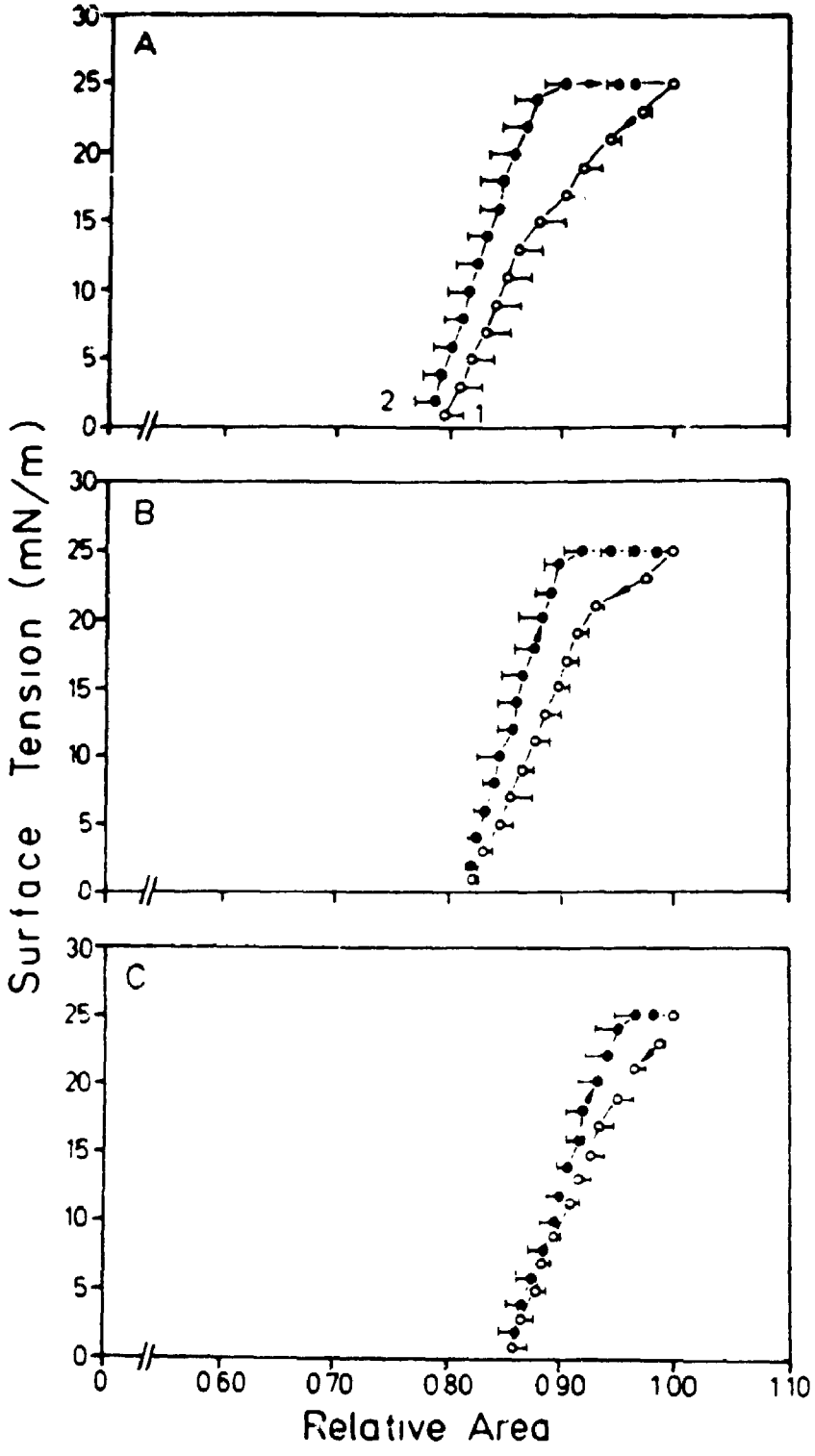

A
B
C
1
2
Surface Tension (mN/m)
Relative Area
0
5
10
15
20
25
30
0.60
0.70
0.80
0.90
1.00
1.10

Figure 5.4 Quasi-static isotherms of Lipid Extract Surfactant supplemented with two different concentrations of SP-A. Surface tension (mN/m) is plotted versus relative area for LES at 200 ug/mL. Samples are suspended in 0.9% NaCl, 1.5 mM $CaCl_2$. Open symbols represent measurements made during compression, filled symbols represent those made during expansion. Values are the means $\pm$ S.D., n=4. Parts A., B., and C. represent the addition of 0.5% SP-A on the first, second and fourth cycles respectively. Parts D., E., and F. (page 168) represent the addition of 1.0% SP-A on the first, second and fourth cycles respectively.


A
B
C
2
1
Surface Tension (mN/m)
Relative Area
0
5
10
15
20
25
30
0
060
070
080
090
100
110

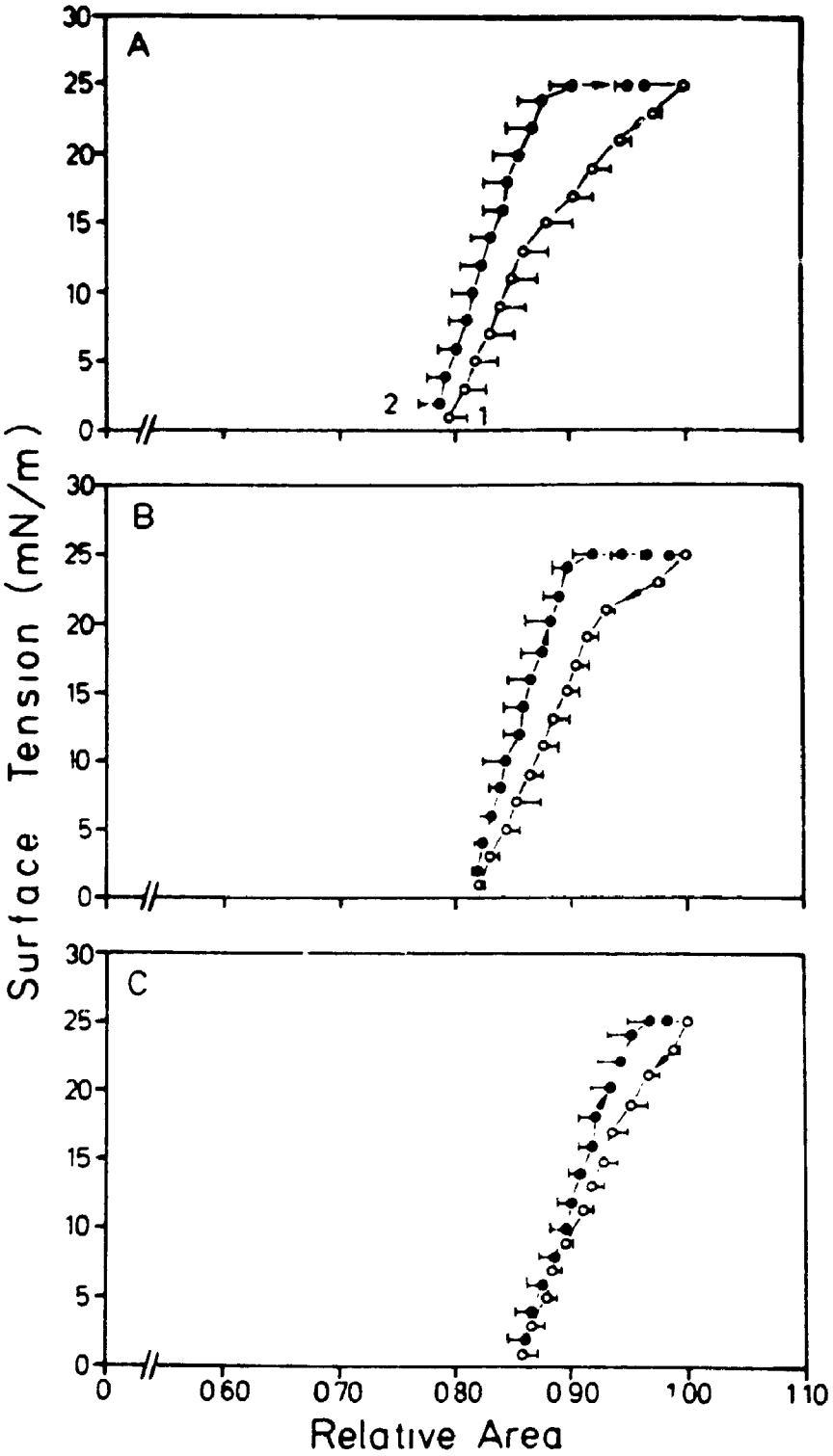

A
B
C
1
2
Surface Tension (mN/m)
Relative Area
0
5
10
15
20
25
30
060
070
080
090
100
110

Figure 5.5 The effect of concentration and SP-A on dynamic hysteresis loops of Lipid Extract Surfactant. Surface tension (mN/m) is plotted versus relative area. The plots are the summaries of four consecutive dynamic cycles centering on the 20th cycle. Open circles represent measurements made during compression, filled circles represent those made during expansion. Samples are suspended in 0.9% NaCl, 1.5 mM $CaCl_2$. A. LES at 200 ug/mL. B. LES at 1000 ug/mL. C. LES at 200 ug/mL supplemented with 4.0% SP-A.

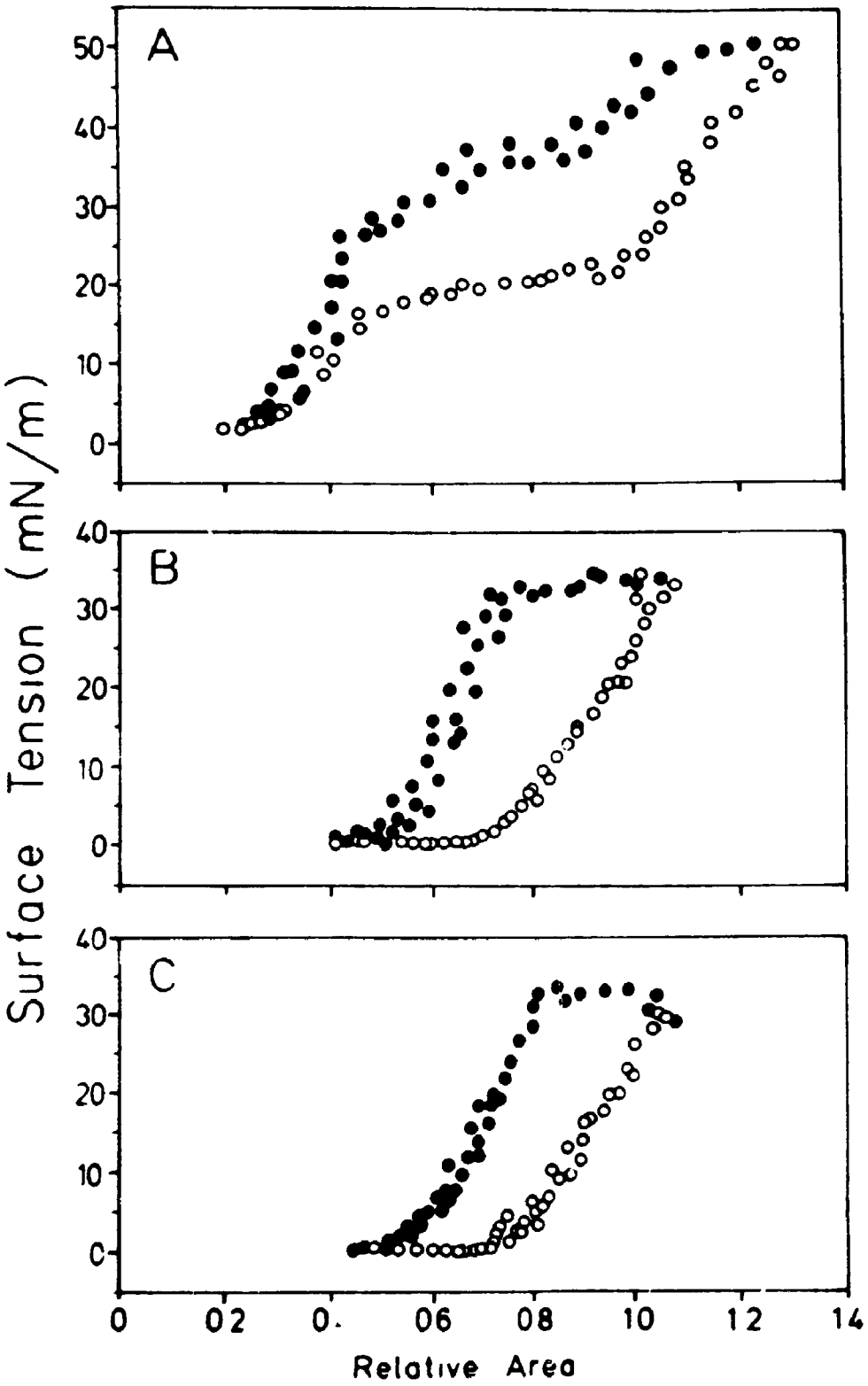
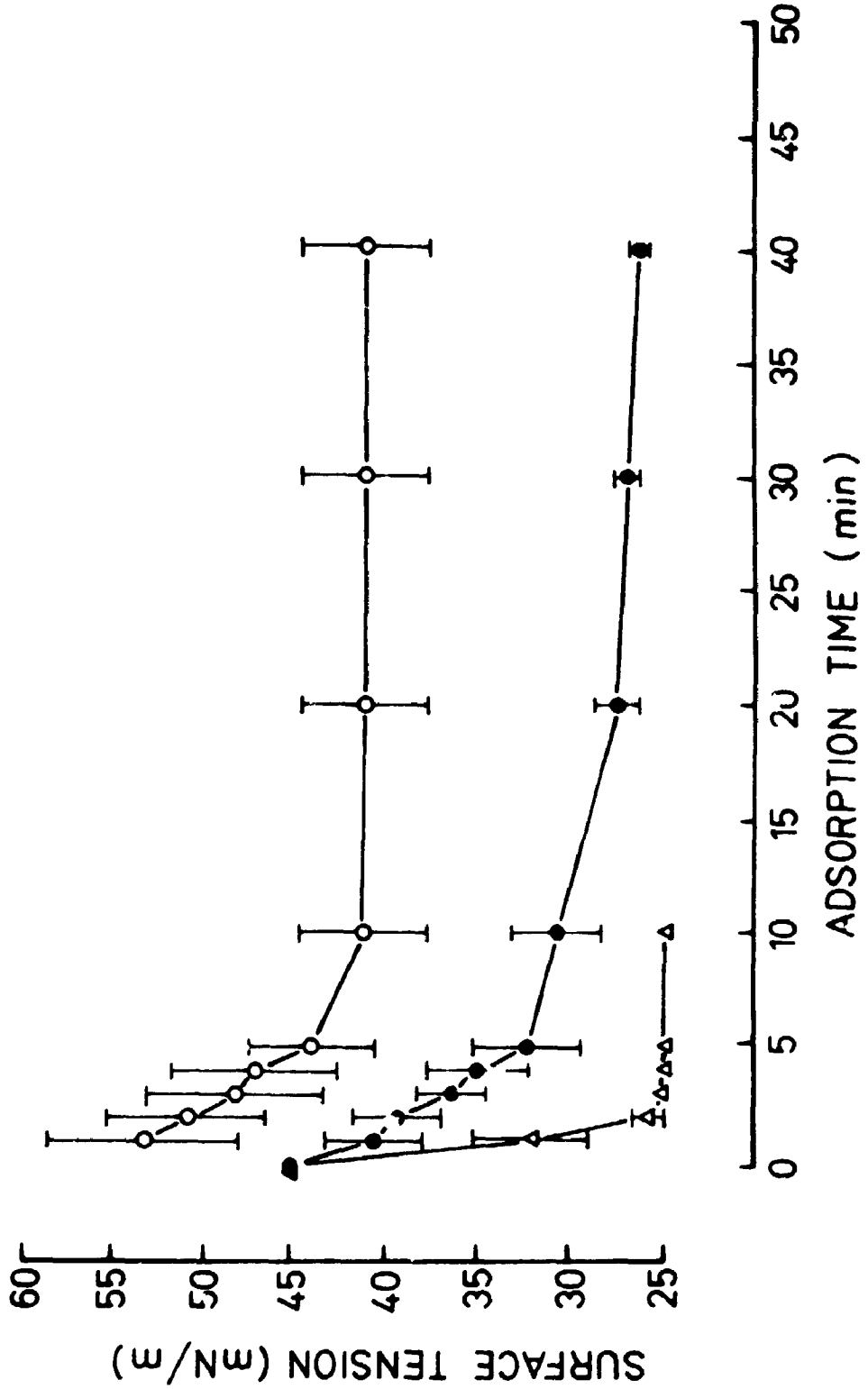

A
B
C
50
40
30
20
10
0
40
30
20
10
0
40
30
20
10
0
0
02
0.
06
08
10
12
14
Surface Tension (mN/m)
Relative Area

Figure 5.6 Time courses for the adsorption of Lipid Extract Surfactant in the presence and absence of SP-A following overnight incubation in EDTA. Surface tension (mN/m) is plotted versus adsorption time (minutes) for LES at 200 ug/mL. ○, 0% SP-A; ●, 1.0% SP-A; △, 4.0% SP-A. Samples are suspended in 0.9% NaCl, 1.5 mM EDTA and incubated overnight in the presence of 0.01% sodium azide. Values are the means ± S.D., n=4.


SURFACE TENSION (mN/m)
60
55
50
45
40
35
30
25
ADSORPTION TIME (min)
0
5
10
15
20
25
30
35
40
45
50

5.5 Discussion

The results of the first series of studies performed using the captive bubble method indicated differences between rabbit natural surfactant and bovine lipid extract surfactant with respect to adsorption kinetics and monolayer stability (Schürch et al, 1989). These data suggested that SP-A was responsible for the enhancement of adsorption and the stabilization of compressed monolayers. In the present investigation these suggestions have been verified using samples of bovine lipid extract surfactant supplemented with SP-A.

Results of adsorption studies indicate that in the presence of calcium, SP-A dramatically enhances the adsorption of LES at low surfactant concentrations. The strong concentration dependence of adsorption in the absence of SP-A suggests that the process is largely limited by diffusion of the lipids in suspension to the air-liquid interface and the subsequent conversion from bilayer to monolayer configuration. SP-A is therefore behaving as a catalyst for adsorption, somehow overcoming the diffusion and conversion limitations. SP-A causes the aggregation of phospholipids in the presence of calcium (King, 1984), therefore it is unlikely that SP-A actually increases the rate of diffusion. A model which involves the restructuring of the lipids in the aqueous subphase leading to facilitated entry onto the monolayer is more likely (Benson et al, 1984; King, 1984).

The addition of the low molecular weight surfactant proteins SP-B and SP-C to lipid mixtures containing phosphatidylcholine and phosphatidylglycerol leads to a large increase in the rate of phospholipid adsorption when measured in a surface balance or pulsating bubble surfactometer (Hawgood et al, 1987; Yu & Possmayer, 1988). The subsequent addition of SP-A further accelerates the adsorption process although it has very little effect on its own. The interaction of SP-A with SP-B appears to be particularly important for adsorption efficiency. When combined with the recent findings that tubular myelin also requires phosphatidylcholine, phosphatidylglycerol, SP-B and SP-A (Suzuki et al, 1989), the obvious interpretation is that the formation of tubular myelin or a related structure correlates with increased adsorptivity of the surfactant lipids. Such correlation has been made previously in studies by Benson et al (Benson et al, 1984). Conclusive evidence for tubular myelin formation in the present system would have to be confirmed by electron microscopy.

If the effect of SP-A on LES were confined to a simple enhancement of surfactant lipid adsorption, the area-surface tension characteristics observed during compression following adsorption to equilibrium would be expected to be unchanged by the addition of SP-A. Results of quasi-static cycling experiments indicate that the addition of SP-A does in fact alter the behaviour of the monolayer upon compression. When first quasi-static cycles are compared in the presence and absence of SP-A it is clear that SP-A limits the hysteresis area, lowers the amount of compression necessary to achieve low surface tensions,

reduces bubble clicking, and leads to lower minimum surface tensions. When the film compressibilities at 15 mN/m are compared it appears as though monolayers formed in the presence of SP-A are already enriched in DPPC prior to or during the first compression cycle. This suggests that SP-A either causes the selective adsorption of DPPC onto the interface or has a compression-independent mechanism for non-DPPC lipid removal. A related possibility is that there is a phase separation of non-DPPC lipids such that they are patched or organized in the monolayer so that only a very small compression is required to extrude them from the monolayer. The loss of the clicking phenomenon and the achievement of lower surface tensions in the presence of SP-A indicate a monolayer stabilizing function. Simple enrichment of DPPC would have this result (Notter, 1984).

Dynamic cycling and quasi-static cycling generate very different isotherms. Much larger area compressions are required to achieve low surface tensions during dynamic compression. In particular, during dynamic compression of LES at low concentrations (200 ug/mL) a number of interesting features are observed. During compression a plateau is observed below 25 mN/m, where compression of ~ 30% of the surface area results in almost no reduction in surface tension. We have termed this a "purification plateau" since it appears as though non-DPPC lipids must be removed during this phase as opposed to the packing of DPPC molecules, which results in reduction of surface tension below the equilibrium value. That this plateau disappears upon addition of SP-A is also indicative that SP-A causes selective adsorption of DPPC.

Another feature of the dynamic loop is a shoulder in the expansion portion of the curve. In this phase of the expansion, increases of surface area of 10-20% result in only modest increases in surface tension. Since adsorption from the bulk suspension is very slow at this lipid concentration, adsorption during this phase must represent adsorption from a distinct pool of lipids that we refer to as the "surface reservoir". The nature of this surface reservoir is unknown. This shoulder is not observed with higher surfactant lipid concentrations or when SP-A is present suggesting that rapid adsorption masks or eliminates this phenomenon.

A particularly interesting feature of the loops performed in the presence of SP-A, and to a lesser extent those performed at high LES concentrations, is the change in slope of the expansion curve of highly compressed monolayers. Rather than the surface tension increasing quickly as expansion commences (as is observed during quasi-static cycling), the surface tension actually remains at ~ 1 mN/m even though the surface area is increased by as much as 10%. This observation suggests a structure, perhaps a multi-layer of nearly pure DPPC, is formed in this over compression situation which can expand by unfolding upon expansion.

Clearly, the differences between the quasi-static and dynamic cycling observed with LES at low concentration demonstrate that the nature of the cycling procedure affects the monolayer structures, even when performed with the same apparatus. Much greater compression is required during dynamic cycling than quasi-static suggesting that over compression and rapid compression may lead to

monolayer collapse or instability, such that subsequent cycles continue to require large compressions. These findings may explain why, in addition to leakage, measurements made on the surface balance, the pulsating bubble and the captive bubble differ.

The dependence of the SP-A mediated effects on calcium was examined by incubating samples in EDTA prior to evaluation. Adsorption of LES alone is drastically impaired by EDTA incubation suggesting that interactions between the surfactant lipids and SP-B and/or SP-C may require calcium for optimization of adsorption efficiency (Kobayashi & Robertson, 1983; Weber & Possmayer, 1984). Supplementation with SP-A does lead to an enhancement of adsorption even in the presence of EDTA, however, 3 minutes as opposed to 1 second is required to reach equilibrium. Clearly, some of the effects of SP-A are not absolutely dependent upon the presence of calcium, however, calcium markedly enhances the SP-A effects. It has been demonstrated that SP-A binds calcium with high affinity (Haagsman et al, 1990), so the possbility exists that a small amount of calcium may remain associated with the SP-A even after incubation with EDTA. The results of dynamic cycling in the presence of EDTA also suggest that the DPPC enriching function of SP-A is impaired since a large area compression is required and the purification plateau and adsorption shoulder are still observed. Calcium is required for the formation of tubular myelin *in vitro* (Suzuki et al, 1989) and is required for the enhancement of surface activity and

reversal of blood protein inhibition observed with the pulsating bubble surfactometer (Cockshutt et al, 1990; Venkitaraman et al, 1990).

If the formation of tubular myelin is considered as the effector through which the actions of SP-A are transmitted, then a mechanism for tubular myelin enhancement of adsorption and monolayer purification is required. Results presented here and elsewhere (Kobayashi & Robertson, 1983; Yu & Possmayer, 1988) indicate that organic extracts of surfactant, which do not contain SP-A but do contain SP-B and SP-C, have surface activities essentially indistinguishable from that of surfactants containing SP-A when assayed at high concentrations. Thus it would appear that many of the functions of tubular myelin may be replaced by high bulk phase surfactant concentrations. As the concentration of surfactant is increased, adsorption probably increases as a result of the increased frequency of collisions of vesicles with the interface that result in conversion to a surface film. Purification of a monolayer arising in this fashion may proceed via mechanical squeeze-out during the compression process mediated by the low molecular weight hydrophobic proteins. Tubular myelin on the other hand, is presumed to act even when the bulk lipid concentration is low. This structure must increase the efficiency of the conversion event which moves bilayer lipids to the monolayer. The lattice-like structure typical of tubular myelin could be important for orienting surfactant lipids and proteins such that adsorption is facilitated. When the organization of this structure is considered it is not hard to imagine how DPPC and non-DPPC lipids may be segregated into different

regions, and become adsorbed differentially. The experiments presented here cannot address these questions, however it has been demonstrated that SP-A can bind specifically to DPPC (Kuroki & Akino, 1991) and SP-B may have a selective association with phosphatidylglycerol (Yu & Possmayer, 1990).

In the present investigation we have demonstrated that SP-A has the capacity, when combined with SP-B, SP-C and surfactant lipids of accelerating the adsorption process and enriching this monolayer in DPPC, thereby reducing compression requirements and stabilizing the monolayer. When the various functions of SP-A are considered it becomes clear that this protein is performing an essential function when surfactant concentrations are limiting, such as in neonatal respiratory distress syndrome, or when the surfactant is compromised by the presence of inhibitory agents (Cockshutt et al, 1990; Venkitaraman et al, 1990). As well, SP-A maintains appropriate alveolar levels of surfactant by controlling secretion from and uptake into type II cells (Wright & Clements, 1987). SP-A also mediates infection of the lung by pathogens by activating alveolar macrophages (Tenner et al, 1989; van Iwaarden et al, 1990). All of these roles of SP-A depict it as a multifunctional protein which maintains the homeostasis of the alveolus.

CHAPTER 6 - GENERAL DISCUSSION

Different aspects of surface tension reduction by pulmonary surfactant have been examined in the four data chapters of this thesis. A unifying theme has been the modulation of surface activity by the addition of components native and foreign to natural surfactant preparations, and the interactions of these additives.

6.1 The Effects of SP-A: Its Role in Surfactant

SP-A enhances the surface activity of lipid extract surfactant in the presence of calcium. It is clear that part of this enhancement is the result of the acceleration of adsorption of surfactant lipids at low bulk phase concentrations (Hawgood et al, 1987; Benson et al, 1984). It is likely, although not conclusively demonstrated, that this enhancement is caused by the reorganization of the lipids from bi- or multi-layered vesicles to tubular myelin or a related complex structure. SP-A also causes selective adsorption of DPPC and/or the rapid removal of non-DPPC lipids form the surface film formed from LES at low concentrations.

The formation of tubular myelin is favoured over simple aggregation of surfactant lipids for a number of reasons. Aggregation would cause a reduction in the number of particles in suspension which would most likely slow the adsorption process. The aggregated particles formed upon SP-A addition to LES tend to

settle (precipitate) in the tube, which would physically move them away from the bubble surface. Finally, it has been demonstrated with resonant energy transfer techniques that biotin-avidin induced aggregation of surfactant vesicles does not accomplish lipid mixing (fusion) the way SP-A does (J. Silvius, personal communication). Thus, the formation of a more complex structure to facilitate adsorption and monolayer purification is required.

The reversal of blood protein inhibition by SP-A may operate through a distinct mechanism, however, it is possible that the formation of tubular myelin is also responsible for this function. It has long been realized that inhibition by blood proteins can be counteracted simply by increasing the surfactant concentration in the assay mixture (Holm et al, 1985a; Ikegami et al, 1984). This process is apparently not stoichiometric, such that increasing the concentration of both surfactant and protein similarly results in loss of inhibition. Experiments with the captive bubble apparatus demonstrate that the addition of SP-A to LES at low concentrations gives very similar results to LES analysed at high concentrations. Thus, SP-A may decrease the threshold concentration required for reversal of inhibition, possibly by increasing the "functional" concentration through the formation of tubular myelin. As well, when the competition model for surfactant inhibition by protein is considered (Holm et al, 1988), the acceleration of surfactant phospholipid adsorption and the formation of a protective layer of tubular myelin around the bubble surface may prevent the inhibitory protein from accessing the interface. This is supported by the

observation of normal adsorption characteristics when SP-A is added to LES inhibited by protein. Indirect support for this hypothesis comes from the data on reversal of inhibition by palmitic acid. At the concentrations of palmitic acid used, adsorption in the absence of inhibitors is very rapid. However, in the presence of inhibitor, adsorption is impaired, and repeated pulsation is required to reverse the inhibition. This palmitic acid effect also varies depending on the inhibitor examined, suggesting that an interaction between palmitic acid and the inhibitor may be responsible for the counteraction of inhibition. The difference in the behaviour of palmitic acid supplemented and SP-A supplemented surfactant (LES) suggests that more specific effects of SP-A (tubular myelin formation and selective DPPC adsorption) may be necessary. The adsorption characteristics of LES with palmitic acid and SP-A in the presence and absence of inhibitors are currently being examined with the captive bubble apparatus. The results of these experiments will contribute to our understanding of the mechanisms of adsorption and inhibition by blood proteins, by establishing the kinetics of film formation in the presence of the inhibitor.

The unresponsiveness of Survanta to SP-A addition is perplexing. In the non-challenged case, the presence of palmitic acid in Survanta would presumably mask any effects of SP-A by optimizing adsorption. Survanta does not achieve very low surface tensions like LES, and surface tensions at maximum bubble radius are also higher than those observed with LES. These data suggest that another component of Survanta prevents the monolayer enrichment of DPPC

high surface tensions, approaching that of the pure protein, and all are counteracted by the addition of SP-A. Lyso-PC on the other hand, results in a more moderate increase in surface tension, and does not respond to SP-A addition, but can be essentially reversed by the inclusion of palmitic acid. It also appears as though combinations of these two types are potentially the most potent inhibitory mixtures. In the current investigations the combination of lyso-PC with fibrinogen results in a more than additive inhibition, and serum is observed to be a very potent inhibitor. The results of others indicate that lipids from cell membranes and proteins inhibit surfactant (Holm et al, 1987), and meconium is an extremely potent surfactant inactivator (Moses et al, 1991).

It is possible that both types of inhibitors act by occupying the surface instead of true surfactant components, but that their properties at the interface, and their removal from the surface may differ. The protein inhibitors are presumably spatially separate from the surfactant lipids, the lipids existing in lamellar structures and the proteins remaining in solution. The lyso-PC, on the other hand, would be mixed with the surfactant lipids in vesicles. This difference in organization and the different molecular nature of the inhibitors may partly explain why SP-A reverses inhibition by one and not the other. Lyso-PC may interfere with the interactions between the surfactant lipids and proteins, such that the effects of SP-A are lost.

The conical shape of lyso-PC is most likely perturbing to the surface film. Such a structure would not pack conveniently with DPPC upon compression

unless another component was present which could fill the space in the hydrophobic region caused by the absence of an acyl chain at the 2 position. The addition of palmitic acid would be expected to have this effect, therefore it is not entirely surprising that lyso-PC inhibition is not observed when palmitic acid is added. Inhibition of Survanta by lyso-PC is inconsistent with this hypothesis, but the surface activity of Survanta appears to be limited even in the absence of added inhibitor.

That the presence of lyso-PC in the monolayer might also interfere with surfactant-associated protein interactions is supported by the finding that lyso-PC sensitizes the preparation to inhibition. If the addition of SP-A to LES acts by increasing the "functional" surfactant concentration by the formation of tubular myelin, then the addition of lyso-PC may act by decreasing the functional concentration, possibly by disrupting critical structures, thereby stripping the gears of the surfactant machinery. That SP-A has no effect on this process and that palmitic acid has only a partial effect is highly suggestive that this may be taking place.

6.3 Suggestions for Surfactant Replacement Therapy

The experiments presented in this thesis have been performed to elucidate the mechanisms of surface tension reduction by pulmonary surfactant. The results can also be applied to the design of superior preparations for surfactant

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6.3 Suggestions for Surfactant Replacement Therapy

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replacement therapy. Recent evidence suggests that surfactant preparations differ in efficacy when assayed *in vitro* and *in vivo* (Cummings et al, 1991). The inclusion of palmitic acid and/or SP-A in surfactant in some circumstances (especially those where alveolar edema is suspected or demonstrated) may be useful. Indeed, it has recently been demonstrated that a temporal correlation exists between an increase in SP-A in tracheal aspirates and and an improvement of clinical features in infants recovering from respiratory failure (Lotze et al, 1990). In addition, the addition of SP-A to organically extracted surfactants increases dynamic compliance and maximal lung volumes in a prematurely delivered rabbit fetus model (Yamada et al, 1990). The amount of lyso-PC in preparations should be limited to prevent inhibiton and sensitization to blood protein inhibition.

6.4 General Conclusions

These experiments have demonstrated the important role which SP-A plays in the reduction of surface tension by pulmonary surfactant. In the past many of the roles described here have been overlooked, presumably because high surfactant concentrations were examined. Since the surfactant concentration appears to be functionally limiting in disease conditions such as NRDS of ARDS, and is not in vast excess in the normal lung, these roles of SP-A apparent at low concentrations may be very important in the lung.

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