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Aspects Of Pulmonary Surfactant Metabolism

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Aspects of pulmonary surfactant metabolism.

by

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ABSTRACT

Pulmonary surfactant stabilizes the lung by reducing the surface tension at the air liquid interface of the alveolus. Phosphatidylcholine represents the principal component of surfactant (70% by weight), other lipids account for 20% of surfactant and the remaining 10% is protein. Four surfactant-associated proteins have been identified, surfactant associated protein (SP) SP-A, SP-B, SP-C and SP-D.

This thesis describes the investigations on the following aspects of surfactant metabolism; 1) phosphatidylcholine synthesis, 2) alterations in surfactant after lung transplantation and 3) mechanisms of alveolar surfactant subtype conversion.

1) The *de novo* synthesis of acyl-specific phosphatidylcholine via the acylation of glycerol-3-phosphocholine (GPC) has been investigated in lung and liver. Attempts to acylate radioactive GPC were unsuccessful. It appears the previously reported evidence in favour of the GPC dependent pathway was based on inaccurate identification of radioactive products. In liver, glycerol and glucose were identified as the true reaction products. Lung also formed some glycerol and another unidentified product which was shown not to be GPC.

2) In many types of lung injury, respiratory failure is associated with alterations in pulmonary surfactant. Prolonged storage of a donor lung before transplantation leads to respiratory failure after reperfusion. Analysis of lung lavage

after transplantation revealed several alterations in surfactant; phosphatidylglycerol was decreased, sphingomyelin was increased, SP-A was decreased and the ratio of large to small surfactant aggregates was increased. Furthermore, the amount of blood proteins in lung lavage was increased. It was concluded that the effects of surfactant supplementation in lung transplantation should be investigated.

3) The large surfactant aggregate subtype, obtained as a 40,000g pellet after centrifugation of lung lavage, can be converted to the small surfactant aggregate subtype by an *in vitro* method known as surface area cycling. Subtype conversion leads to the degradation of SP-B, as demonstrated by dot blot analysis, resulting in a loss of biophysical activity. It is proposed that this degradation occurs during lipid adsorption initiated by a changing surface area.

Surface area cycling of different surfactant preparations led to the conclusion that SP-A and SP-B are necessary for large aggregate integrity.

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On a nice 15°C autumn day in November 1987, I arrived in my new thick wintercoat in Canada. My self-imposed, 2-year mission was to learn something about Canada, Canadians and science (in random order). I would like to acknowledge the many people who have contributed to this learning process.

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ABBREVIATIONS

ARDS, Adult Respiratory Distress Syndrome

ATP, adenosine-5′-triphosphate

bLES, bovine lipid extract surfactant

CDP, cytidine-5′-diphosphate

CMP, cytidine-5′-monophosphate

CTP, cytidine-5′-triphosphate

DG, diacylglycerol

DHAP, dihydroxyacetonephosphate

DPPC, dipalmitoylphosphatidylcholine

DTT, dithiothreitol

EC, Euro-Collins solution

EDTA, ethylenediaminetetraacetic acid

GP, glycerol-3-phosphate

GPC, glycerol-3-phosphocholine

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

LES, lipid extract surfactant

LPA, left pulmonary artery

LPC, lysophosphatidylcholine

NRDS, neonatal respiratory distress syndrome

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

PI, phosphatidylinositol

PMSF, phenylmethylsulfonyl fluoride

PS, phosphatidylserine

R_{max}, maximum bubble size

R_{min}, minimum bubble size

RPA, right pulmonary artery

SEM, standard error of the mean

SM, sphingomyelin

SP-A, surfactant-associated protein A

SP-B, surfactant-associated protein B

SP-C, surfactant-associated protein C

Tes, N-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid

TLC, thin layer chromatography

UTP, uridine 5'-triphosphate

UW, University of Wisconsin solution

CHAPTER 1 INTRODUCTION.

1.1. General introduction

Pulmonary surfactant prevents alveolar collapse by reducing the surface tension at the air liquid interface of the terminal airways. Dipalmitoylphosphatidylcholine (DPPC) is thought to be the major surface tension reducing agent of surfactant. Other components of surfactant are other phospholipids, neutral lipids and the surfactant-associated proteins: these components are necessary for the formation and maintenance of the monolayer enriched in DPPC.

The physiological importance of surfactant is apparent in the neonatal respiratory distress syndrome (NRDS) in which a deficiency of surfactant is associated with impaired lung function. Surfactant supplementation of infants at risk of NRDS results in a remarkable improvement in gaseous exchange. Surfactant impairment has been implicated in many other types of lung injuries.

1.2. Historical perspective

Based on the observation that the pressure needed to distend a lung to a certain volume is greater when the lung is filled with air as compared to saline, Von Neergard was the first to suggest the importance of surface tension in

the lung (Von Neergard 1929). This finding was ignored until the 1950's when a series of important observations were reported. While attempting to calculate the surface area of the lung, Radford conducted pressure-volume studies which confirmed von Neergards observations (Radford 1954). Unfortunately, Radford misinterpreted his data and concluded that the lung had a smaller surface area then had previously been estimated by histological methods. In the same year, Macklin proposed the secretion of a thin aqueous mucoid material by what is now called, the Type II pneumocyte. Furthermore, Macklin hypothesized that this material formed a surface film covering the alveolar wall which could assist in maintaining appropriate surface tension (Macklin 1954). Pattle reported that foam bubbles obtained from lung lavage material were remarkably stable indicating very low surface tensions (Pattle 1955). In 1957, Clements used a Langmuir-Wilhelmy surface balance to show for the first time, the reduction of surface tension during compression of lung extract films (Clements 1957). The clinical significance of this surface active material became apparent after Avery and Mead discovered that lung extracts from infants dying from hyaline membrane disease had higher minimum surface tensions than infants dying of other causes (Avery and Mead 1959). This correlation between surfactant deficiency and neonatal respiratory distress syndrome (NRDS) initiated a large increase in research in pulmonary surfactant.

1.3. Composition of pulmonary surfactant

Pulmonary surfactant can be defined functionally as the material that reduces the surface tension at the air liquid interface of the alveolus. However, determination of the composition of the surface film has been limited by the methods available. Therefore, for compositional analysis, surfactant is defined as the surface tension-reducing material that can be isolated by density centrifugation or differential centrifugation of alveolar lavages. This isolated material is composed of a mixture of lipids and proteins which is highly conserved among species (Shelly et al 1984, King 1984, Possmayer 1984).

Figure 1.1 shows the composition of bovine pulmonary surfactant (Yu et al 1983). The main component is phosphatidylcholine (PC), a large percentage of which is the disaturated species, dipalmitoylphosphatidylcholine (DPPC). Phosphatidylglycerol (PG) represents approximately 10% of surfactant by weight. In some species or during perinatal development, another acidic phospholipid, phosphatidylinositol (PI), is present at relatively high amounts instead of PG. Phosphatidylethanolamine (PE), sphingomyelin (SM), lyso-PC and lyso-*bis*-phosphatidic acid are present in low amounts (Yu et al 1983, King 1984).

Neutral lipids are also present in low amounts but this can vary with the isolation method or species.

Figure 1.1. The composition of bovine pulmonary surfactant. (Data obtained from Yu et al 1983)

PA, phosphatidic acid

PC, phosphatidylcholine

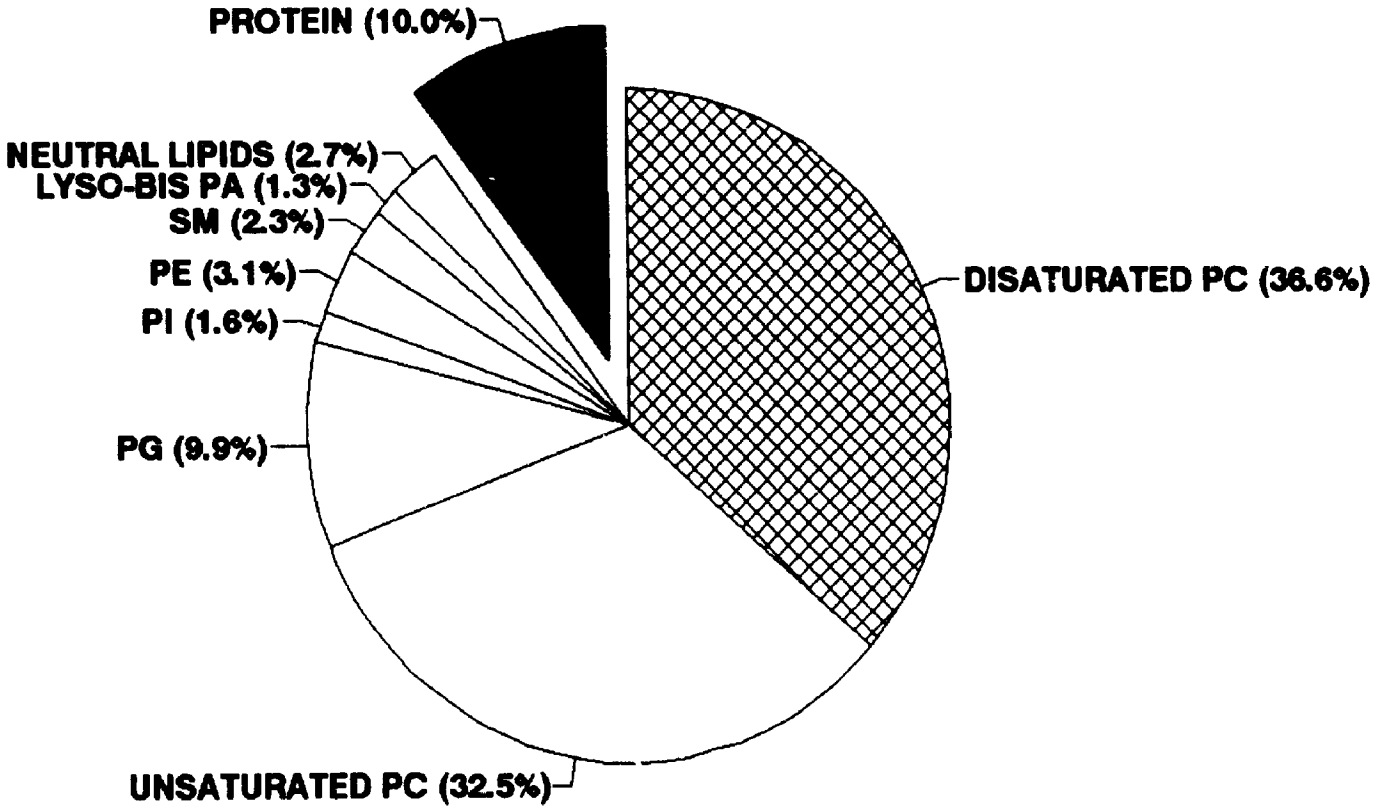
PE, phosphatidylethanolamine

PG, phosphatidylglycerol

PI, phosphatidylinositol

SM, sphingomyelin

The composition of pulmonary surfactant



Cholesterol is the most abundant neutral lipid present (approx 90% of neutral lipid), the remainder is mainly diacylglycerol.

In bovine pulmonary surfactant, surfactant-associated proteins represent approximately 10% by weight. The percentage of protein varies with the species and possibly with the isolation method. Four surfactant-associated proteins (SP-), SP-A, SP-B, SP-C and SP-D have been reported to date. SP-A is a relatively large water soluble glycoprotein. SP-B and SP-C are two small hydrophobic proteins which are soluble in organic solvent upon lipid extraction. The recently identified SP-D, is a watersoluble glycoprotein (Persson et al 1988, 1989). As will be discussed later, the association of SP-D with surfactant has yet to be established.

1.4. Surfactant synthesis

It is generally accepted that the synthesis of pulmonary surfactant occurs in the Type II pneumocyte (Haagsman and Van Golde 1991). The Type II cells, which represent approximately 10% of the cells in the lung, can be recognized as cuboidal cells containing the characteristic surfactant storage organelles, the lamellar bodies. The phospholipid composition of lamellar bodies is similar to surfactant (Haagsman and Van Golde 1991). The surfactant-

associated proteins SP-A, SP-B and SP-C have also been found in lamellar bodies (Oosterlaken-Dijksterhuis et al 1991a, Bakewell et al 1991). However, SP-A was relatively low in lamellar bodies compared to extracellular surfactant indicating that this protein might also be secreted independently (Oosterlaken-Dijksterhuis et al 1991a). SP-D protein has been detected in Type II cells (Persson et al 1989), but an association with lamellar bodies has not yet been reported.

It has been reported that Clara cells can produce SP-B mRNA (Phelps and Floros 1988). *In situ* hybridization studies have detected SP-A mRNA in Clara cells from rat and dog but not from human lung (Phelps and Floros, 1988, Weaver and Whitsett 1991). Recently immunoreactive SP-D has also been detected in rat Clara cells (Crouch et al 1992). Many aspects relating to the production of surfactant components by Clara cells are still unclear. The relative contribution of Clara cells to the alveolar pool sizes of surfactant components, whether these Clara cell derived surfactant components are secreted with lipid and whether these components have a different function than the Type II cell secreted surfactant needs further investigation.

Synthesis of surfactant proteins in other lung cells or in other tissues has not been reported. The subsequent sections focus on the synthesis of surfactant components by the Type II cell.

1.4.1. Phospholipid biosynthesis

The *de novo* synthesis of surfactant phospholipids occurs according to the Kennedy pathway (Kennedy 1962). This pathway (figure 1.2) initiates with the formation of phosphatidic acid (PA) from glycerol-3-phosphate or dihydroxyacetonephosphate. PA marks an important branchpoint in phospholipid synthesis, it can be utilized for the formation of CDP-diacylglycerol or for the formation of diacylglycerol. CDP-diacylglycerol represents an intermediate for the formation of PG and PI. Diacylglycerol can react with CDP-choline to form PC.

Pulmonary surfactant contains a high percentage of disaturated PC. The enzymes involved in the *de novo* pathway are not selective towards DPPC formation (Batenburg et al 1986, Batenburg 1992). The acyl composition of PC formed by the *de novo* pathway is therefore dependent on the availability of the acyl substrates. The high percentage of palmitoyl-CoA found in isolated Type II cells suggests that these cells can produce a relatively high percentage of this disaturated PC by the *de novo* pathway (Breejen et al 1989). Remodelling of PC containing unsaturated fatty acids at the 2-position is thought to be responsible for the formation of more DPPC.

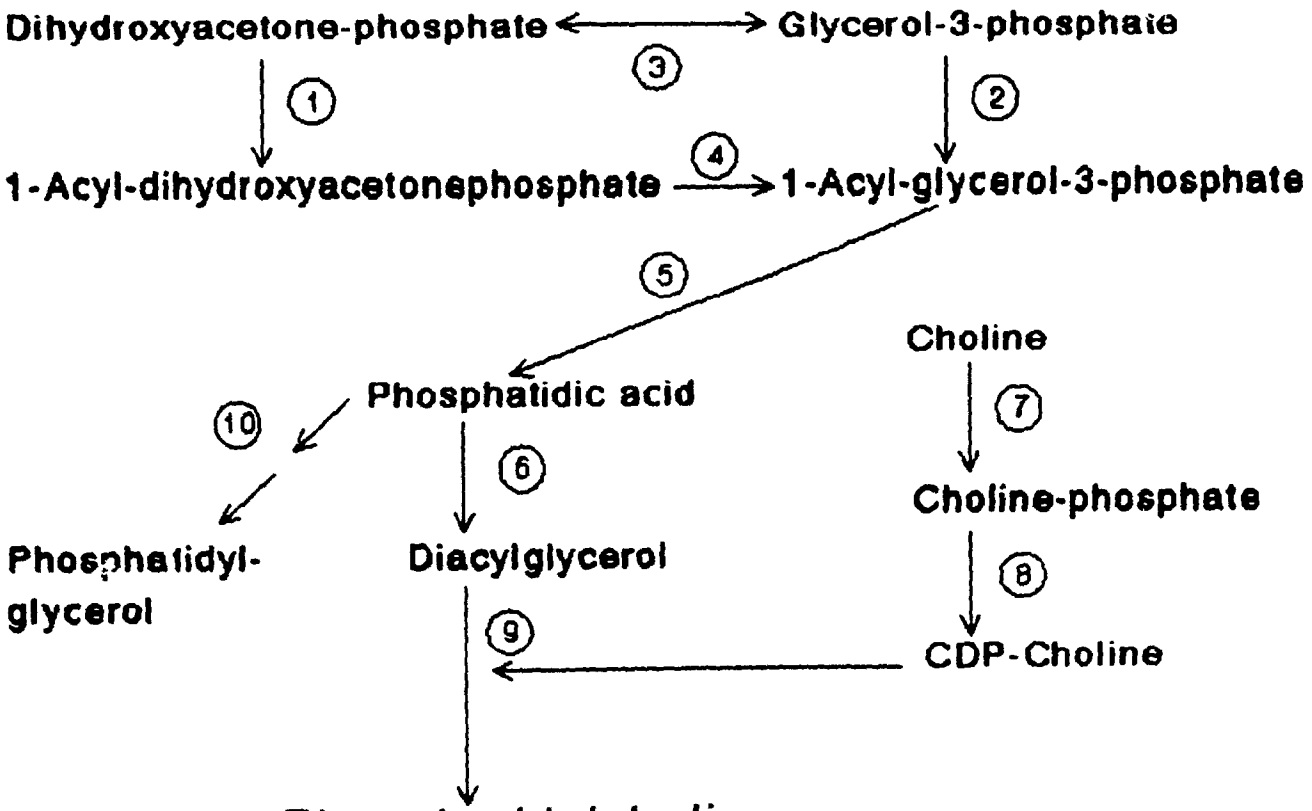
Remodelling of PC to form DPPC could occur by several different mechanisms (Possmayer 1989, Batenburg 1992).

Figure 1.2. The *de novo* pathway for phosphatidylcholine synthesis.

Enzymes:

1. Dihydroxyacetone-phosphate acyltransferase
2. Glycerol-3-phosphate acyltransferase
3. Glycerol-3-phosphate dehydrogenase
4. Acyldihydroxyacetone-phosphate reductase
5. 1-Acylglycerol-3-phosphate acyltransferase
6. Phosphatidate phosphohydrolase
7. Choline kinase
8. Choline-phosphate cytidyltransferase
9. Cholinephosphotransferase
10. Glycerophosphate phosphatidyltransferase and phosphatidylglycerolphosphatase

The *de novo* pathway for phosphatidylcholine



One remodelling pathway is the deacylation-reacylation mechanism in which phospholipase A_2 removes a fatty acid at the 2 position, the resulting lyso-PC is then reacylated with palmitoyl-CoA. There are indeed several indications that this pathway occurs in Type II cells (De Vries et al 1985, Post and Van Golde 1988). A second proposed remodelling mechanism is the deacylation-transacylation reaction. In this pathway two 1-palmitoyl-lyso-PC molecules react to form DPPC and glycerol-3-phosphocholine. This pathway, however, does not appear to contribute significantly to surfactant synthesis (De Vries et al 1985, Post and Van Golde 1988, Possmayer 1989). A modification of the deacylation-reacylation remodelling pathway has been proposed by Stymne and Stobart (Stymne and Stobart 1985) and Nijssen and Van den Bosch (Nijssen and Van den Bosch 1986a, 1986b). This pathway involves a transfer of the unsaturated fatty acid at the 2 position of PC to lyso-PE by the reverse reaction of lysophosphatidylcholine acyltransferase. The LPC generated is subsequently acylated with palmitoyl-CoA to form DPPC (Stymne and Stobart 1985, Nijssen and Van den Bosch 1986a, 1986b). This remodelling pathway has been demonstrated in whole lung (Stymne and Stobart 1985, Nijssen and Van den Bosch 1986a, 1986b). Indirect evidence for the occurrence of this pathway in the Type II cell has recently been described (Rüstow et al 1992). Lyso-PE appears to be the preferred substrate for acylation by the reverse

reaction of lysophosphatidylcholine acyltransferase (Nijssen and Van den Bosch 1986a, 1986b). Interestingly, a pulmonary phospholipase A_2 with a higher affinity for PE than PC has been described (Filgueiras and Possmayer 1987).

An alternative *de novo* pathway for acyl-specific phospholipid synthesis has been proposed by Infante (Infante 1986a, 1987, Infante and Huszagh 1987). This pathway will be discussed in more detail in chapters 2 and 3.

1.4.2. Synthesis and structure of SP-A

SP-A has a primary translation product of 248 amino acids in the human. In the rat and rabbit SP-A contains 247 amino acids. There is about 70% homology between the three sequences (Weaver and Whitsett 1991). The primary sequence consists of a signal peptide, a N-terminal collagen-like domain and a carbohydrate recognition region (Hawgood 1989, Weaver and Whitsett 1991).

Post translational modifications of SP-A include cleavage of the signal peptide, N-linked glycosylation, proline hydroxylation and oligomerization (Haagsman and Van Golde 1991). Glutamic acid carboxylation, acetylation and sulfation have also been reported, however, these latter modifications have not yet been observed in the native protein obtained from lung lavages (Haagsman and Van Golde 1991, Hawgood and Shiffer 1991). The monomeric molecular

weight of SP-A is 26-36 kDa depending on the degree of glycosylation. Oligomerization of SP-A results in an 18'mer, made up out of 6 collagen-linked trimers (Voss et al 1988). This structure resembles a bouquet of flowers (figure 1.3A) and is analogous to the complement component C1q.

SP-A contains a phospholipid binding domain, a carbohydrate binding domain and high and low affinity calcium binding sites. Preferential binding of SP-A to DPPC compared to other species of PC and other lipids has been reported by Kuroki and Akino (Kuroki and Akino 1991). The carbohydrate binding by SP-A is calcium dependent (Haagsman et al 1987). As will be described in more detail later, it is thought that self aggregation of SP-A involving carbohydrate binding is important in the formation of an alveolar form of surfactant known as tubular myelin (Haagsman et al 1991, Voorhout et al 1991). SP-A contains high and low affinity calcium binding sites and binds two to three calcium ions per monomer (Haagsman et al 1990).

1.4.3. Synthesis and structure of SP-B

The SP-B gene encodes a precursor protein of approximately 375 amino acids which is 67% conserved among different species (Hawgood 1989). This 40 kDa SP-B precursor undergoes N-linked glycosylation and dimerization (Haagsman and Van Golde 1991, Hawgood and Shiffer 1991).

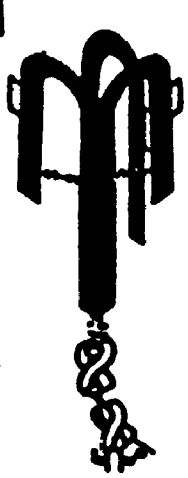
Figure 1.3. A schematic representation of the structures predicted for SP-A, SP-B and SP-C. (Modified from Weaver and Whitsett 1991)

A) SP-A trimer and 18'mer structures

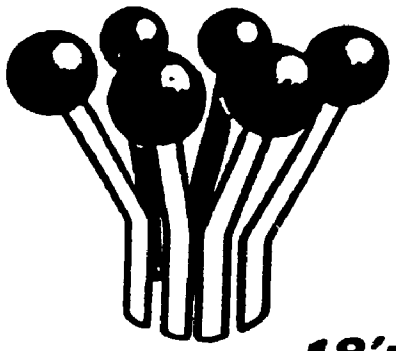
B) SP-B in a lipid bilayer

C) palmitoylated SP-C

A. SP-A

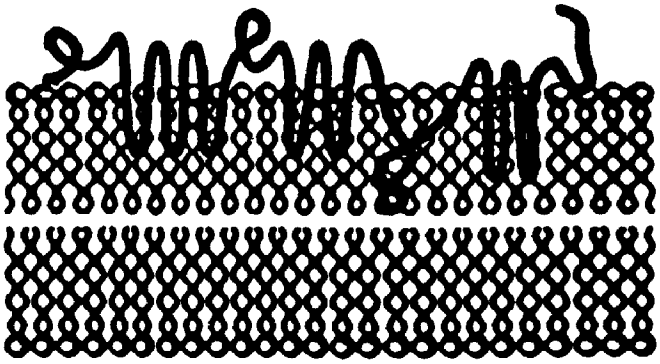


Trimer



18'mer

B. SP-B



C. SP-C

Subsequent N and C-terminal proteolytic cleavages of this SP-B precursor result in the mature SP-B protein. The enzyme involved in cleavage of the N-terminal of the SP-B precursor has recently been identified as a cathepsin D-like protease (Weaver et al 1992).

The mature SP-B is a 79 amino acid long peptide containing 7 cysteines (Hawgood 1989). These 7 cysteines are involved in 3 intrachain disulfide bonds and 1 interchain cysteine bond. The predicted structure of SP-B is shown in figure 1.3B. An amphipatic helix has been proposed as the site of lipid-SP-B interaction (figure 1.3B) (Weaver and Whitsett 1991).

1.4.3. Synthesis and structure of SP-C

Although SP-C is found in the alveolar space, its cDNA sequence does not contain a typical signal peptide (Weaver and Whitsett 1991). The SP-C precursor consists of 197 amino acids and appears to be a palmitoylated transmembrane protein (Vorbroker et al 1992, Keller et al 1991). Cleavage of the precursor results in the extremely hydrophobic mature peptide of 33-35 amino acids. Residues 13 to 33 of this protein consist mainly of valine, leucine and isoleucine and presumably forms an α-helix that can span a bilayer (figure 1.3C). In addition, SP-C contains palmitates at two adjacent cysteines near the N-terminus (Curstedt et al 1990)

providing another possible membrane anchor. Canine SP-C is an exception in that it contains a single palmitoylated cysteine residue (Johansson et al 1991).

1.4.4. Synthesis and structure of SP-D

Although SP-D is only weakly associated with surfactant lipids (Kuroki et al 1991), it appears to be at least partly synthesized in the Type II cell (Persson et al 1988). The mature protein is 43 kDA which undergoes cleavage of a signal peptide, glycosylation, proline hydroxylation, lysine hydroxylation and oligomerization (Hawgood and Shiffer 1991, Persson et al 1988, 1989, 1990). Like SP-A, SP-D contains a collagen-like domain and a calcium dependent carbohydrate recognition site (Persson et al 1990, Crouch et al 1991a).

1.5. Surfactant assembly and secretion

Synthesis of the surfactant components occurs mainly in the endoplasmic reticulum (Batenburg 1992). Electron microscopy autoradiographic studies with several different lipid precursors have demonstrated that the bulk of surfactant lipid is synthesized in the endoplasmic reticulum, transferred to the Golgi and then assembled into lamellar bodies (Chevalier and Collet 1972). Subcellular localization studies on the enzymes involved in surfactant

phospholipid synthesis are consistent with these findings (Batenburg 1992). It is thought that the remodelling of unsaturated PC to DPPC also occurs in the endoplasmic reticulum (Voelker and Snyder 1979).

The surfactant associated proteins are also synthesized on the endoplasmic reticulum and transferred to the Golgi (Van Golde et al 1988). Using immunogold labelling of precursor SP-B and mature SP-B, Voorhout et al have demonstrated that the intracellular proteolytic processing of SP-B occurs in multivesicular bodies which are located between the golgi and the lamellar bodies (Voorhout et al 1992). Intracellular trafficking of the other surfactant proteins has not yet been reported.

It is thought that the multivesicular bodies containing surfactant-associated proteins SP-B and SP-C might assemble with the small lipid-containing lamellar bodies (Van Golde et al 1988, Voorhout et al 1992). SP-A might be secreted independently since isolated lamellar bodies contain mature SP-B and SP-C but only relatively low amounts of SP-A (Oosterlaken-Dijksterhuis et al 1991a).

Secretion of lamellar bodies occurs by exocytosis. Fusion of the lamellar body with the plasma membrane of the Type II cell has been demonstrated using electron microscopy (Ryan et al 1975, Kliewer et al 1985). *In vitro* fusion of lamellar bodies with plasma membrane can be increased by synexin (Chander and Wu 1992). The exact mechanisms involved

in the secretion of the lamellar bodies are not fully understood.

Considerably more information is available on factors that stimulate or inhibit surfactant secretion. A variety of substances can stimulate surfactant secretion; adrenergic agonists, cholinergic agonists, mechanical stress, prostaglandins and other agents (Wright and Clements 1987, Wright and Dobbs 1991). It should be noted however, that many of the studies on surfactant secretion have been performed *in vitro*. The importance of these factors *in vivo* needs further investigation. SP-A appears to inhibit surfactant secretion *in vitro* (Wright and Dobbs 1991) but whether this occurs *in vivo* is not clear.

1.6. Extracellular metabolism of surfactant

Alveolar surfactant exists in several morphologically distinct forms. The relationship between these structures is depicted in figure 1.4. Newly secreted lamellar bodies appear as densely packed multilamellar structures. This structure can be converted into a unique lattice-like structure called tubular myelin (Wright et al 1984, Ryan et al 1975, Sanders et al 1980, Wright and Clements 1987). Tubular myelin has been suggested to be the direct precursor of the surface tension reducing monolayer (Magoon et al 1983, Goerke and Clements 1985). It is thought that small

Figure 1.4. A schematic representation of the extracellular metabolism of surfactant. (Modified from Weibel 1984, Cockshutt 1991)

ER = endoplasmic reticulum

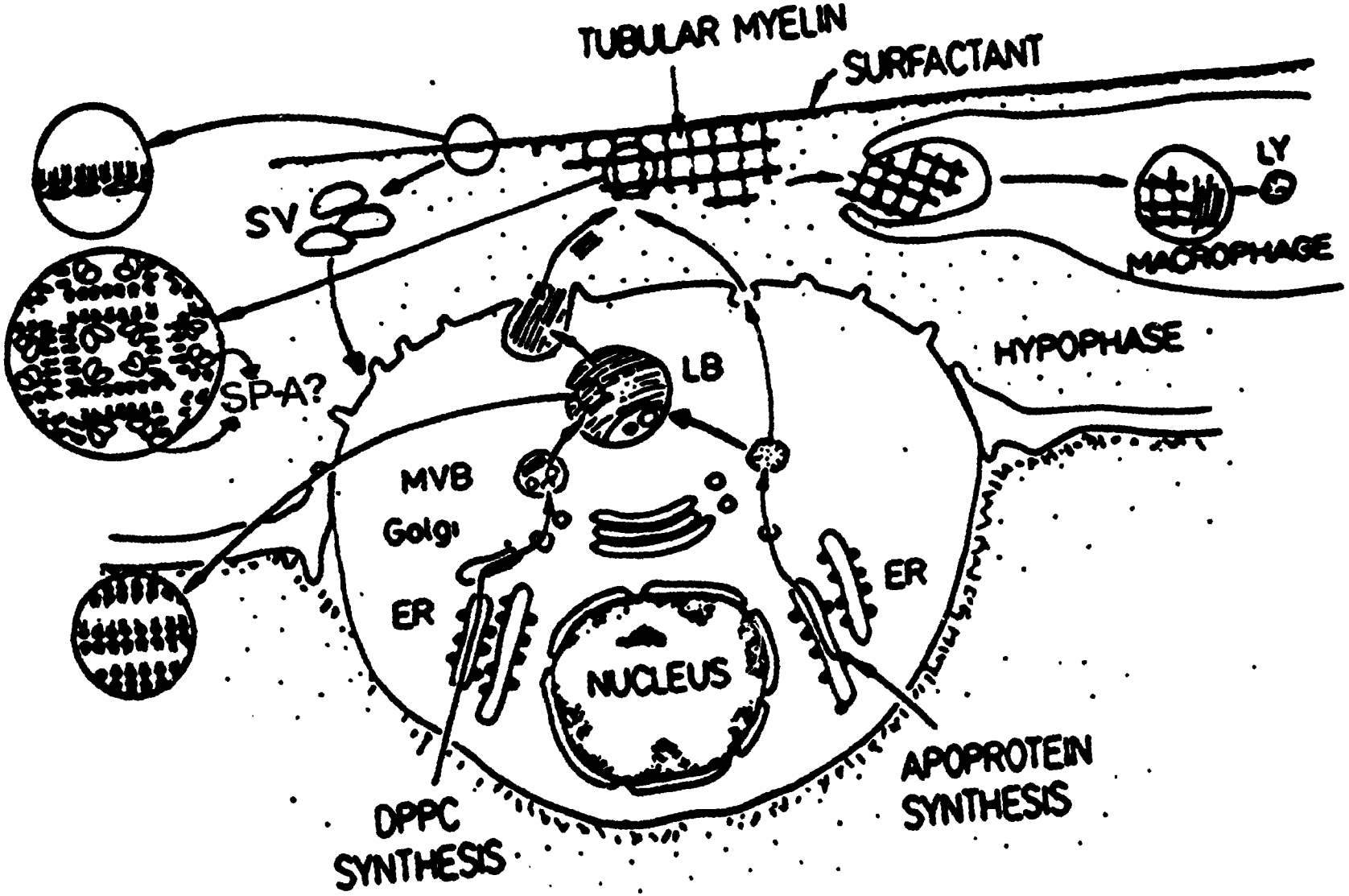
MVB = multivesicular body

LB = lamellar body

LY = lysosome

SV = small vesicle

SP-A = surfactant-associated protein A


TUBULAR MYELIN
SURFACTANT
SV
LY
MACROPHAGE
SP-A?
LB
HYPOPHASE
MVB
Golgi
ER
ER
NUCLEUS
APOPROTEIN
SYNTHESIS
DPPC
SYNTHESIS

vesicles are generated from the monolayer for reuptake by the Type II cell or for degradation by alveolar macrophages. These small vesicles are of a lower density than the tubular myelin and larger vesicles and remain in the supernatant during centrifugation of lung lavages at 40,000g. With the exception of pulse chase studies which showed that small aggregates are the metabolic product of the heavier surfactant subtype (Magoon et al 1983, Baritussio et al 1984), metabolism of this light surfactant subfraction has largely been ignored.

The inability to isolate subfractions of a single morphological appearance from lung lavage has limited the information available on the molecular mechanisms involved in the extraalveolar metabolism of surfactant. The possibility that alveolar lavage could alter the surfactant structures further complicates this issue. The *in vitro* formation of morphologically uniform surfactanc structures has proven difficult. Tubular myelin can be formed from purified constituents but these preparations are still not homogeneous (Suzuki et al 1989, Williams et al 1991).

To avoid some of the problems described above, Gross and Narine have developed an *in vitro* method for the conversion of a dense surfactant subfraction into a lighter subfraction (Gross and Narine 1989a). In this *in vitro* system, surfactant subtype conversion appears to be dependent on a change in surface area (Gross and Narine

1989a) and the activity of a serine protease (Gross and Schultz 1990). Subtype conversion was blocked by cycling at low temperatures (Gross and Narine 1989a). Recently, Gross and Schultz described the tentative identification of the serine protease involved in the conversion of surfactant aggregates (Gross and Schultz 1992).

1.7. Surfactant clearance

Surfactant pool-sizes appear to be under tight control and surfactant is continuously taken up by lung cells for degradation or recycling (Wright and Clements 1987, Wright and Dobbs 1991). This regulation of alveolar pool-sizes might be important for adaptation to changing circumstances, such as in exercise (Nicholas et al 1982).

Surfactant clearance has been studied, both *in vitro* and *in vivo*. The *in vitro* studies have mainly focused on factors that can stimulate or inhibit reuptake. SP-A appears to enhance the uptake of surfactant lipids by Type II cells (Wright et al 1987). This process might be mediated by a specific SP-A receptor on the Type II cell (Ryan et al 1989, Wright et al 1989, Kuroki et al 1988). The binding of SP-A to the type II cell is calcium dependent and the SP-A appears to be effectively recycled to the lamellar body (Kuroki et al 1988). SP-B and SP-C have also been reported to enhance reuptake of phospholipids by the Type II cell

(Rice et al 1989, Bates et al 1992). Whether this enhancement of phospholipid reuptake by these hydrophobic proteins occur in a cell specific, receptor-mediated manner needs further investigation.

Alveolar macrophages might also participate in the clearance of surfactant from the alveolar space (Grabner and Meerbach 1991, Stern et al 1987). Degradation of surfactant phospholipid by alveolar macrophages has been reported (Stern et al 1987, Miles et al 1988). Like the Type II cells, macrophages also appear to have a specific SP-A receptor (Manz-Keinke et al 1991, Pison et al 1992). However, this receptor might be important in host defence mechanisms (Tenner et al 1989, Van Iwaarden et al 1990,1991) rather than in the uptake and degradation of surfactant.

Most of the *in vivo* studies on surfactant clearance have used radio-labelled surfactant preparations to monitor surfactant turnover. Surfactant half-life times obtained by these methods are, in general, 5 to 10 hours, but this can vary depending on the species and the age studied (Wright and Clements 1987). Surfactant lipids taken up by the lung can be effectively recycled (Wright and Clements 1987, Post and Van Golde 1988). Rider et al showed that 24 hours after intratracheal injection of surfactant containing a nondegradable radioactive analogue of DPPC, over 65% of the radioactivity taken up by the lung was found in the Type II cells and is presumably recycled (Rider et al 1992).

Radioactivity in the macrophages accounted for 20% (Rider et al 1992).

The fate of alveolar surfactant associated proteins has not been studied as extensively as phospholipids. Uptake and recycling of SP-A has been reported (Snyder et al 1988, Young et al 1989, Fisher et al 1991). The rate of SP-A reuptake appears similar to that of surfactant phospholipids (Fisher et al 1991). SP-C is also taken up by the lung and appears to be effectively recycled (Baritussio et al 1989).

1.8. Function of the surfactant components

Surface tension arises from the attractive forces between molecules in solution. Molecules at the air-liquid interface of such a solution lose some of these positive interactions thus giving rise to surface tension. Pulmonary surfactant reduces the surface tension at the air-liquid interface of the alveoli. Although a monolayer enriched in DPPC is thought to be the principal surface tension reducing agent, other phospholipids and the surfactant associated proteins are necessary to generate and maintain this monolayer (Possmayer et al 1988).

1.8.1. Phospholipids

A monolayer of phospholipid can reduce the surface tension across an air-water interphase by displacing water molecules from the interphase as well as by binding water with the charged phospholipid head groups. Upon lateral compression of a surface monolayer of DPPC, the surface tension can be reduced to near zero. The straight, saturated acyl chains of DPPC allow it to pack tightly upon compression. Monolayers of kinked, unsaturated phospholipids are unable to pack tightly and will collapse upon compression. Lipids cannot adsorb rapidly at the air-liquid interface. This is particularly true of disaturated lipids in the gel phase. The surfactant associated proteins are necessary for lipid adsorption. It is thought that compression of the adsorbed surfactant monolayer causes subsequent squeeze-out of unsaturated phospholipids thus enriching the monolayer in DPPC. This monolayer purification process is also facilitated by surfactant associated proteins.

PG is the second most abundant phospholipid in surfactant. The role of this lipid in surfactant is not clear. It is thought to interact with SP-B (Yu and Possmayer 1992, Baatz et al 1991) and has been shown to be required for the formation of tubular myelin *in vitro* (Suzuki et al 1989, Williams et al 1991).

1.8.2. SP-A

Surfactant-associated protein A is the best characterized of the surfactant proteins. It is a collagen-like glycoprotein which can form 18'mers structurally analogous to the complement component C1q (Voss et al 1988). SP-A contains a collagen-like region, a phospholipid binding domain, a carbohydrate binding domain and high and low affinity calcium binding sites.

Proposed functions of SP-A include the formation of tubular myelin (Suzuki et al 1989, Williams et al 1991), the enhancement of lipid adsorption (Chung et al 1989, Cockshutt et al 1990, Schürch et al 1992) and the ability to counteract surfactant inhibition by serum proteins (Cockshutt et al 1990, Venkitaraman et al 1990). These functions are all dependent on the presence of SP-B and calcium.

Tubular Myelin is enriched in SP-A (Hawgood 1989) and SP-A is also essential for the formation of tubular myelin *in vitro* (Suzuki et al 1989 and Williams et al 1991). Immunogold electron microscopy studies by Voorhout et al (Voorhout et al 1991) showed that SP-A is probably localized at the corners of the tubular myelin lattice. Kuroki et al showed that SP-A binds specifically to DPPC (Kuroki and Akino 1991). It is hypothesized that SP-A interacts with the lipids and/or SP-B in the corners of tubular myelin, and that self aggregation by carbohydrate binding site

stabilizes this structure (Haagsman et al 1991, Voorhout et al 1991).

Tubular myelin is proposed to adsorb rapidly to form a monolayer (Goerke and Clements 1985). The formation of tubular myelin could be responsible for the increased adsorption and for the counteraction of surfactant inhibition by blood proteins by addition of SP-A to lipid extracts of surfactant *in vitro* (Cockshutt et al 1990).

There are also several regulatory functions described for SP-A. SP-A is thought to be responsible for receptor-mediated reuptake of surfactant by the Type II cell (Wright et al 1987, Ryan et al 1989, Wright et al 1989, Kuroki et al 1988) Inhibition of surfactant secretion by SP-A has also been reported (Wright and Dobbs 1991, Dobbs et al 1987). These latter functions of SP-A are not dependent on the hydrophobic surfactant associated proteins. Both the inhibition of surfactant secretion and the increase in reuptake by SP-A have been demonstrated with Type II cells *in vitro* (Wright and Dobbs 1991) but have not yet been confirmed *in vivo*.

Soon after Voss et al showed a structural analogy between SP-A and the complement component C1q (Voss et al 1988), several different groups investigated the effects of SP-A in host defence mechanisms (Tenner et al 1989, Van Iwaarden et al 1990, 1991). These studies have demonstrated a possible role of SP-A in enhancing phagocytosis by

alveolar macrophages (Tenner et al 1989, Van Iwaarden et al 1990, 1991). Other studies have shown that alveolar macrophages contain a specific SP-A receptor (Manz-Keinke et al 1992, Pison et al 1992).

In view of the variety of functions described for SP-A it would be very interesting to establish whether these different functions can be attributed to different alveolar pools of SP-A and/or different oligomeric structures of SP-A.

1.8.3. SP-B

The observation that antibodies against SP-B can induce respiratory failure clearly demonstrates the importance of SP-B in surfactant's ability to stabilize the lung (Kobayashi et al 1991, Robertson et al 1991). Several different kinds of *in vitro* studies have demonstrated that SP-B can enhance adsorption (Yu and Possmayer 1990, Oosterlaken-Dijksterhuis et al 1991b) and monolayer purification (Yu and Possmayer 1992). Results from pulsating bubble surfactometer experiments by Yu and Possmayer showed that SP-B can promote squeeze-out of PG during compression of a DPPC-PG monolayer (Yu and Possmayer 1990, 1992). Fluorescence anisotropy measurements by Baatz et al also suggest a specific interaction of SP-B with PG (Baatz et al 1990).

As mentioned in the preceding section, SP-B together with SP-A, DPPC, PG and calcium can form tubular myelin *in vitro* (Suzuki et al 1989, Williams et al 1991). A role for SP-B in the reuptake of surfactant by the Type II cell has been reported (Rice et al 1989) but this potential role of SP-B needs further study.

1.8.4. SP-C

SP-C is clearly the least understood of the surfactant associated proteins. In addition to containing only 2 charged amino acids, SP-C is palmitoylated at two adjacent cysteine residues (Curstedt et al 1990). This extreme hydrophobicity makes SP-C difficult to purify, insoluble in aqueous solvents and make it a poor antigen for generating antibodies (Beers and Fisher 1992). Biophysical studies with purified SP-C suggest a role in the adsorption of lipid at the air liquid interface (Yu and Possmayer 1988, Oosterlaken-Dijksterhuis et al 1991b, Notter et al 1987). SP-C appears to be less effective in monolayer purification than SP-B (Yu and Possmayer 1988).

The precursor of SP-C appears to be a palmitoylated transmembrane protein (Keller et al 1991, Vorbroker et al 1992). The intracellular role of this SP-C precursor requires further investigation.

1.8.5. SP-D

As yet, there are no reports attributing a biophysical property to SP-D. In alveolar lavages obtained from rats most of the SP-D was found in the soluble fraction and not in the surfactant fraction (Kuroki et al 1991). A proposed function for SP-D is a role in mediating host defence mechanisms (Hawgood and Schiffer 1991).

1.9. Clinical significance of surfactant

The lung becomes functional during the latter stages of gestation. As a consequence, prematurely delivered infants often develop respiratory problems due to surfactant deficiency (Avery and Mead 1959). Surfactant supplementation of these surfactant deficient lungs has resulted in remarkable improvement in gas exchange (Jobe and Ikegami 1987, Enhorning et al 1985, Collaborative European Multicenter Study Group 1988). Different surfactant preparations have been used for these studies. Most of these preparations contain surfactant associated proteins SP-B and SP-C. However the immunological consequences of treating infants with a preparation containing these hydrophobic surfactant proteins appears negligible (Chida et al 1991, Whitsett et al 1991).

Surfactant abnormalities are also implicated in the development of the adult respiratory distress syndrome (ARDS) (Hallman et al 1982, Enhorning 1989, Seeger et al 1990). ARDS can be caused by a variety of insults such as, sepsis, shock, pneumonia, liquid aspiration, metabolic disorders and many others (Bersten and Sibbald 1984, Metz and Sibbald 1991). It appears that in most cases, the surfactant abnormalities in ARDS are secondary to a systemic reaction resulting from the insults described above. The surfactant abnormalities include an decrease in PG, an increase in SM and LPC and a decrease in SP-A and SP-B in the surfactant lipid fraction (Holm and Matalon 1990, Gregory et al 1991). Lung permeability also increases resulting in an increase in the amount of blood proteins in the alveolar space (Holm and Matalon 1990, Jobe 1989). The increase in blood protein can result in surfactant inhibition (Seeger et al 1990, Jobe 1989). The increase in LPC and the decrease in SP-A could further sensitize surfactant to blood protein inhibition (Cockshutt and Possmayer 1991). Surfactant inhibition can cause alveolar collapse or a further increase in blood protein leakage which can further inhibit surfactant (Holm and Matalon 1989). This vicious circle of surfactant inactivation is thought to be a major factor in the pathogenesis of ARDS (Seeger et al 1990, Holm and Matalon 1989, Jobe 1989). It has been suggested that surfactant supplementation could be

useful in the treatment of ARDS (Enhorning 1989, Gregory et al 1991). Studies on several animal models of ARDS show a marked improvement of gaseous exchange with surfactant treatment (Enhorning 1989, Robertson 1991). Some success with surfactant supplementation of patients with ARDS has been reported in two uncontrolled clinical trials (Richman et al 1989, Lachmann 1989). Many aspects of surfactant supplementation in ARDS require further study. The effects of different surfactant preparations, dose, mode of delivery and the timing of the supplementation all need to be investigated further. The multifactorial cause of ARDS might complicate these studies.

A large increase in surfactant is found in silicosis and in alveolar proteinosis (Hook 1991). In a sheep silicosis model, the phospholipid composition, SP-A and SP-B content and surface tension reducing activity of the lipid extracts were similar to nontreated animals (Bégin et al 1987, Lesur et al in press). Crouch et al found that SP-D was increased in a rat silicosis model (Crouch et al 1991b).

1.10. Thesis outline

The work described in this thesis consists of several distinct projects relating to surfactant metabolism. Chapter 2 and 3 describe investigations on the glycerol-3-phosphocholine (GPC) dependent pathway proposed by Infante

for acyl-specific phosphatidylcholine (PC) synthesis (Infante 1984). This pathway was investigated in lung and liver. The main conclusion from these studies is that the GPC pathway does not occur to any significant extent in liver and lung.

In chapter 4, alterations in surfactant occurring after experimental lung transplantation are reported. It is concluded that surfactant supplementation might be useful in lung transplantation.

The observation that the ratio of small to large surfactant aggregates is increased after transplantation led to the experiments described in chapters 5 and 6. The results in chapter 5 demonstrate the degradation of SP-B during the conversion of large to small surfactant aggregates *in vitro*. Aggregate conversion experiments with lipids plus surfactant proteins, lipid extract surfactant and bovine natural surfactant are reported in chapter 6. These experiments demonstrate that both SP-A and SP-B are necessary for the integrity of large surfactant aggregates.

In the last chapter, chapter 7, general conclusions are listed and some ideas for future directions are given.

CHAPTER 2 EXAMINATION OF THE POTENTIAL ROLE OF THE GLYCEROL-3-PHOSPHOCHOLINE (GPC) PATHWAY IN THE BIOSYNTHESIS OF PHOSPHATIDYLCHOLINE BY LIVER AND LUNG.

2.1. Summary

The potential involvement of the glycerol-3-phosphocholine (GPC) pathway for the synthesis of phosphatidylcholine (PC) has been examined in rat liver and lung and in a human cell line, the A549 cell which possesses characteristics representative of mature alveolar Type II epithelial cells. Although mitochondrial and microsomal fractions from the above sources readily incorporated radioactive glycerol-3-phosphate into lipids, the only incorporation observed with radioactive GPC was a small variable labelling with the mitochondrial and microsomal fractions from rat lung. Even with these fractions, no radioactivity from GPC was incorporated into PC or lyso-PC. Attempts to increase the incorporation of GPC into lipids by manipulating the incubation conditions were unsuccessful. It was concluded that the occurrence of the GPC pathway in liver and lung is unlikely.

2.2. Introduction

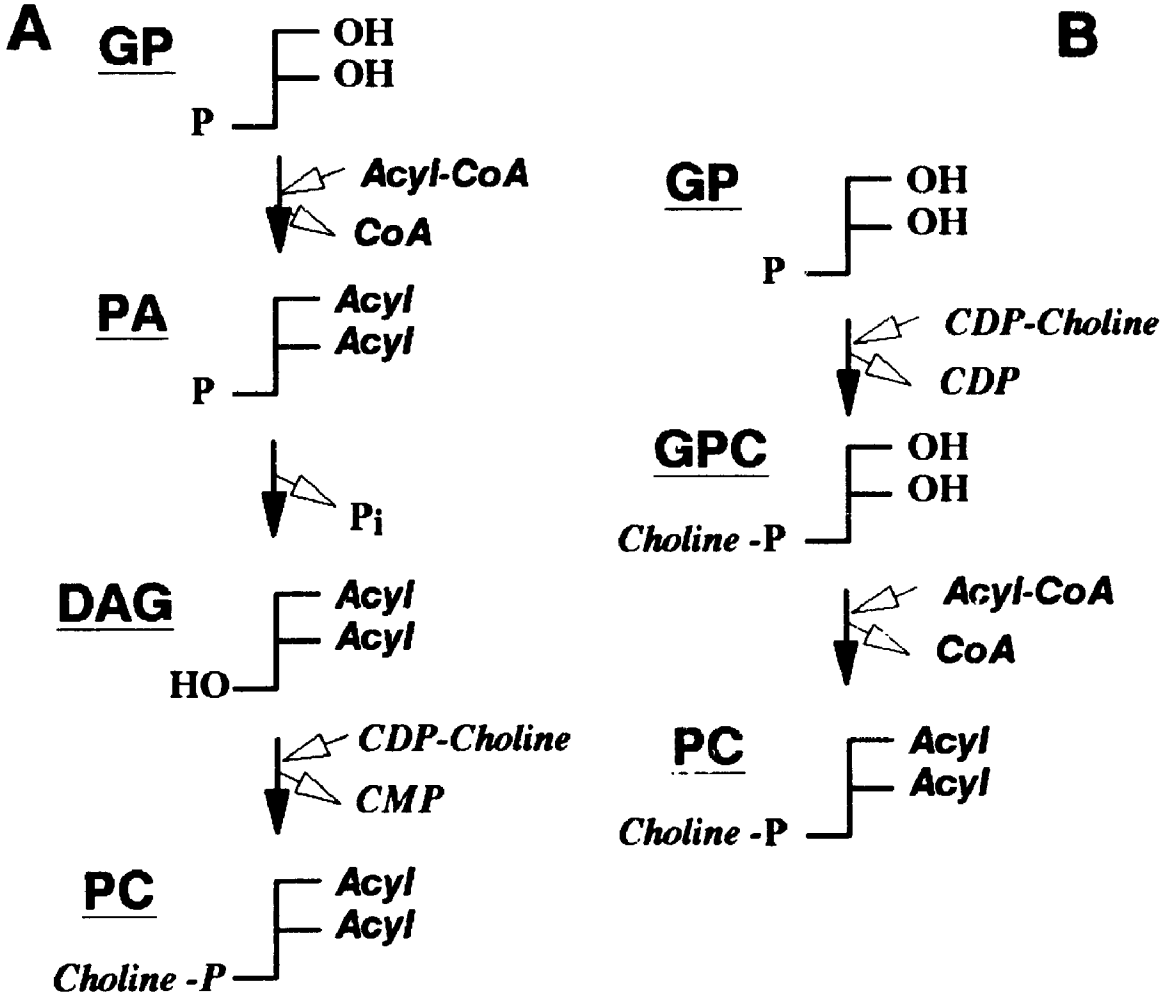
It is generally acknowledged that phosphatidylcholine (PC) is produced via the *de novo* pathway established by Kennedy and his associates during the 1950's and early 1960's (Kennedy 1962). The Kennedy pathway involves the acylation of either glycerol-3-phosphate (GP) or dihydroxyacetonephosphate (DHAP) to yield phosphatidic acid (PA), followed by the hydrolysis of PA to diacylglycerol (DG), and the subsequent reaction of DG with CDP-choline to yield PC (figure 2.1A). PC can also be formed through the methylation pathway which involves a sequential methylation of phosphatidylethanolamine (PE) to PC. The PE for this pathway also arises from DG produced via the Kennedy *de novo* pathway (Kennedy 1962) .

Recently, Infante has postulated a novel *de novo* pathway for PC synthesis (Infante 1984). According to this pathway, GP first reacts with CDP-choline to form glycerol-3-phosphocholine (GPC) plus CDP. The GPC is then acylated twice to produce PC (figure 2.1B). This pathway will be referred to as the GPC pathway. Infante has suggested that the GPC pathway is acyl-specific (Infante 1984). Furthermore, Infante has hypothesized that this GPC pathway makes a significant contribution to PC synthesis in muscle, liver, brain and lung (Infante 1985a, 1985b, 1986a, 1986b and 1987).

Figure 2.1 Schematic representation of the Kennedy and GPC pathway for *de novo* phosphatidylcholine synthesis.

A) Kennedy pathway

B) GPC dependent pathway


A
GP
OH
OH
P
Acyl-CoA
CoA
PA
Acyl
Acyl
P
Pi
DAG
Acyl
Acyl
HO
CDP-Choline
CMP
PC
Acyl
Acyl
Choline -P
B
GP
OH
OH
P
CDP-Choline
CDP
GPC
OH
OH
Choline -P
Acyl-CoA
CoA
PC
Acyl
Acyl
Choline -P

The experimental support for the GPC pathway consists mainly of measurements of GPC synthesis and the incorporation of radioactive GP into PC (Infante 1985a, 1985b, 1986a and 1987). The actual acylation of GPC has never been demonstrated.

The purpose of the present study was to investigate whether this GPC pathway indeed occurs, and, if so, in what percentage it contributes to the synthesis of PC in different tissues. Our experimental approach was to measure the incorporation of labelled GPC into PC by subcellular fractions of different tissues. The results were compared to those obtained with the same assay using labelled GP as a substrate instead of GPC.

The assays were conducted with subcellular fractions of rat liver and lung, two tissues which are known to produce considerable amounts of PC, and tissues in which Infante has suggested the GPC pathway to occur (Infante 1985a, 1986a and 1987). Finally, we examined the acylation of GPC in subcellular fractions of cells derived from A549 cells. A549 is a continuous cell line derived from a human pulmonary adenocarcinoma that has been shown to have many morphologic and biochemical features of the lung alveolar Type II cell including the presence of characteristic lamellar bodies, representing stored surfactant, which are enriched in

dipalmitoylphosphatidylcholine (DPPC) (Shapiro et al 1978, Nardone and Andrews 1979, Lieber et al 1976).

No evidence for the production of PC through the acylation of GPC was obtained in any of these preparations. It was concluded that the significance of a GPC-dependent pathway for the formation of PC in liver and lung is doubtful.

2.3. Materials

[^{3}H-choline]DPPC (76 Ci/mMol) was obtained from Amersham, [^{14}C]GP (144 mCi/mMol) and [^{3}H]GP (7.1 Ci/mMol) were purchased from New England Nuclear. Biochemicals were obtained from Sigma. Scintillation fluid was New England Nuclear 963. Cell culture media and materials were purchased from GIBCO. A549 cells were a gift from Dr. Jacob Finkelstein, Department of Pediatrics, University of Rochester.

2.4. Methods

2.4.1. Production of [^{3}H]GPC

[^{3}H-choline]DPPC was hydrolysed to [^{3}H-choline]GPC by the method described by Dittmer and Wells (Dittmer and Wells 1969). The radioactive DPPC (50 μCi) was dried under

nitrogen and dissolved in 1 mL chloroform/methanol (1:4). After adding 100 μL 1.2 N NaOH in methanol/H_2O (1:2), the DPPC was incubated at 37°C for 30 min. The solution was then neutralized with acetic acid and the GPC was extracted by adding 2 mL chloroform/methanol (9:1), 1 mL isobutanol and 2 mL H_2O. The lower aqueous phase containing the GPC was dried under nitrogen, then redissolved in water, and subsequently washed 2 times with chloroform. The recovery of radioactive GPC was always greater than 90%.

2.4.2. Preparation of subcellular fractions

Rat liver subcellular fractions were prepared by differential centrifugation (McMurray and Dawson 1968, Fleischer and Kervina 1974). In brief, after the tissue had been removed from the rat, it was homogenized in a 1mM Hepes-0.1mM EDTA (pH 7.0) buffer in 0.25M sucrose using a Dounce homogenizer. The suspension was then centrifuged at 480g for 10 min. The mitochondrial pellet was obtained by centrifuging the supernatant at 4,300g for 10 min., the pellet was washed with buffer and recentrifuged. The supernatant was centrifuged for 20 min. at 12,000g to remove lysosomes. The microsomal pellet was obtained by centrifugation for 60 min. at 100,000g.

The preparations of lung and A549 cell subcellular fractions were conducted as described previously (Harding et

al 1983). Lungs were perfused with saline before they were removed from the rats. After homogenization in the Hepes-EDTA buffer, the centrifugation steps in this method were 1,500g for 10 min. (crude nuclear pellet), 12,000g for 10 min. (mitochondrial pellet), 17,000g for 20 min. (to remove lysosomes) and 100,000g for 60 min. (microsomal pellet). The mitochondrial and microsomal pellets were resuspended in the Hepes-EDTA buffer and aliquots were either used fresh or frozen at -20°C. All steps in this procedure were carried out at 4°C.

2.4.3. Assay procedure for GP and GPC acylation

Unless otherwise indicated, the acylation of GP and GPC was measured at 37°C in 0.5 mL incubation medium containing 40 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES) (pH 7.5), 2 mM $MgCl_2$, 0.5 mg bovine serum albumin, 0.15 mM dithiothreitol (DTT) and either 0.2 mM palmitoyl-CoA (homogenates, microsomes) or 0.25 mM palmitoyl-carnitine with 0.15 mM CoA (mitochondria). The acylation substrate was either 2.5 mM GP containing 0.2 μCi [^{3}H] or [^{14}C]GP or 1 mM GPC containing 0.3 μCi [^{3}H]GPC. The protein concentration was in the range of 50-200 μg per assay.

At specific times the reaction was stopped by adding 0.75 mL n-butanol. After adding 1.5 mL H_2O, the mixture was

centrifuged at 250g for 15 min. and the butanol phase was removed and washed twice with 1.5 mL butanol-saturated H_2O. Radioactivity in the butanol phase was measured by liquid scintillation counting. Phospholipids were separated by thin layer chromatography (TLC) either on silica gel G plates impregnated with 0.5 N oxalate using chloroform:methanol:H_2O:HCl (87:10:0.1:0.1) or on Whatman LK5D plates using chloroform:ethanol:H_2O:triethylamine (30:34:8:35). With the latter system, the plates were run twice. After staining with iodine, appropriate areas were scraped into scintillation vials for counting.
All experiments were performed with at least three different preparations of subcellular fractions, typical results are shown. The results obtained from experiments with freshly isolated subcellular fractions did not differ significantly from the results obtained with frozen subcellular fractions.

2.4.4. Other methods

Protein was measured by the method of Lowry et al. (Lowry et al 1951) with 0.1% sodium dodecylsulphate using bovine serum albumin as the standard. A549 cells were grown to confluence in 75 cm^2 flasks in minimum essential medium with 10% fetal calf serum. Cells were harvested by trypsinization.

2.5. Results

The time course of the incorporation of radioactive GP and GPC into the lipids of the mitochondrial and microsomal fractions from rat liver was examined up to 30 min. at 37°C (figure 2.2). With the mitochondrial fraction there was a rapid incorporation of GP into the lipids followed by a gradual decline (figure 2.2A). The microsomal lipids were labelled more slowly (figure 2.2B). Examination of the distribution of the incorporated radioactivity with the oxalate-impregnated TLC plate system revealed PA as the major radioactive product (approx. 65%). The remaining radioactivity was associated with lyso-PA (25% mitochondria, 10% microsomes) and the neutral lipid fraction (10% mitochondria, 25% microsomes). In contrast to the rapid acylation observed with GP, incorporation of GPC was not detected at any time point (figure 2.2).

The results of a similar time course experiment using the subcellular fractions from rat lung are depicted in figure 2.3 As in liver, GP was rapidly incorporated into mitochondrial lipids and more slowly into the microsomes. The approximate distribution of the radioactivity in the subcellular fractions as determined on oxalate plates was: PA (70% mitochondria, 80% microsomes), lyso-PA (30% mitochondria, 10% microsomes), and neutral lipids (<1% mitochondria, 10% microsomes).

Figure 2.2 Time course for the incorporation of radioactive GP and GPC into lipids by liver subcellular fractions.

A) Mitochondrial fraction

B) Microsomal fraction

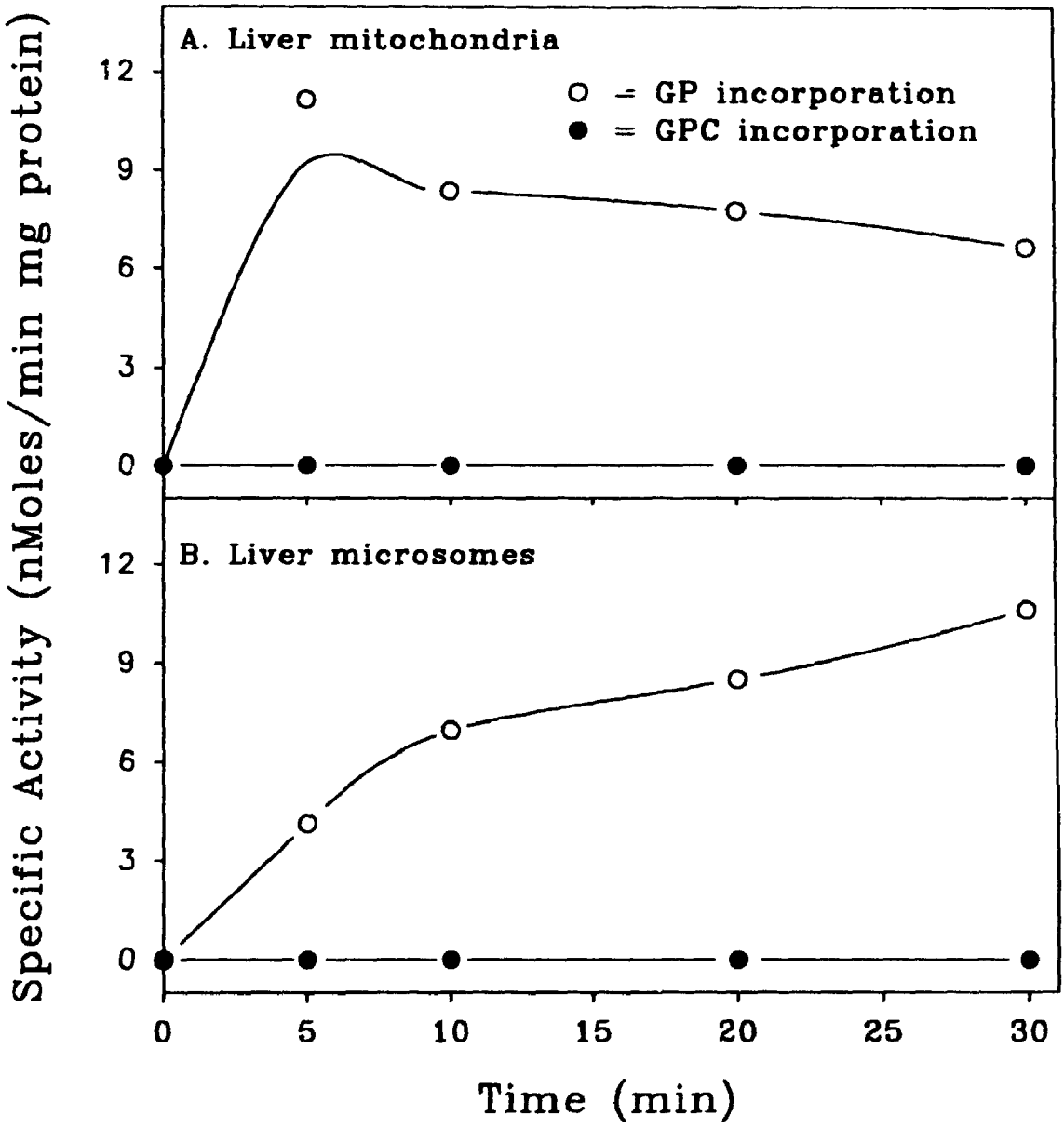
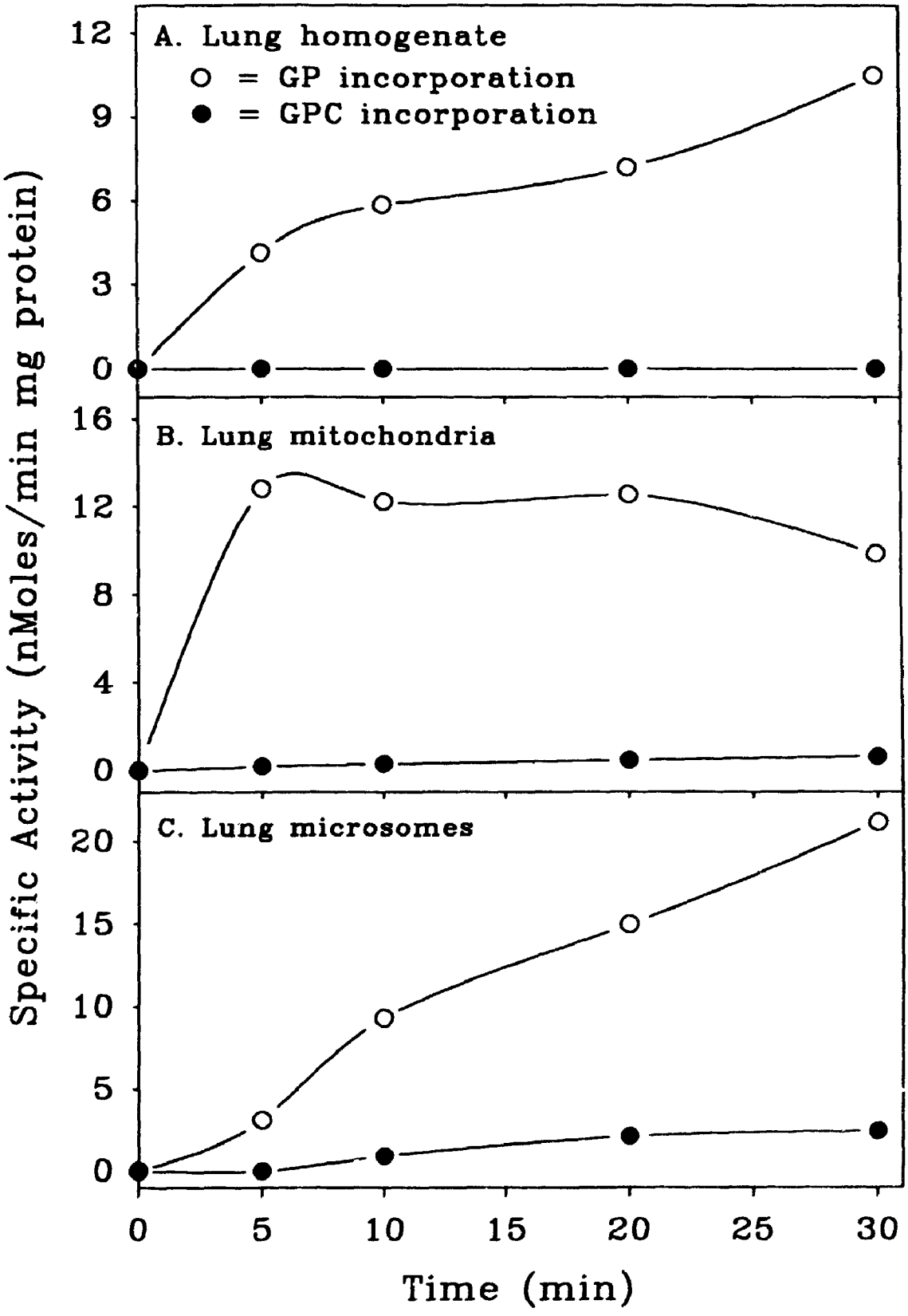

A. Liver mitochondria
○ = GP incorporation
● = GPC incorporation
B. Liver microsomes
Specific Activity (nMoles/min mg protein)
Time (min)
0
3
6
9
12
0
5
10
15
20
25
30

Figure 2.3 Time course for the incorporation of radioactive GP and GPC into lipids by lung homogenate and subcellular fractions.

A) Homogenate

B) Mitochondrial fraction

C) Microsomal fraction


A. Lung homogenate
○ = GP incorporation
● = GPC incorporation
B. Lung mitochondria
C. Lung microsomes
Specific Activity (nMoles/min mg protein)
Time (min)
0
3
6
9
12
0
4
8
12
16
0
5
10
15
20
0
5
10
15
20
25
30

No detectable incorporation of GPC was observed with the whole lung homogenate (figure 2.3A). However, themitochondrial and microsomal fractions appeared to show some incorporation of GPC into the butanol layer (figure 2.3B, 2.3C). The experiment was repeated several times but the incorporation was not very consistent and could often only be detected after incubation for 20 min. Addition of nonradioactive choline did not affect this incorporation.

In order to investigate whether this observed activity was due to the actual acylation of GPC, the radioactivity in PC and other lipids in the butanol phase after 0 and 30 min was estimated by TLC using the triethylamine-containing system. There was no significant difference in radioactivity in PC or lyso-PC between the 0 and 30 min time points (figure 2.4). The difference was mostly found near the origin and is presumably due to GPC or breakdown products. These results confirm that in lung mitochondria and microsomes the acylation of GPC is not detectable under the conditions used.

A number of different conditions was examined in order to determine whether the acylation of GPC could be encouraged through modification of the incubation system. Figure 2.5 shows that measuring GPC incorporation in the presence and absence of adenosinetriphosphate (ATP) (1mM), DTT and $MgCl_2$ in all possible combinations did not affect the acylation of GPC significantly. The same experiment was

Figure 2.4 Thin layer chromatography of the butanol phase from GPC acylation assays conducted with lung mitochondrial and microsomal fractions. The butanol phases from 5 samples were combined and chromatographed. The lanes were divided in 12 equal parts, scraped off into vials, and counted.

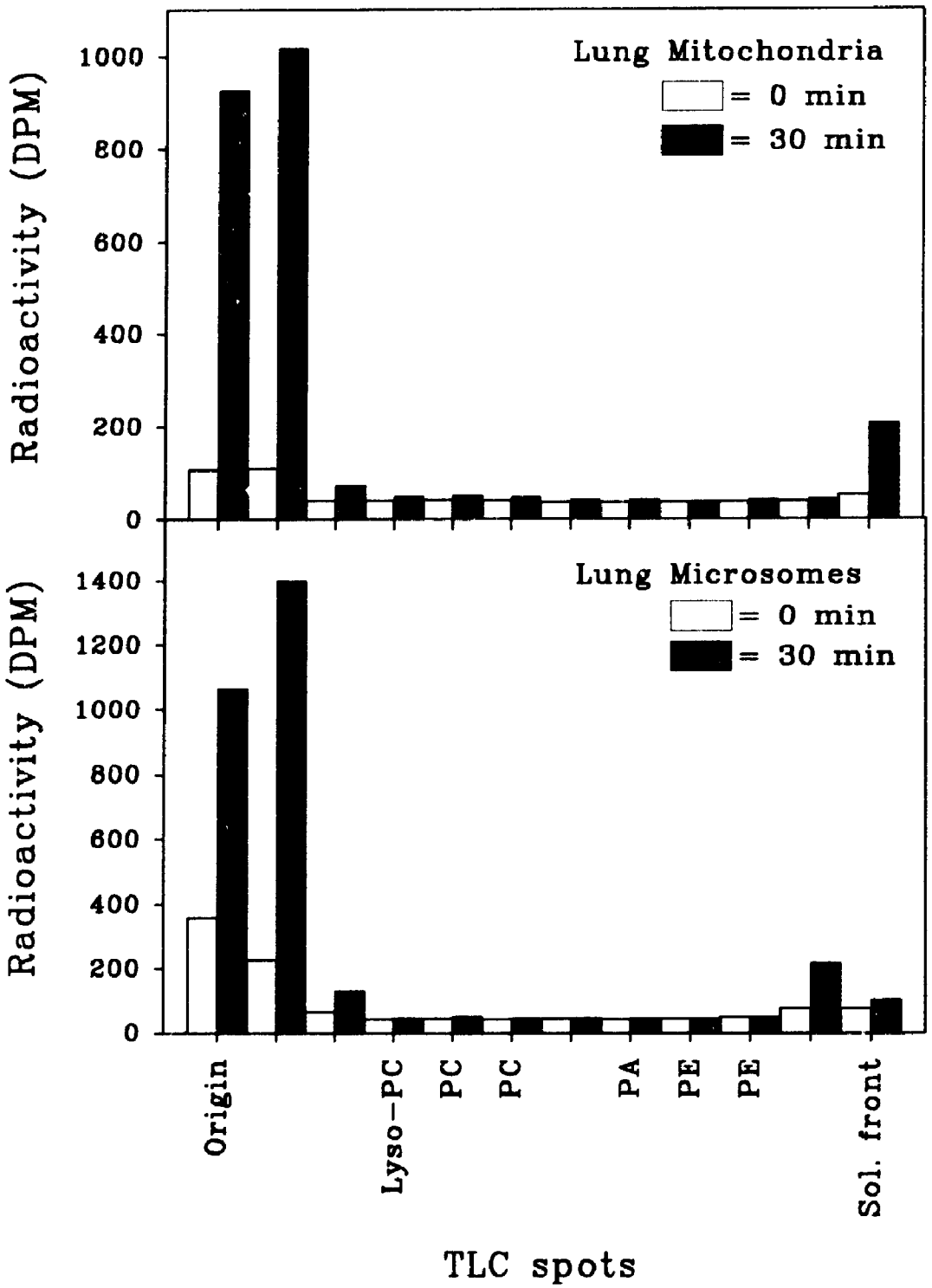
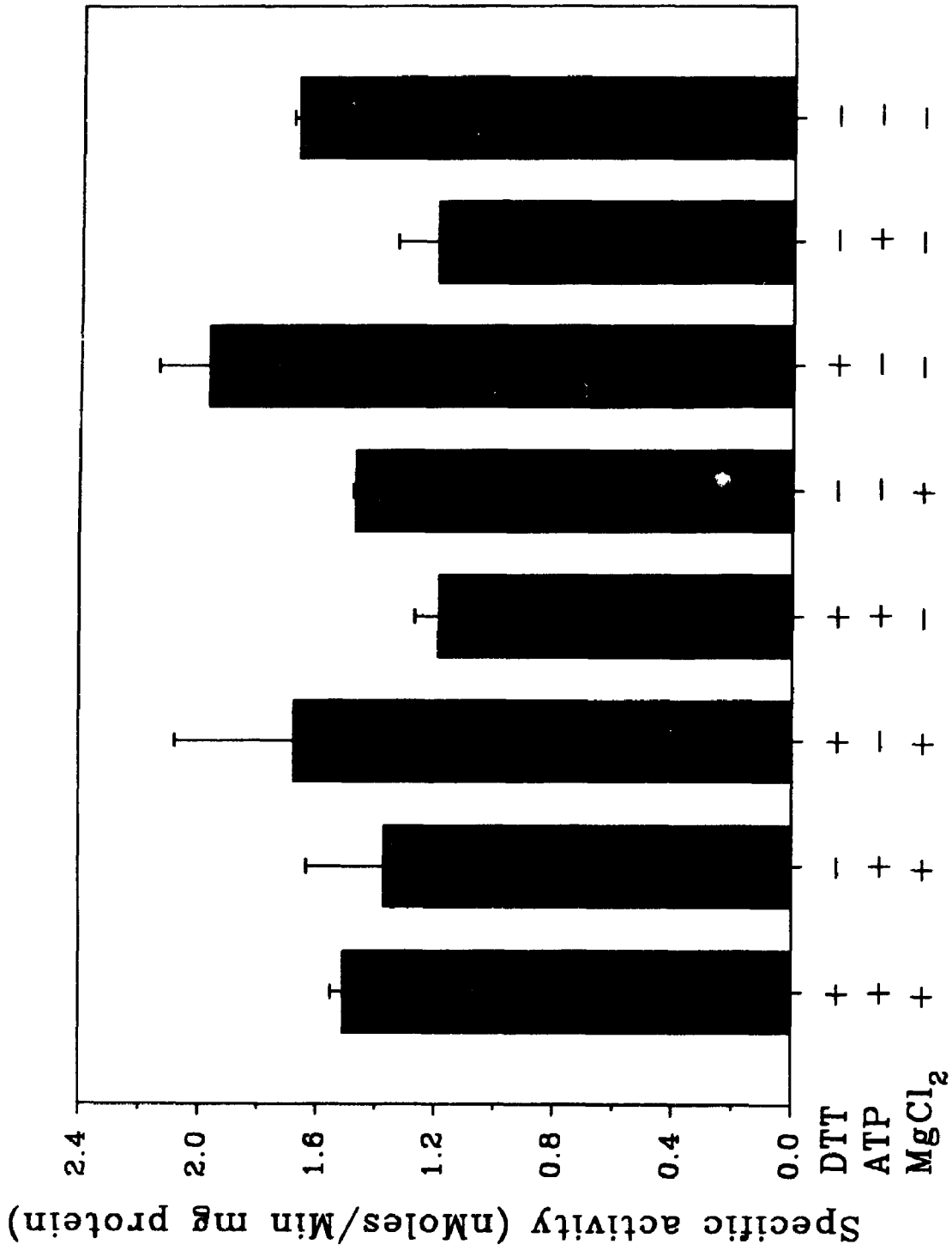

Lung Mitochondria
= 0 min
= 30 min
Radioactivity (DPM)
1000
800
600
400
200
0
Lung Microsomes
= 0 min
= 30 min
Radioactivity (DPM)
1400
1200
1000
800
600
400
200
0
Origin
Lyso-PC
PC
PC
PA
PE
PE
Sol. front
TLC spots

Figure 2.5 The effects of various modifications of the incubation mixture on the incorporation of labelled GPC into the butanol phase after 30 min. Condition 3 represents the standard incubation condition. The ATP concentration, where indicated, was 1.0 mM.


Specific activity (nMoles/Min mg protein)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
DTT
ATP
MgCl2
+ + +
- + +
+ - +
+ + -
+ - -
- + -
- - +
- - -

performed using microsomes instead of mitochondria with similar overall results (not shown).

Finally, we examined the acylation of GPC using subcellular fractions from A549 cells. The result of a time course experiment (figure 2.6) shows that GPC acylation was not detectable in either the mitochondrial or microsomal fractions. The GP acylation in these fractions was similar to that observed in lung and liver, indicating that these cells have the potential to produce a large amount of lipid.

2.6. Discussion

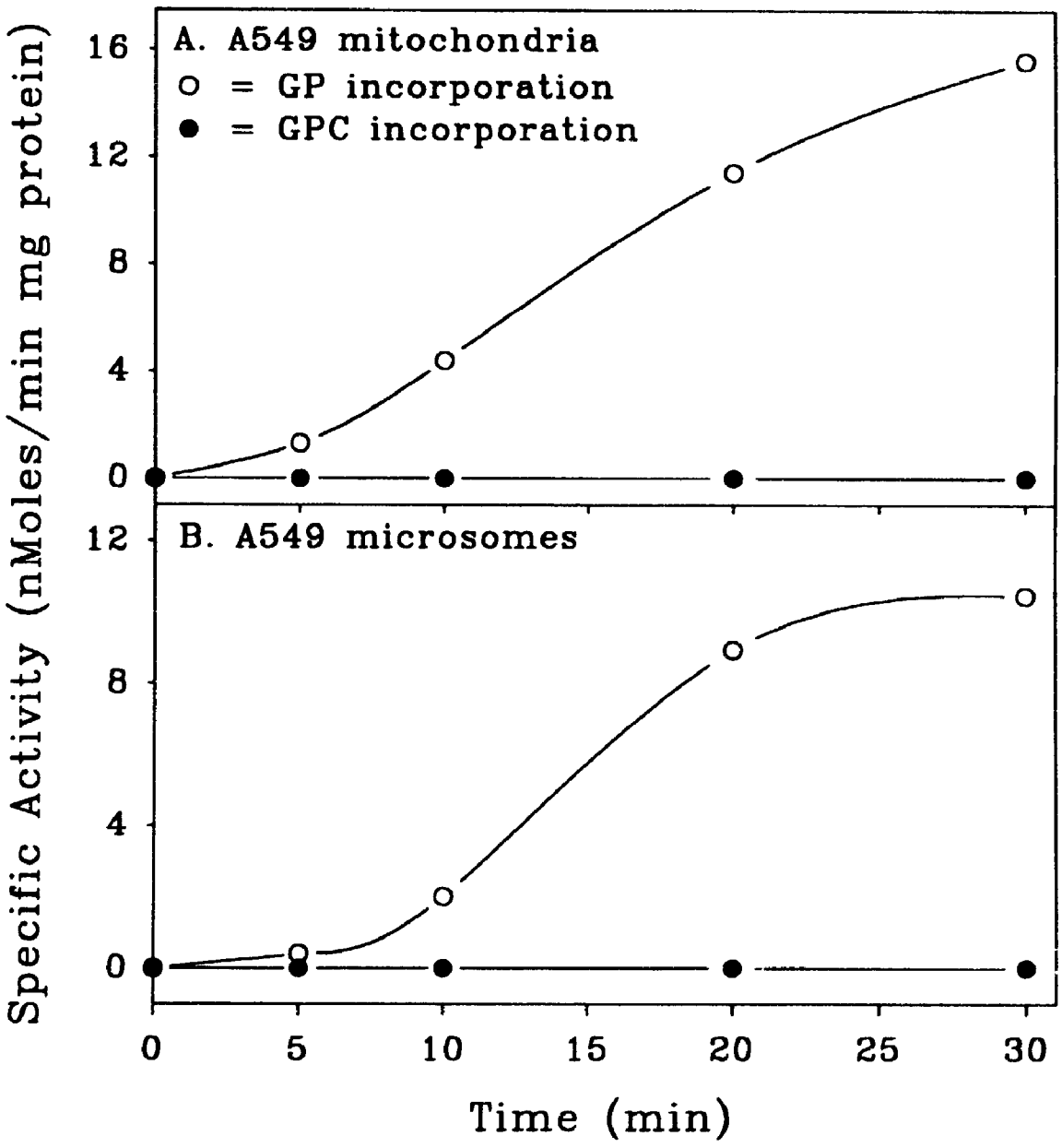
It has been postulated that PC synthesis may occur via a pathway involving the acylation of GPC (Infante 1984). Experimental evidence for this GPC pathway consists only of measurements of GPC synthesis (Infante 1985a, 1985b, 1986a). We have attempted to obtain more direct evidence for the existence of this pathway by measuring GPC acylation. The GPC pathway has been suggested to occur in liver and GPC synthesis has been measured in that tissue (Infante 1984). In our experiments, however, GPC acylation was not detectable in any of the liver fractions indicating little or no PC synthesis by the GPC pathway in this tissue.

Pulmonary surfactant is synthesized by Type II cells (Post and Van Golde 1988). The high percentage of DPPC in

Figure 2.6 Time course for the incorporation of radioactive GP and GPC into lipids by subcellular fractions from A549 cells.

A) Mitochondrial fraction

B) Microsomal fraction


A. A549 mitochondria
○ = GP incorporation
● = GPC incorporation
B. A549 microsomes
Specific Activity (nMoles/min mg protein)
Time (min)
0
4
8
12
16
0
5
10
15
20
25
30

pulmonary surfactant led to the suggestion that the acyl-specific GPC pathway is important for DPPC productionin Type II cells (Infante 1986a, Infante and Huszagh 1987). The synthesis of GPC has been detected in the lung (Infante 1986a). However, in our measurements of GPC acylation in lung we found no detectable production of PC or lyso-PC from radioactive GPC in any of the lung fractions. The small amounts of radioactivity incorporated into lipid extracts after prolonged incubation appear to be due to contamination by unreacted GPC or breakdown products.

Since the acylation of GPC may proceed maximally under different conditions than GP acylation, we attempted to optimize the incubation conditions with respect to GPC acylation using lung fractions. The presence or absence of $MgCl_2$, DTT or ATP did not significantly change the incorporation of GPC. The use of freshly isolated subcellular fractions resulted in similar values for GP and GPC incorporation as was observed with the frozen aliquots. The possibility that this reaction may be demonstrable under other conditions must still be considered. However it is not evident under conditions where GP is acylated rapidly.

To test for the potential involvement of the GPC pathway in surfactant synthesis, we attempted to measure GPC acylation in A549 subcellular fractions. The A549 cell line is a tumour cell line derived from a human lung benign tumour. Some reports have questioned whether these cells are

useful for Type II cell studies (Rooney et al 1977, Mason and Williams 1980). Several other reports, however, have shown that the A549 cell-line has Type II cell properties, including morphologic similarities such as the presence of characteristic lamellar bodies, analogous phospholipid composition and rates of lipid precursor incorporation, and the presence of the surfactant-associated proteins (Shapiro et al 1978, Nardone and Andrews 1979, Lieber et al 1976, Balis et al 1984, Smith 1977). In preliminary experiments we observed the specific activity of the acylation of GP by the microsomal fractions from A549 cells was high compared to microsomes from whole lung but have more routinely observed incorporation similar to those depicted in figure 2.3. The reason for the variability is not understood. Van Golde and coworkers (Van Golde et al 1985) have observed that sonicates of freshly isolated Type II cells were more active in acylating GP than lung homogenates. In the studies reported herein GPC acylation was never detected suggesting that little or no synthesis of PC in A549 cells occurs by the GPC pathway.

In the investigations depicted in figures 2.2 to 2.6, the whole homogenates and microsomal fractions were incubated with palmitoyl-CoA, while the mitochondrial fractions were incubated with palmitoyl-carnitine plus CoASH as suggested by Daae and Bremer (Daae and Bremer 1970). In other studies in which the nature of the acyl donors was

reversed, a considerable incorporation of radioactive GP into lipids was also observed. However, the incorporation of radioactive GPC was similar to that reported in figures 2.2 to 2.6.

Incubation of HeLa cells with [^{3}H-choline]GPC leads to an uptake of GPC by the cells [Vance DE, personal communication]. At early time points, radioactivity is observed in GPC, choline and phosphocholine: only at later time points does radioactivity accumulate in PC. These findings suggest that the Kennedy pathway is responsible for PC synthesis in HeLa cells.

Since we could not find any evidence for GPC acylation in liver, lung or A549 cells, we conclude that the existence of the GPC pathway in these tissues is highly doubtful. Infante has suggested that the GPC pathway is involved in muscular dystrophy (Infante 1985a, 1985b, 1985c and 1986b). We have not examined muscle but note that the evidence presented for the GPC pathway in muscle is the same as for liver and lung. It has recently been suggested that glycerol-3-phosphoserine can be acylated to form phosphatidylserine in rat liver microsomes (Baranska 1988) but, as in the case of the GPC pathway, no direct evidence was presented for the acylation of glycerol-3-phosphoserine.

The experimental evidence for the GPC pathway consists of the labelling of GPC from GP. The proposed mechanism involves the reaction of GP plus CDP-choline to produce GPC

and CDP. This reaction seems very interesting because it would be the first known reaction in which CDP acts as a donor of the choline moiety; in reactions of the Kennedy pathway CMP is the donor of a phosphocholine moiety. However, in a report by Morash et al. it was demonstrated that PC degradation occurs via GPC in five cell types (Morash et al 1988). They concluded that the major part of the GPC pool in these cells came from degradation of PC rather than *de novo* synthesis.

CHAPTER 3 RAT LIVER AND LUNG MITOCHONDRIA DO NOT INCORPORATE RADIOACTIVITY FROM GLYCEROL-3-PHOSPHATE OR CDP-CHOLINE INTO GLYCEROL-3-PHOSPHOCHOLINE.

3.1. Summary

The proposed formation of glycerol-3-phosphocholine (GPC) from glycerol-3-phosphate (GP) and CDP-choline catalysed by the enzyme GPC-synthetase has been examined in liver and lung subcellular fractions. Previous observations on the incorporation of radioactive GP into the GPC-spot on paper chromatograms have been interpreted as evidence for the GPC-dependent synthesis of phosphatidylcholine. Although we could reproduce this incorporation of GP, we could not detect any incorporation of radioactive CDP-choline into the GPC-spot using the same paper chromatographic method. TLC separation of the substrate and products showed no detectable formation of GPC with either radioactive substrate. These results strongly suggest that the previously reported formation of GPC in liver and lung was due to an inaccurate identification of the true radioactive products. We demonstrate that the major radioactive products formed in liver mitochondria are glucose and glycerol. Lung mitochondria incorporate radioactive GP into glycerol and into another unidentified compound or compounds. It is

concluded that the occurrence of the GPC dependent formation of PC is unlikely.

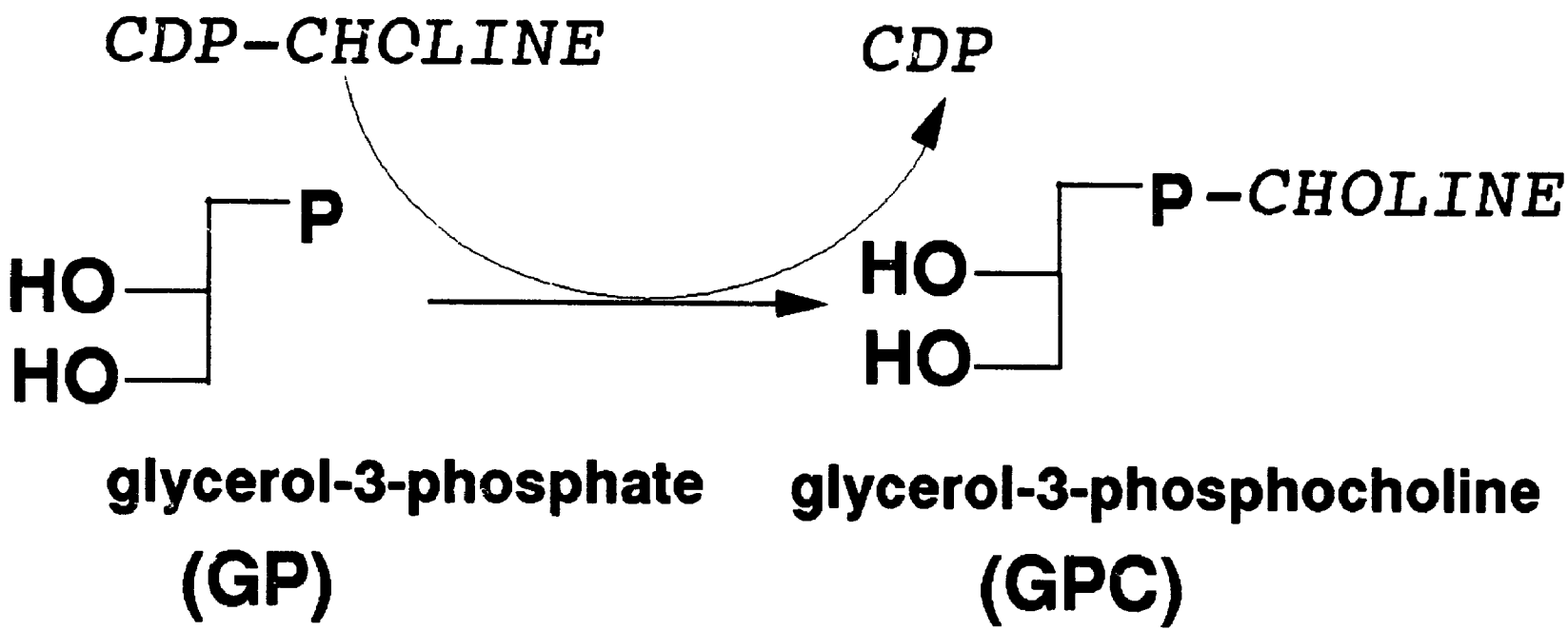
3.2. Introduction

It is well recognized that the phospholipid acyl-patterns vary in different tissues (Infante 1984, Holub and Kuksis 1978). Liver, for example, contains high proportions of 1-stearoyl, 2-arachidonyl phosphatidylcholine and 1-stearoyl, 2-docosahexanoyl phosphatidylcholine, while lung contains a considerable amount of dipalmitoylphosphatidylcholine (DPPC) a molecular species not found in high proportions in other tissues (Post and Van Golde 1988, Possmayer 1989). With both liver and lung, the *de novo* "Kennedy" pathway incorporates mainly palmitate at the *sn*-1 position and large amounts of oleate or linoleate at the *sn*-2 position (Holub and Kuksis 1978, Possmayer 1982, Batenburg et al 1986, Breejen et al 1989). Consequently, the *de novo* pathway can only account in part for the acyl-specific species of phosphatidylcholine (PC). Several remodelling pathways have been proposed and investigated in a number of tissues. Nevertheless, our understanding of all of the mechanisms involved and the manner in which they interact is clearly incomplete. As a result we were interested to learn of the novel hypothesis of Infante (Infante 1984, 1985a, 1986a, 1987, Infante and Huszagh 1987)

for a new *de novo* pathway for acyl-specific synthesis of phospholipids. According to this pathway, PC formation proceeds in two steps. Glycerol-3-phosphate (GP) and CDP-choline react to produce glycerol-3-phosphocholine (GPC) which is followed by the acylation of GPC to form specific species of PC. This "GPC" pathway is postulated to occur in a variety of tissues including liver and lung. Evidence for this pathway consists primarily of measurements of the incorporation of radioactive labelled GP and CDP-choline into GPC. This proposed reaction (figure 3.1) constitutes a unique biosynthetic reaction involving CDP as the "base-donating" group, whereas in all previously described reactions involving cytidine nucleotides, CMP acts as "donor" of a phosphorylated base.

We have examined this unique reaction for the formation of GPC using Infante's previously published paper chromatographic method for measuring the synthesis of radioactive GPC (Infante 1985a). Inconsistencies in our data led to a more vigorous identification of the products formed by this postulated reaction. Our results suggest that the previously published measurements of the production of radioactive GPC may be based on an inaccurate identification of the actual radioactive products. The results are consistent with the view that the true products of the "GPC-synthetase" assay in liver are glucose and glycerol. Glycerol is also produced in lung but another unidentified

Figure 3.1 The proposed reaction for GPC synthesis from GP and CDP-choline.


CDP-CHOLINE
CDP
P
HO
HO
P-CHOLINE
HO
HO
glycerol-3-phosphate
(GP)
glycerol-3-phosphocholine
(GPC)

product is also formed. These observations, taken together with the previous inability to demonstrate the acylation of GPC in liver and lung (Veldhuizen et al 1989, chapter 2) suggest that the GPC dependent pathway postulated by Infante does not function to a significant extent in these two tissues.

3.3. Materials

Radioactive compounds were obtained from New England Nuclear with exception of [^{14}C]-glucose which was purchased from Amersham. Biochemicals and enzymes were purchased from Sigma. Scintillation fluids were obtained from Beckman. Adsorbosil soft layer thin layer chromatography plates were purchased from Mandel Scientific Company. Other reagents were of the highest grade available and were obtained from Canlab or Fisher.

3.4. Methods

3.4.1. Preparation of subcellular fractions

Rat liver subcellular fractions were prepared by differential centrifugation essentially as previously described (McMurray and Dawson 1968, Fleisher and Kervina 1974). The liver was removed from the rat and homogenized in

a 1mM HEPES/0.1mM EDTA (pH 7.0) buffer in 0.25 M sucrose using a glass homogenizer with a teflon pestle. The suspension was centrifuged at 480g for 10 min to obtain a crude nuclear pellet. The supernatant was centrifuged at 4,300g for 10 min to obtain a crude mitochondrial pellet. The mitochondria were dispersed with the sucrose-EDTA buffer and recentrifuged as above to obtain the mitochondrial pellet. Lysosomes were removed from the initial mitochondrial supernatant by centrifugation at 12,000g for 20 min. The resulting supernatant was centrifuged for 60 min at 100,000g to obtain the microsomal fraction.

The lung subcellular fractions were prepared as described previously (Harding et al 1983). Lungs were perfused with saline before being removed from the rats. After homogenization in the sucrose-EDTA buffer, the suspension was centrifuged for 10 min at 1,500g (crude nuclear pellet), followed by 10 min at 12,000g (mitochondrial pellet), 20 min at 17,000g (lysosomal pellet) and 60 min at 100,000g (microsomal pellet) respectively. The mitochondrial pellet was washed once with the sucrose-EDTA buffer.

The mitochondrial and microsomal pellets from both tissues were resuspended in the sucrose-EDTA buffer and aliquots were either stored at -70°C or used fresh. All steps in this procedure were carried out at 4°C.

3.4.2. GPC-synthetase assay

The GPC synthase reaction was performed as described by Infante (Infante 1985a). The incubation mixture contains; 80 mM HEPES (pH 7.0), 2.3 mM $MgCl_2$, 5.0 mM UTP, 0.54 mM GP, 1mM CDP-choline, containing either 0.2 μCi [^{14}C]-GP or 0.2 μCi CDP-[^{14}C]choline. An aliquot of a subcellular fraction suspension containing 0.25-1.5 mg protein was added to start the reaction. The final volume was 0.375 ml and the mixture was incubated for 30 min at 37°C. In initial studies the reaction was stopped with 7% perchloric acid, the mixture was centrifuged (5 min at 600g) and the supernatant was used for analytical determinations of the radioactive product (Infante 1985a). As explained further in the results, it was necessary to modify this assay in order to identify the radioactive products enzymatically. Therefore, the reaction was stopped by heating the mixture (5 min at 95°C) and 300 μL of the supernatant were passed through a 2 mL Biorad AG1-X9 anion exchange column in the formate form (Dittmer and Wells 1969) to remove the unreacted GP. The radioactive products were eluted with H_2O.

3.4.3. Separation methods for the GPC synthetase assay

The radioactive substrates and products were separated by a paper chromatographic system described previously by

Infante for the identification of GPC (Infante 1985a). An aliquot of the supernatant was spotted on Whatman 3MM paper and developed in 1.0 M ammonium acetate in 67% ethanol. The Rf values with this system are; 0.24, 0.68, 0.74, 0.68 and 0.40 for GP, GPC, glycerol, glucose and gluconic acid respectively. This system was also used for incubations with CDP-[^{14}C]choline. Rf values for the choline metabolites are; CDP-choline 0.35, phosphocholine 0.75, choline 0.32 and GPC 0.68. Radioactive standards were used to identify the mobilities of the compounds. The spots co-migrating with the radioactive GPC standard were cut out of the paper and counted by liquid scintillation.

In addition, a TLC method was modified to separate the products (Morash et al 1988). For the [^{14}C]GP incubations, Adsorbosil soft layer plates were developed twice with methanol:H_2O:NH_4OH (70:70:7) as the solvent system. The Rf values are: GP 0.78, GPC 0.6, glycerol 0.95, glucose 0.90. The same plates were used for the CDP-[^{14}C]choline incubations. In this case the solvent system was the same as above for the first run, but the plates were developed a second time in methanol:0.6% NaCl:NH_4OH (70:70:7). The Rf values are CDP-choline 0.8, phosphocholine 0.54, choline 0.20 and GPC 0.64. The choline containing products were visualized by spraying with 1% phosphomolybdic acid in ethanol:chloroform (1:1) followed by 1% stannous chloride in

3 M HCl. The GPC spots were scraped off the plates and counted by liquid scintillation counting.

3.4.4. Identification of glucose and glycerol

The flowthrough from the anion exchange column (as described above) was dried under N_2 and redissolved in 500 μL H_2O. For identification of glucose, a 100 μL aliquot was incubated in 200 μl 0.1 M sodium acetate buffer with and without the enzyme glucose oxidase at 30°C for 1 hour. Glucose oxidase reacted and unreacted samples were taken to dryness under nitrogen, redissolved in 25 μL H_2O and analyzed using the paper chromatographic system.

For identification of glycerol, 100 μL aliquots were reacted with glycerol kinase in 200 μl 0.2 M glycine buffer (pH 9.8) containing 2 mM $MgCl_2$ and 1 mM ATP at 37°C for 1 hour. The samples were dried, redissolved and analyzed by paper chromatography.

Preliminary studies demonstrated that radioactive glucose, glycerol and GPC, added to the GPC synthetase incubation system, were almost quantitatively (>90%) eluted from the column. However, only 60% of the eluted counts were recovered after the paper chromatographic steps, the counting of radioactivity on the chromatography paper was found to be ~ 80-85% effective, the other losses were possibly due to solubilization and spotting. Since similar

losses were observed with all three radioactive standards, with both the glucose oxidase and glycerol kinase incubations, this was not investigated further.

3.4.5. Other procedures

Protein concentration was determined by the method of Lowry et al with 0.1% sodium dodecyl sulphate using bovine serum albumin as the standard (Lowry et al 1951). Radioactive GPC was synthesized as previously described (Dittmer and Wells 1969, Veldhuizen et al 1989).

All experiments were performed with at least three different preparations of subcellular fractions, typical results are shown.

3.5. Results

Since the proposed reaction for GPC synthesis utilizes both GP and CDP-choline, we examined the incorporation of both substrates into GPC. The results of a GPC synthetase assay with these substrates using different subcellular fractions of liver and lung is shown in Table 3.1. Both the previously described paper chromatographic method of Infante (Infante 1985a) and the TLC system described in the methods were used for the separation of substrates and products. When [^{14}C]GP was used as the radioactive substrate,

Table 3.1. Incorporation of radioactive glycerol-3-phosphate (GP) or CDP-Choline into the glycerol-3-phosphocholine (GPC) spot as isolated by paper chromatography or TLC.

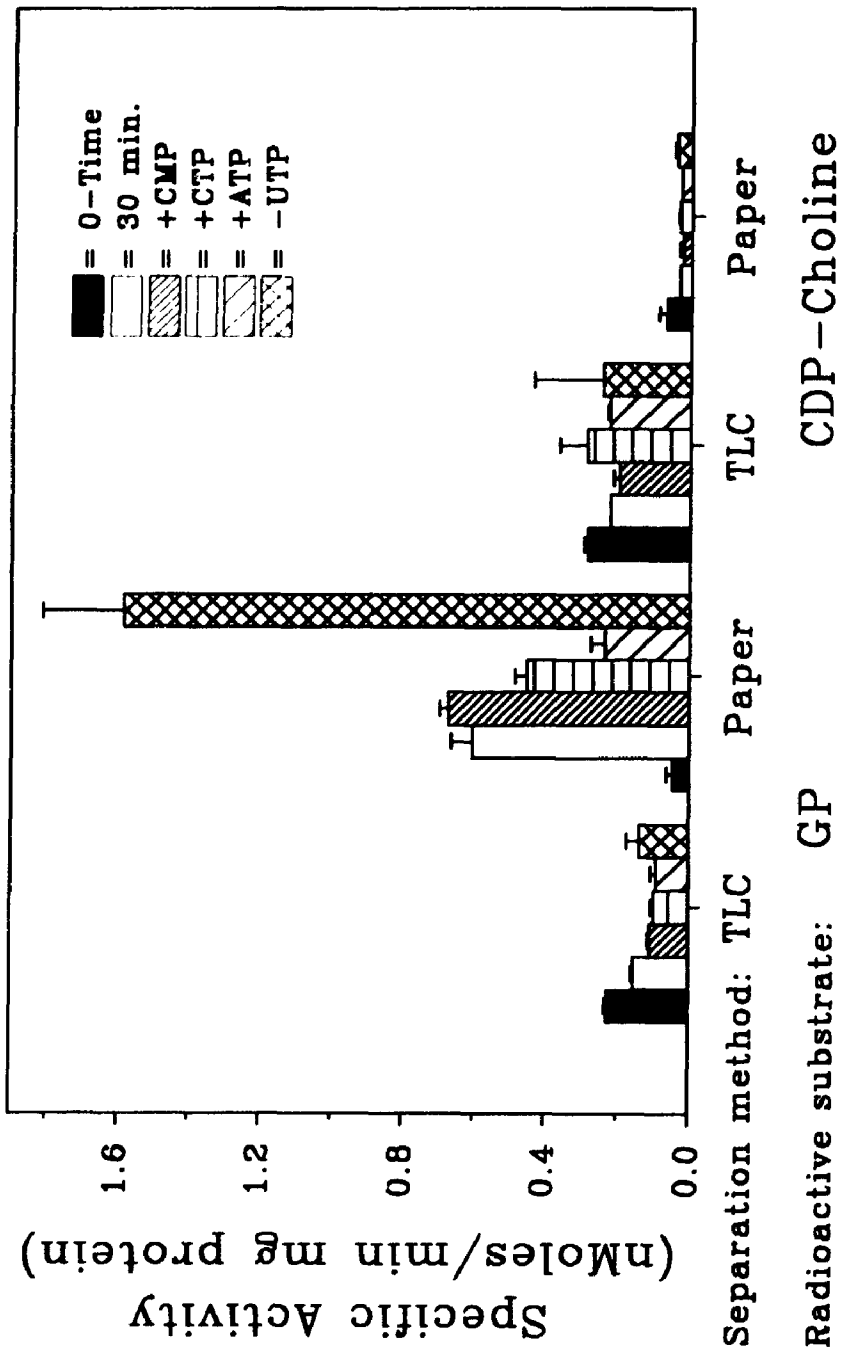
	Specific activity (nMoles/min mg protein)			
Separation Method	Paper		TLC	
Radioactive substrate:	GP	CDP-Choline	GP	CDP-Choline
A. Liver				
Microsomes	0.02	<0.001	<0.001	<0.001
Mitochondria	0.59	<0.001	<0.001	<0.001
B. Lung				
Homogenate	0.22	<0.001	0.001	<0.001
Microsomes	0.07	<0.001	<0.001	<0.001
Mitochondria	0.40	<0.001	<0.001	<0.001

radioactivity co-migrating with GPC was detected with the paper chromatographic system but not with the TLC separation method. When CDP-[^{14}C]choline was used as a substrate, no detectable radioactivity was associated with the GPC-spot with either system. With both liver and lung the highest activity was found in the mitochondrial fraction.

The standard incubation system described by Infante contains 5.0 mM UTP. The effect of addition of several other phosphonucleotides to the incubation system is shown in figure 3.2 for liver mitochondria. In agreement with Table 3.1, incorporation greater than with the non-incubated controls was only found with radioactive GP and the paper chromatographic system. This particular activity is decreased in the presence of ATP and increased in the absence of the UTP normally present in the incubation mixture. When [^{14}C]CDP-choline was used as a substrate, radioactive incorporation above the non-incubated controls was not observed with any of the phosphonucleotides. When this experiment was conducted with lung mitochondria, similar results were obtained (data not shown).

The experiments described above indicated that the radioactivity co-migrating with GPC on paper is not due to GPC but to another product or products derived from [^{14}C]GP. We attempted to identify this other radioactive product (or products). Consideration of the nature of the radioactive substrate and the mobility of the various compounds on the

Figure 3.2 The effects of various phosphonucleotides on the GPC synthetase reaction with liver mitochondria. Radioactive GP or radioactive CDP-choline were used as substrates. Paper chromatography and TLC were used for separation of substrates and products.


Specific Activity
(nMoles/min mg protein)
1.6
1.2
0.8
0.4
0.0
= 0-Time
= 30 min.
= +CMP
= +CTP
= +ATP
= -UTP
Separation method: TLC Paper TLC Paper
Radioactive substrate: GP CDP-Choline

paper chromatographic systems used by Infante led to the conclusion that glucose and glycerol were potential products. Glucose co-migrates with GPC in the paper chromatographic system. Glycerol, which can be produced by phosphatase activity, possesses a slightly higher Rf value but some overlap occurs. Both glucose and glycerol are separated by the TLC system. The next step was to identify or eliminate these potential products through specific enzymatic procedures. Since the perchloric acid used by Infante (Infante 1985a) to terminate the reaction could be inhibitory, incubations were stopped by heat treatment (see methods). In addition, preliminary experiments revealed that the radioactive GP used as a substrate interfered with detection of the products of the enzymatic reaction. Therefore the supernatants of the heat inactivated GPC synthetase reactions were applied to anion exchange columns and eluted with water. While the radioactive GP was virtually quantitatively removed by this procedure, the radioactive product(s) co-migrating with GPC were recovered. In addition, control experiments demonstrated that radioactive GPC is also quantitatively recovered in the column eluents.

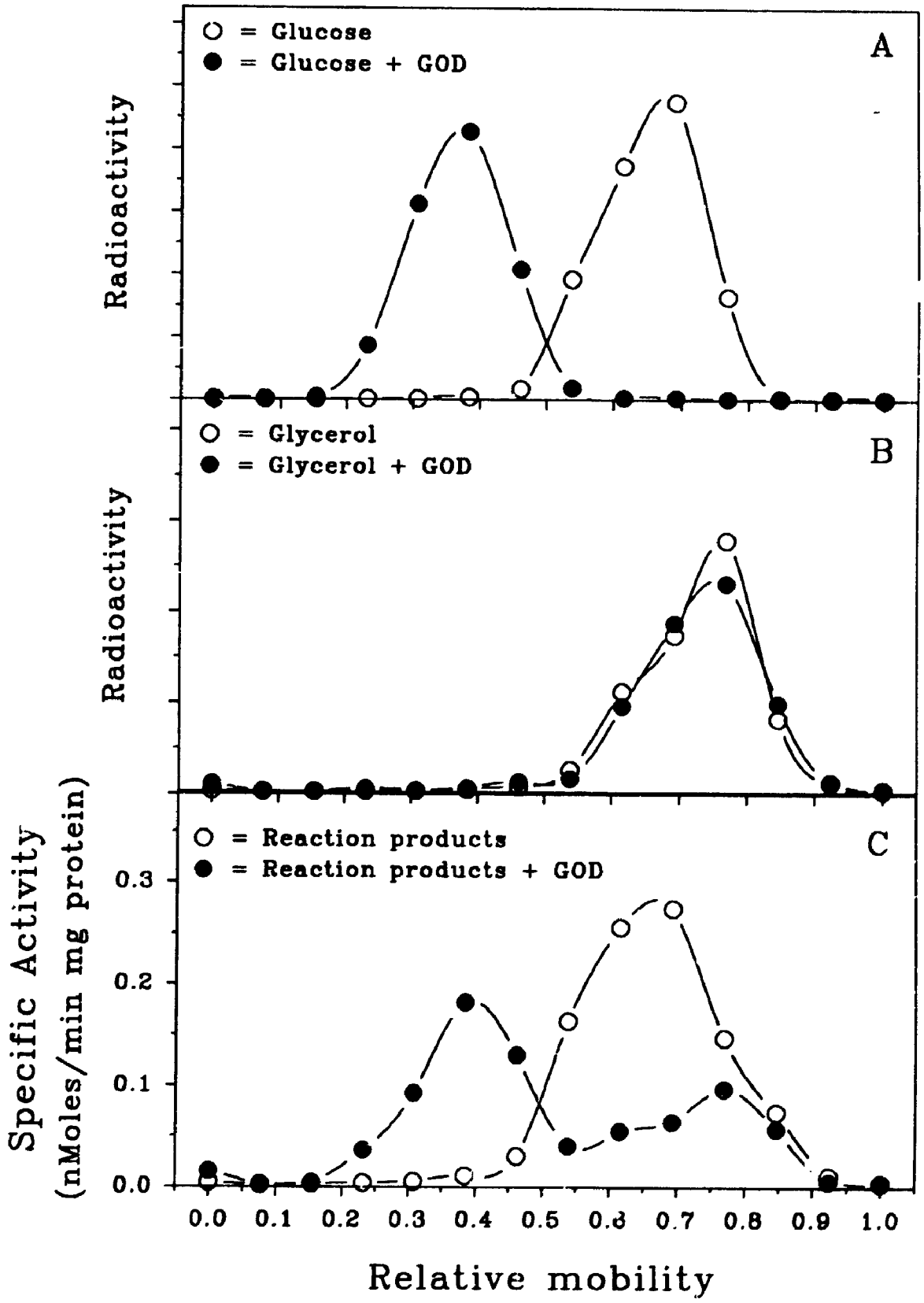
Figure 3.3 illustrates the effect of reaction with glucose oxidase on the mobility on paper chromatography of the reaction products from the incubation of [^{14}C]GP with liver mitochondria. Due to the formation of gluconic acid,

Figure 3.3 The effects of incubation with glucose oxidase (GOD) on the paper chromatographic mobility of glucose, glycerol, and GPC-synthetase reaction products.

A) Radioactive glucose

B) Radioactive glycerol

C) GPC-synthetase reaction products


○ = Glucose
● = Glucose + GOD
A
Radioactivity
○ = Glycerol
● = Glycerol + GOD
B
Radioactivity
○ = Reaction products
● = Reaction products + GOD
C
Specific Activity
(nMoles/min mg protein)
0.0
0.1
0.2
0.3
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Relative mobility

reaction with glucose oxidase led to a marked decrease in the mobility of the glucose standard (figure 3.3A). The migration of glycerol was not affected (figure 3.3B). Incubation of the supernatant from the GPC synthetase assay with glucose oxidase led to a shift of most of the radioactivity from the glucose (GPC) area to a spot with the same Rf as gluconic acid (figure 3.3C). Some radioactivity still migrated with a Rf similar to glycerol and not all of the radioactivity was lost from the glucose-GPC spot. This experiment, which was conducted several times, suggested that in different incubations glucose accounted for 60-80% of the total radioactive products. When radioactive GPC was reacted with glucose oxidase under the same conditions, there was no alteration in the relative mobility on paper chromatography (not shown).

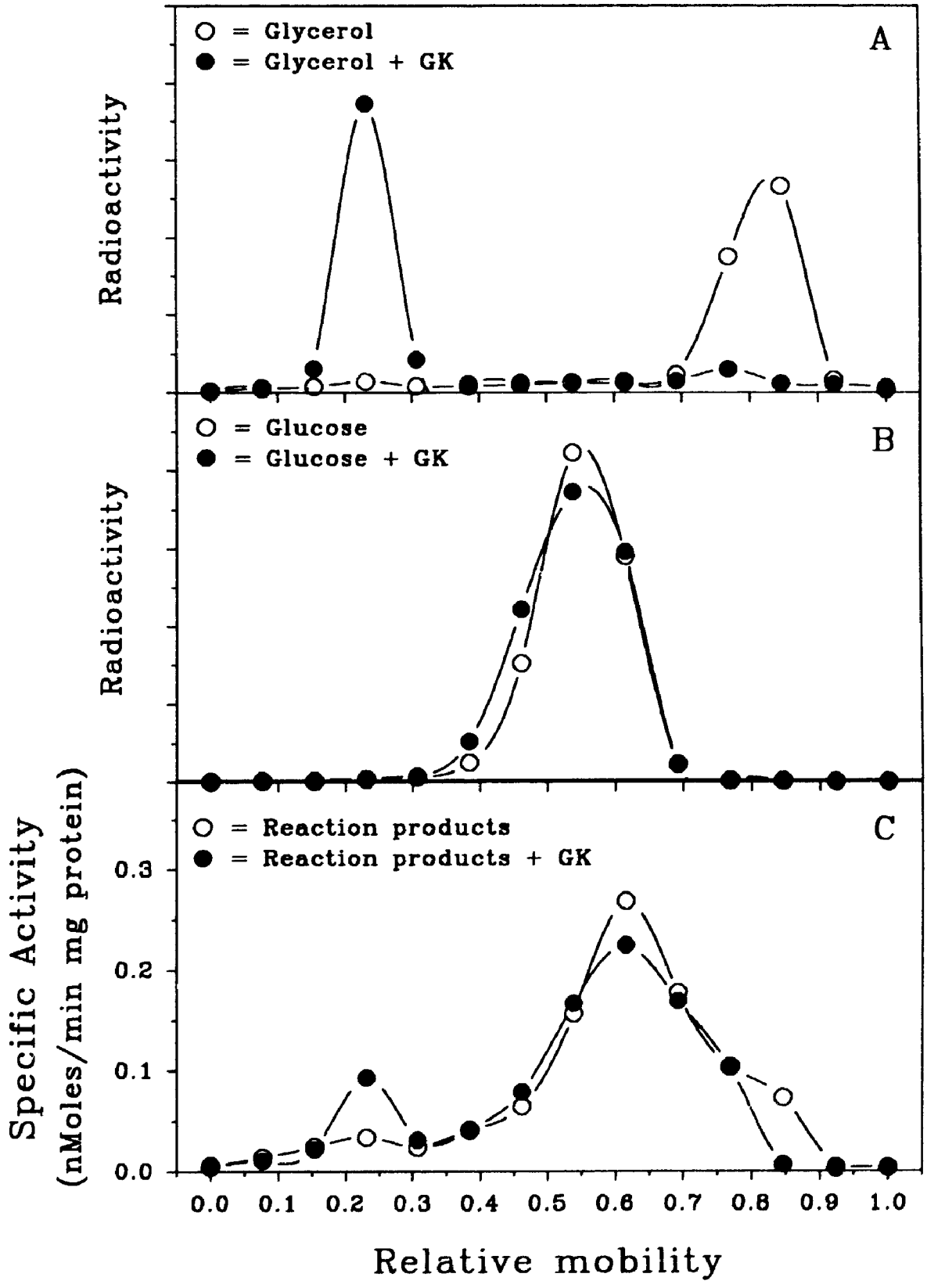
Reaction of the glycerol standard with glycerol kinase results in a marked decrease in Rf (figure 3.4A). The mobility of glucose was not affected (figure 3.4B). Nor was the migration of GPC altered (not shown). Paper chromatography of glycerol kinase-reacted and glycerol kinase-unreacted supernatants from the GPC synthetase assay with liver mitochondria revealed a decrease in the radioactivity with an Rf similar to glycerol and an increase in the radioactivity of the GP spot (figure 3.4C). These studies suggest that 20-40% of the radioactive products in

Figure 3.4 The effects of incubation with glycerolkinase (GK) on the paper chromatographic mobility of glycerol, glucose and GPC-synthetase reaction products.

A) Radioactive glycerol

B) Radioactive glucose

C) GPC-synthetase reaction products


○ = Glycerol
● = Glycerol + GK
A
Radioactivity
○ = Glucose
● = Glucose + GK
B
Radioactivity
○ = Reaction products
● = Reaction products + GK
C
Specific Activity
(nMoles/min mg protein)
0.0
0.1
0.2
0.3
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Relative mobility

the heat inactivated anion exchange eluants is associated with glycerol.

In other experiment we attempted to identify the products arising from incubation of [^{14}C]GP in the GPC reaction conditions with rat lung mitochondria. The bulk of the radioactive products migrated with an Rf value ranging from 0.60 to 0.90. Reactions with glucose oxidase or glycerol kinase demonstrated the presence of small amounts of radioactive glycerol but glucose formation was not detectable in the supernatant from these reactions (data not shown). The nature of the radioactive products with lung mitochondria needs to be investigated further.

3.6. Discussion

If the reaction depicted in figure 3.1 for the synthesis of GPC occurs, it should be possible to label GPC using either radioactive GP or radioactive CDP-choline. However, even when the published paper chromatographic method (Infante 1985a) for measuring the synthesis of GPC was employed, radioactive incorporation into the GPC spot was only observed with labelled GP and not with labelled CDP-choline. In addition, when TLC was used to separate the substrates and products, neither GP nor CDP-choline showed incorporation of radioactivity into the GPC spot. This clearly indicates that under the conditions used, the

reaction proposed by Infante does not occur to a significant extent. Rather, the activity reported for this assay appears to arise from the presence of unidentified radioactive products which co-migrate with GPC with the paper chromatographic separation system. In agreement with Infante, the highest specific activity for this assay was observed with the mitochondrial fractions. Therefore further experiments were conducted with the mitochondrial fractions.

Infante included UTP in his standard incubation to suppress the formation of free glycerol from labelled GP by phosphohydrolases present in the subcellular fractions (Infante 1985a). Baranska reported that the incorporation of the phosphate moiety of glycerol-3-phosphate into phosphatidylserine is dependent on CMP in liver (Baranska 1988). Addition of several phosphonucleotides to the GPC incubation system did not provide evidence for the formation of GPC by liver or lung mitochondria (figure 2). Labelling of the GPC spot above the 0-time controls was only observed with radioactive GP as the substrate and the paper chromatographic system. No incorporation above control was observed using GP and the TLC system or labelled CDP-choline with either system. In contrast to Infante (Infante 1985a), we observed that incorporation of GP into the GPC spot was stimulated by omitting UTP and inhibited by addition of ATP. These experiments and similar observations with the

mitochondrial fraction from rat lung suggested that dephosphorylation of GP or other components might be involved. On the basis of these experiments and similar studies by Dr. W. Thompson (University of Toronto, personal communication) it was concluded that the earlier reports on GPC synthesis might be due to inaccurate identification of the true radioactive products. We have used enzymatic approaches to identify these products. Glucose exhibits an identical mobility to GPC with the paper chromatographic method employed by Infante, but not with the TLC system used for the present studies. Reaction of the radioactive products from liver mitochondria with glucose oxidase, a highly specific enzyme (Gibson et al 1964), led to the formation of a product which co-migrated with gluconic acid. In different experiments, 60-80% of the radioactivity associated with the GPC-glucose spot was eliminated from the sample reacted with glucose oxidase. A similar approach was used to identify part of the remaining radioactivity associated with the GPC-glucose spot using glycerol kinase. While glycerol exhibits a higher mobility than GPC and glucose, there is considerable overlap. Reaction of the GP incubation supernatant with glycerol kinase demonstrated the presence of radioactive glycerol due to phosphohydrolase activity. Whether the decrease in radioactivity from GP observed with the addition of ATP (figure 3.2) results from a inhibition of phosphohydrolase activity or from glycerol

kinase activity cannot be concluded from the present studies.

These results demonstrate that the bulk of the radioactivity from GP migrating in the general area of the GPC-spot with the paper chromatographic system (Infante 1985a) is due to glucose and glycerol. Under the conditions used in these studies, glucose accounts for 60-80% while glycerol accounts for 20-40% of the radioactivity. However, although glucose, glycerol and GPC eluted quantitatively from the anion exchange column, overall losses of 30-40% were experienced during these procedures. In addition due to differences in pH optimum of glucose oxidase and glycerol kinase, it was not possible to subject a single aliquot of the column eluants to both enzymatic reaction consecutively. Therefore it is possible that small amounts of the radioactivity associated with the GPC-glucose spot may be due to other compounds. However, on the basis of the results obtained with radioactive CDP-choline and the TLC separation such radioactivity cannot represent GPC. It should also be noted that products other than glucose and glycerol could remain associated with the anion exchange column. Such products would be acidic in nature and would undoubtedly have a lower Rf value with the paper chromatographic separation method than GPC. As mentioned earlier GPC is not retained by the column.

In conclusion, these studies have attempted to demonstrate the synthesis of GPC from radioactive GP and CDP-choline by subcellular fractions from liver and lung using the incubation system described by Infante (Infante 1985a). Our results were not consistent with the production of GPC but did establish that in liver mitochondria glucose and glycerol are produced from [^{14}C]GP. D-Glucose was also identified as the product of the GPC-synthetase reaction in liver by Thompson and Belina (Thompson and Belina 1991). Lung mitochondria produced a radioactive compound other than GPC which must still be identified. Although Infante reported that radioactive glycerol was distinct from GPC on paper chromatography (Infante 1985a), we have observed a partial overlap. The Rf value for glucose, which in our hands coincides with GPC, was not reported by Infante. We must caution that it is conceivable that our failure to detect GPC formation could arise from an unforseen difference between Infante's incubation procedures and those used by our group. Nevertheless, our present data are consistent with our previous conclusion (Veldhuizen et al 1989, chapter 2) that the significant synthesis of PC in liver and lung via an acyl-specific GPC pathway is unlikely.

CHAPTER 4 ALTERATIONS IN PULMONARY SURFACTANT COMPOSITION AND ACTIVITY AFTER EXPERIMENTAL LUNG TRANSPLANTATION.

4.1. Summary

Pulmonary surfactant facilitates breathing by reducing the surface tension at the air liquid interface of the alveoli. We have examined the effect of experimental lung transplantation on the phospholipid pool sizes of alveolar surfactant large and small aggregates, the composition of the large aggregates, the surface tension reducing ability of lipid extract surfactant and the leakage of serum proteins into the lung. A double lung block was stored for 2 or 12 hours after perfusion with either Euro-Collins solution or University of Wisconsin solution. The right lung was lavaged immediately after the storage period, while the left lung was transplanted and lavaged 6 hours later. The recipient's native right lung was also lavaged 6 hours after the transplantation.

After an ischemic time of 12 hours impaired gas exchange was observed in the transplanted lung as well as the native lung, during the 6 hours of reperfusion. This impaired gas exchange was associated with several significant changes in pulmonary surfactant: 1) the small/large surfactant aggregate ratio was increased, 2) sphingomyelin was increased in large aggregates, 3)

phosphatidylglycerol content was decreased in large aggregates and, 4) the surfactant-associated protein-A content of large aggregates was decreased. In addition, the total yield of serum proteins in the lung lavage was increased. No significant differences were observed between the results obtained with Euro-Collins and University of Wisconsin solutions. We conclude that prolonged storage of the donor lung before transplantation results in ischemic damage which produces an increase in potential surfactant inhibitors and can alter the small/large surfactant aggregate ratio after lung transplantation. These alterations in pulmonary surfactant resemble those in many ARDS type injuries. We conclude that surfactant supplementation in lung transplantation should be investigated.

4.2. Introduction

Pulmonary surfactant stabilizes the lung by reducing the surface tension at the air-liquid interface of the alveoli. Surfactant is composed of lipid, mainly phospholipids, and protein (King 1984). The major surfactant phospholipid is phosphatidylcholine (PC) which represents about 80% by weight of surfactant lipid; about 60% of PC is the disaturated species, dipalmitoylphosphatidylcholine (DPPC). It is thought that a monolayer enriched in DPPC is

responsible for the reduction of surface tension. The second most abundant phospholipid species is the anionic phospholipid, phosphatidylglycerol (PG) (approximately 10%). Other phospholipids and neutral lipids are present in lower amounts.

At least three proteins are isolated with pulmonary surfactant: surfactant-associated protein A (SP-A), SP-B and SP-C (Possmayer 1988). SP-A is a water soluble glycoprotein of 26-36 kDa which has been implicated in modulating surfactant secretion and recycling, formation of tubular myelin, blocking surfactant inhibition by serum proteins and promoting host defence mechanisms (Wright and Dobbs 1991, Cockshutt et al 1990, Van Iwaarden et al 1990). SP-B and SP-C are small hydrophobic proteins which remain in the organic phase upon lipid extraction. These hydrophobic proteins are involved in enhancing lipid adsorption to the monolayer and in monolayer purification (Yu and Possmayer 1990, 1992).

Due to ischemia/reperfusion injury, long term lung preservation remains a major obstacle in lung transplantation (Novick et al 1992, Haverich et al 1985). Many contributing factors, including leucocyte and platelet activation, complement activation, arachidonic acid metabolites and oxygen free radicals have been implicated in this injury (Novick et al 1992). Considerable research on lung preservation has focused on the effects of these mediators in order to define optimal storage conditions and

appropriate conditions for flushing the donor lungs. The effects of oxygen free radical scavengers, ATP precursors, PAF antagonists, calcium blockers, prostaglandins and corticosteroids have all been investigated (Novick et al 1992). Despite these efforts, only a maximum storage interval of 6-8 hours is possible in clinical lung transplantation before lung preservation becomes unsatisfactory.

Adult respiratory distress syndrome (ARDS) or acute lung injury can occur after a variety of insults (Bersten and Sibbald 1989, Ward et al 1985, Metz and Sibbald 1991). It appears that different types of insults are induced by specific mechanisms in which different mediators or at least a different hierarchy of mediators contribute to the injury cascade (Bersten and Sibbald 1989, Ward et al 1985, Metz and Sibbald 1991). At least some of these mediators have been implicated in the transplant injury (Novick et al 1992). The severe respiratory failure observed in ARDS has been associated with surfactant dysfunction (Jobe 1989, Seeger et al 1990, Holm and Matalon 1989) regardless of the primary injury. It has been well documented that pulmonary surfactant is altered in several ARDS-type injuries, and surfactant supplementation has been shown to be beneficial in several animal models of lung injury (Robertson 1991).

Little is known about the changes in pulmonary surfactant after lung transplantation (Novick et al 1991a).

Klepetko and associates reported a decrease in DPPC in bronchoalveolar lavage from dog lungs after experimental lung transplantation (Klepetko et al 1990). The potential role of surfactant analysis in the diagnosis of rejection and infection after experimental lung transplantation has also been suggested (Jurmann et al 1989).

The purpose of this study was to examine the effect of 2 and 12 hours of storage of donor lung grafts and subsequent transplantation on pulmonary surfactant. After 6 hours of reperfusion, the phospholipid yield of small and large surfactant aggregates, phospholipid composition, SP-A and SP-B content, protein yield and the surface tension reducing ability of surfactant extracts were measured. Lung function was monitored by blood gas determinations. The effect of short-term and long-term storage, as well as the effect of two different flushing solutions, Euro-collins (EC) versus University of Wisconsin (UW) solution, has been investigated.

4.3. Methods

4.3.1. Canine left lung transplantation

Left single lung transplantation and blood gas measurements were performed by Dr Richard Novick and colleagues. The transplantation procedure was performed as

described previously (Novick et al 1991b). Thirty dogs (15-20 kg) were randomly divided into three groups (n=10 per group; 5 donors and 5 recipients): 1) donor pulmonary artery flush with Euro-Collins (EC) solution and 2 hours of ischemia, 2) donor pulmonary artery flush with EC-solution and 12 hours of ischemia, 3) donor pulmonary artery flush with University of Wisconsin (UW) solution and 12 hours of ischemia. All animals were premedicated with xylazine 1 mg/kg and atropine 0.04 mg/kg. Anaesthesia was induced using thiopental sodium 12 mg/kg, and maintained with 1% halothane. Neuromuscular blockade was achieved with pancuronium, 0.1 mg/kg, which was repeated at intervals. After intubation with a cuffed endotracheal tube, mechanical ventilation was commenced at a tidal volume of 20 ml/kg, at a fraction of inspired oxygen (FiO_2) of 0.5 for the donor and 1.0 for the recipient. All animals received humane care in accordance with the "Principles of Laboratory Animal Care" (National Society for Medical Research), the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication No. 85-23, revised 1985), and the specifications of the Council on Animal Care of The University of Western Ontario.

4.3.2. Donor operation

After median sternotomy and anterior pericardiectomy, the superior and inferior venae cavae, ascending aorta, pulmonary artery and trachea were mobilized. The donor animals underwent pulmonary artery flushing with 60 ml/kg of EC or UW solution. The heart was excised followed by the double lung block. The double lung block was stored inflated for either 2 hours (EC flush) or 12 hours (EC or UW flush) in 4°C saline. UW-flushed lungs received prostacyclin infusion (dose of 50-200 ng/kg/min) to counteract the vasoconstrictive effects of the high potassium concentration in UW-solution.

4.3.3. Recipient operation

Weight-matched recipients underwent a left posterolateral thoracotomy through the fifth intercostal space. The right and left pulmonary arteries were encircled with heavy silk ties for subsequent snaring during blood gas measurements. The chest was closed using towel clips, leaving a small opening through which the pulmonary arteries snares were manipulated. The animals were placed in the supine position, and baseline arterial blood gases were measured at an FiO_2 1.0 on a Corning 178 pH/blood gas analyzer. The pulmonary arteries were sequentially snared to obtain baseline blood gas values for the left lung (right pulmonary artery (RPA) snared) and right lung (left

pulmonary artery (LPA) snared) after 10 min of occlusion. The chest was then reopened and a left pneumonectomy was performed.

After the storage time, the double lung block was removed from its 4^0C saline bath. The right lung was separated from the double lung block and lavaged twice through the main bronchus with approximately 1 L 0.15 M NaCl containing 1.5 mM $CaCl_2$. The lavage was analyzed as described below.

The donor left lung was then anastomosed to the recipient. Blood gas measurements on the recipient's native right and the transplanted left lung were performed immediately after transplantation and at 2, 4 and 6 hours of reperfusion using 10 minutes of pulmonary snaring prior to each measurement. The animals were ventilated at an FiO_2 of 1.0.

After 6 hours of reperfusion, animals were sacrificed with an overdose of pentobarbital. The transplanted left lung as well as the native right lung were lavaged twice with approximately 1L 0.9% NaCl containing 1.5 mM $CaCl_2$. Lung lavages were analyzed as described below.

In summary, three experimental groups were examined: 1) EC-solution flush and 2 hour storage, 2) EC-solution flush and 12 hour storage and 3) UW-solution flush and 12 hours storage. Three different lung lavages were obtained from each experimental group: A) a donor lung lavage obtained

from the right stored lung after the storage period, B) a transplanted lung lavage obtained from the left transplanted lung 6 hours after reperfusion, and C) a native lung lavage obtained from the recipient's right lung 6 hours after the left single lung transplantation. In addition, control lung lavages (n=7) for comparison were obtained from normal lungs of animals used for other experiments or from the recipients left lung after left pneumonectomy.

4.3.4. Lung lavage processing and analyses

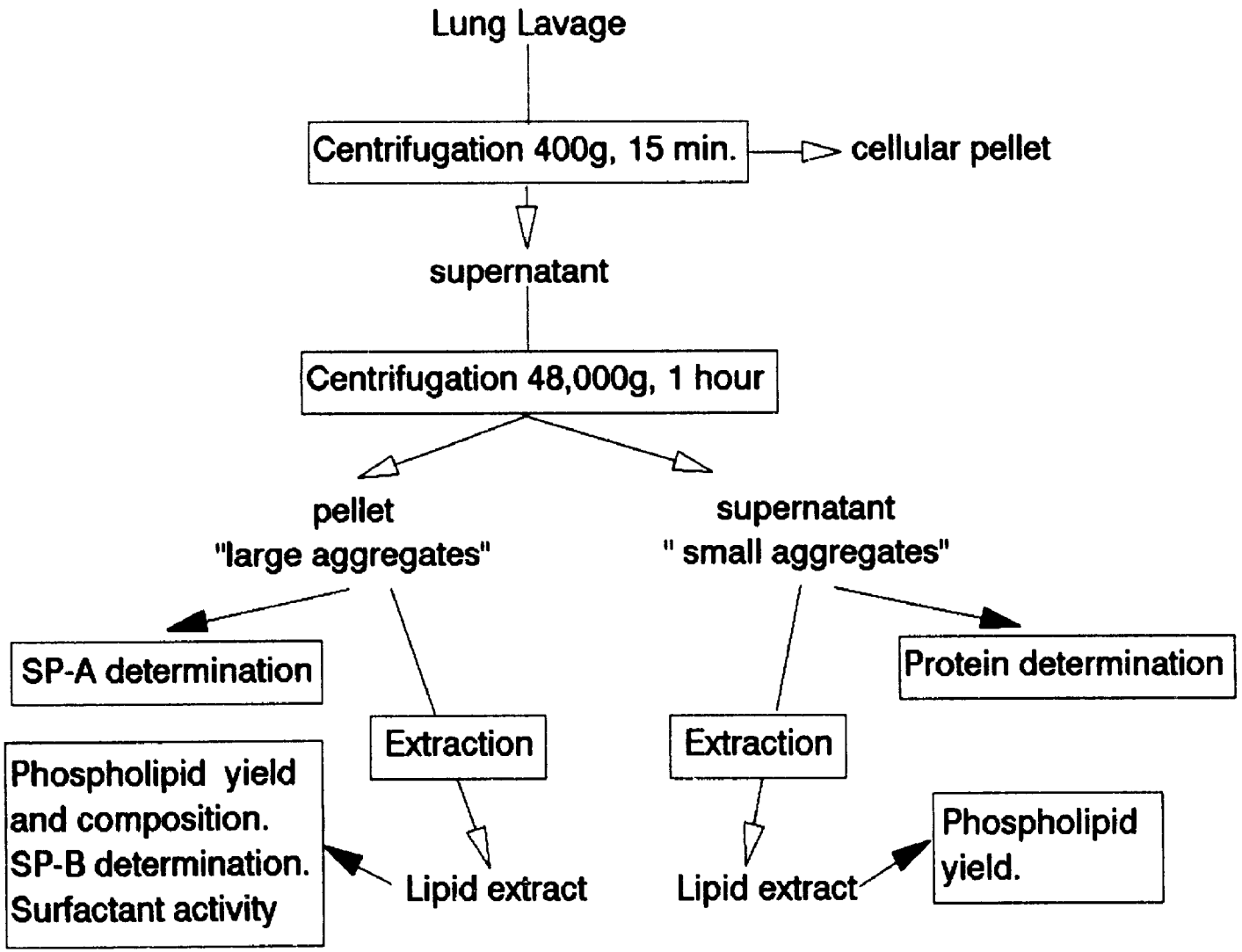
Lung lavages were processed essentially as described by Gregory and associates (Gregory et al 1991). Briefly, lung lavages were centrifuged at 400g for 15 min. to remove tissue and cellular debris. The 400g supernatant was centrifuged for 1 hour at 48,000g to obtain a pellet of the large surfactant aggregates. The small surfactant aggregates remain in the 48,000g supernatant. The large aggregates were resuspended in 0.9% NaCl containing 5 mM $CaCl_2$, and an aliquot used for SP-A determination as described below. Lipid extracts of the large aggregates were prepared by chloroform:methanol extraction according to the method of Bligh and Dyer and subsequent acetone precipitation (Bligh and Dyer 1959, Weber and Possmayer 1984). The lipid extracts were analyzed for phospholipid yield and composition, SP-B content and surface tension reducing ability.

The supernatant of the 48,000g centrifugation was examined for total protein by the method of Lowry et al (Lowry et al 1951) using bovine serum albumin as a standard. An aliquot of the 48,000g supernatant was extracted by the Bligh and Dyer procedure to determine the phospholipid content of the small surfactant aggregates. In some experiments larger volumes of the small aggregates were extracted for determination of phospholipid composition and surface tension reducing ability measurements. The processing and analysis of lung lavages is schematically outlined in figure 4.1. All steps were carried out at 4°C.

4.3.5. Phospholipid yield and composition

Total phospholipid was determined by phosphorus analysis of aliquots of the lipid extracts by the method of Rouser et al (Rouser et al 1970). Phospholipid composition was determined by separating the lipids by thin layer chromatography followed by phosphorus measurements. Aliquots containing either 25 μg or 250 μg phospholipids were separated on Whatman LK6D thin layer chromatography plates using chloroform:ethanol:triethylamine:water (30:34:35:8) as a solvent system (Touchstone et al 1980). The plates were developed twice in the same direction. The 25 μg phospholipid lanes were used for obtaining PC while the

Figure 4.1. Schematic representation of the lavage analysis.


Lung Lavage
Centrifugation 400g, 15 min.
cellular pellet
supernatant
Centrifugation 48,000g, 1 hour
pellet
"large aggregates"
supernatant
" small aggregates"
SP-A determination
Extraction
Protein determination
Extraction
Phospholipid yield
and composition.
SP-B determination.
Surfactant activity
Lipid extract
Lipid extract
Phospholipid
yield.

other lipids were scraped from the 250 μg phospholipid lane. Lanes containing phospholipid standards were sprayed with the Dittmer Lester phosphorus spray and the spots corresponding to the individual phospholipids in the sample lanes were scraped off and used for phosphorus determination (Dittmer and Lester 1964). Phosphorus was measured by a modification of the method of Duck-Chong (Duck-Chong 1979). Briefly, 50 μl of 10% magnesium nitrate in methanol was added to the samples and allowed to dry. The dried samples were ashed in a fume hood on an electric heating rack (on high) for 30 sec. After cooling, 1.0 ml of 1M HCl was added and the samples were heated at 95°C for 15 min. The silica containing samples were centrifuged at 150g for 5 min., a 66 μl aliquot of each sample was pipetted into a 96 well dish and 133 μl dye reagent (3 volumes 0.3% malachite green + 1 volume 4.2% ammonium molybdate in 4.5M HCl) was added to each sample. After 15 min at room temperature the absorbance was read at 650nm on an ELISA reader. A standard curve was created using aliquots of KH_2PO_4 solution corresponding to 0, 0.1, 0.3, 0.5 and 0.7 μg phosphorus.

4.3.6. Measurement of SP-A and SP-B

Quantitation of SP-A and SP-B in large aggregates was kindly performed in Dr Jeffrey Whitsett's laboratory (University of Cincinnati) using an enzyme-linked

immunoabsorbent assay (ELISA). For SP-A, wells were coated with protein dispersed in 0.1 M $NaHCO_3$. The wells were blocked with 5% human serum in 0.15 M NaCl, 0.01 M Tris pH 7.4, 5 mg/ml bovine serum albumin. Samples and standard were preincubated overnight at 37°C with anti-canine SP-A monoclonal antibody in 0.15 M NaCl, 0.01 M Tris pH 7.4, 5 mg/ml bovine serum albumin, 5% human serum, 0.5% nonidet P-40. The samples and standards are then incubated in the coated wells for 1 or 2 hours and the wells were washed and incubated with a secondary horseradish peroxidase conjugated anti-mouse IgG antibody. After washing colour was developed using 100μl of 0.065M $NaKPO_4$,pH 6.3, containing 0.017 M citric acid, 0.05% H_2O_2 and 5 mg/ml ortho-phenylenediamine. The colour reaction was stopped using 50% sulphuric acid and the absorbance at 492 nm was determined.

For SP-B determination, the wells were coated with a rabbit polyclonal anti-SP-B antibody; after blocking the wells were subsequently incubated with standard SP-B (0.5 to 10 ng) or sample, a monoclonal anti-SP-B antibody and a secondary horseradish peroxidase conjugated anti-mouse IgG antibody. Colour was developed and measured as described for SP-A.

4.3.7. Surface tension measurements

Lipid extracts were dried under nitrogen and resuspended in 0.9% NaCl/1.5mM $CaCl_2$ to a final concentration of 2.5 mg/ml (based on total phosphorus). Samples were incubated at least 90 min. at 37°C before being analyzed with a pulsating bubble surfactometer as described by Enhorning (Enhorning 1977). With this technique a bubble is created in a surfactant suspension. The bubble is pulsated between maximum bubble radius (R_{max}) of 0.55mm and a minimum bubble radius (R_{min}) of 0.4mm at 37°C. The pressure across the air liquid interface is measured by a pressure transducer. Surface tension is calculated by the law of Laplace which states that the pressure across a sphere is directly proportional to twice the surface tension and indirectly proportional to the radius. Surface tensions at R_{max} and R_{min} are expressed.

4.3.8. Statistical analysis

Results were expressed as means +/- standard error of the mean. The experimental groups were compared by analysis of variance and Bonferroni's correction using the Instat statistical software program (San Diego, California). A probability value <0.05 was considered significant.

4.4. Results

Lung function at different times after transplantation was assessed by measuring the PO_2 on 100% oxygen with the pulmonary arteries snared sequentially. Gas exchange of the transplanted lung was determined by snaring of the right pulmonary artery (figure 4.2A). With the two hour-stored lungs, the PO_2 decreased slightly but not significantly immediately and 2 hours after transplantation compared to the control values obtained before transplantation. By 4 and 6 hours the PO_2 values returned to baseline conditions. Twelve hour storage resulted in a significantly decreased PO_2 immediately after transplantation, with no recovery in the following 6 hours. The results obtained with UW-solution were not significantly different from lungs flushed with EC-solution.

Gas-exchange of the native lungs (left pulmonary artery snared) of dogs receiving a 2 hour stored lung was not altered significantly (figure 4.2B). However, recipients of 12 hour-stored lungs showed a gradual decrease in PO_2 values from their native right lungs which were significantly different from the 2 hour storage group after 2, 4 and 6 hours of reperfusion. At 4 and 6 hours gas exchange of the native lung was almost as impaired as that of the transplanted lung.

Figure 4.2. PO_2 values before and after transplantation. Measurements were made while the animals were ventilated on 100% oxygen with either the left (LPA) or right pulmonary artery (RPA) snared (the mean value and the standard error of the mean SEM are expressed, * = $p<0.05$ versus 2 hour values). (Measurements made by Dr Richard Novick and associates).

A) Transplanted lungs

B) Native lungs

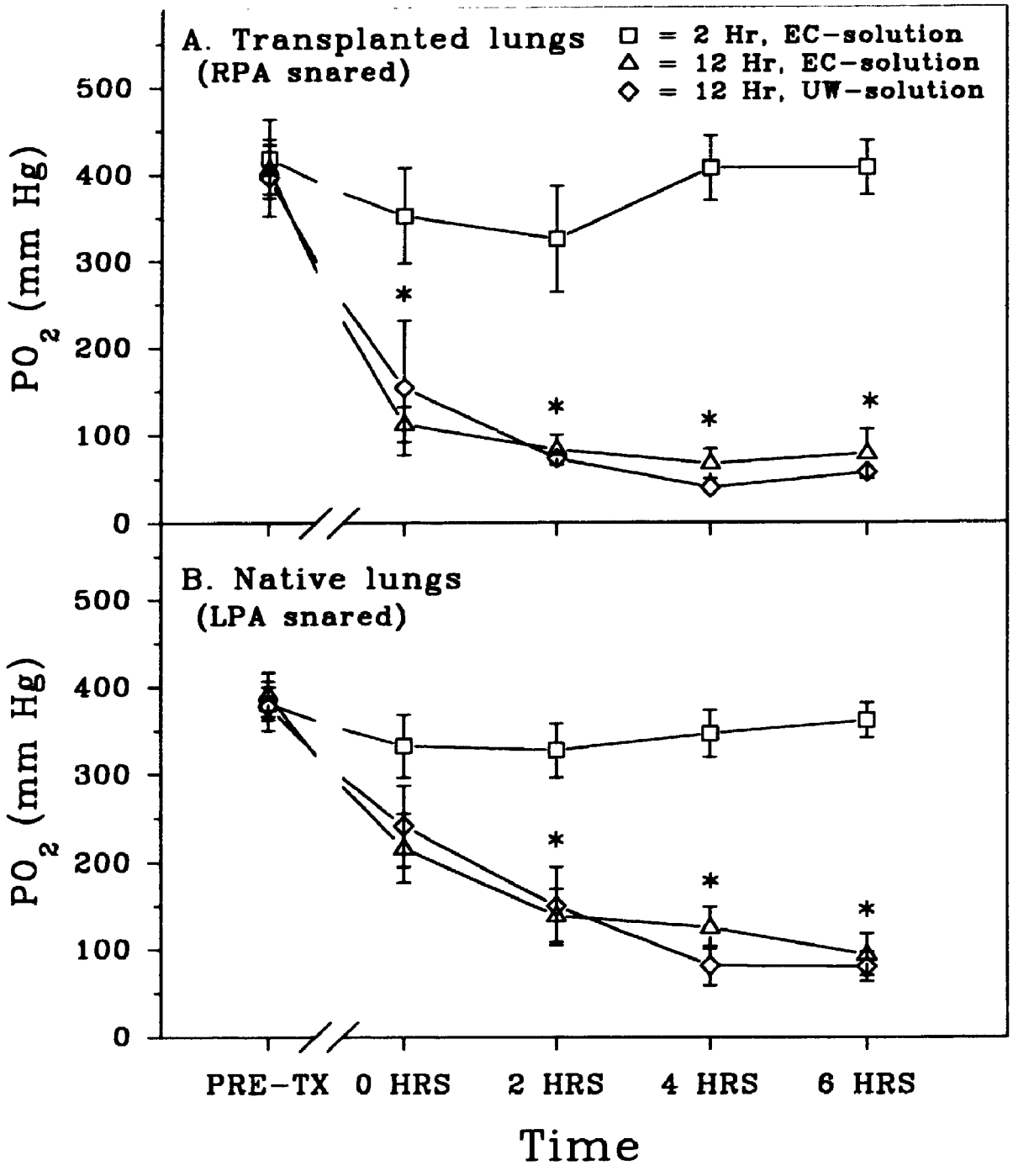
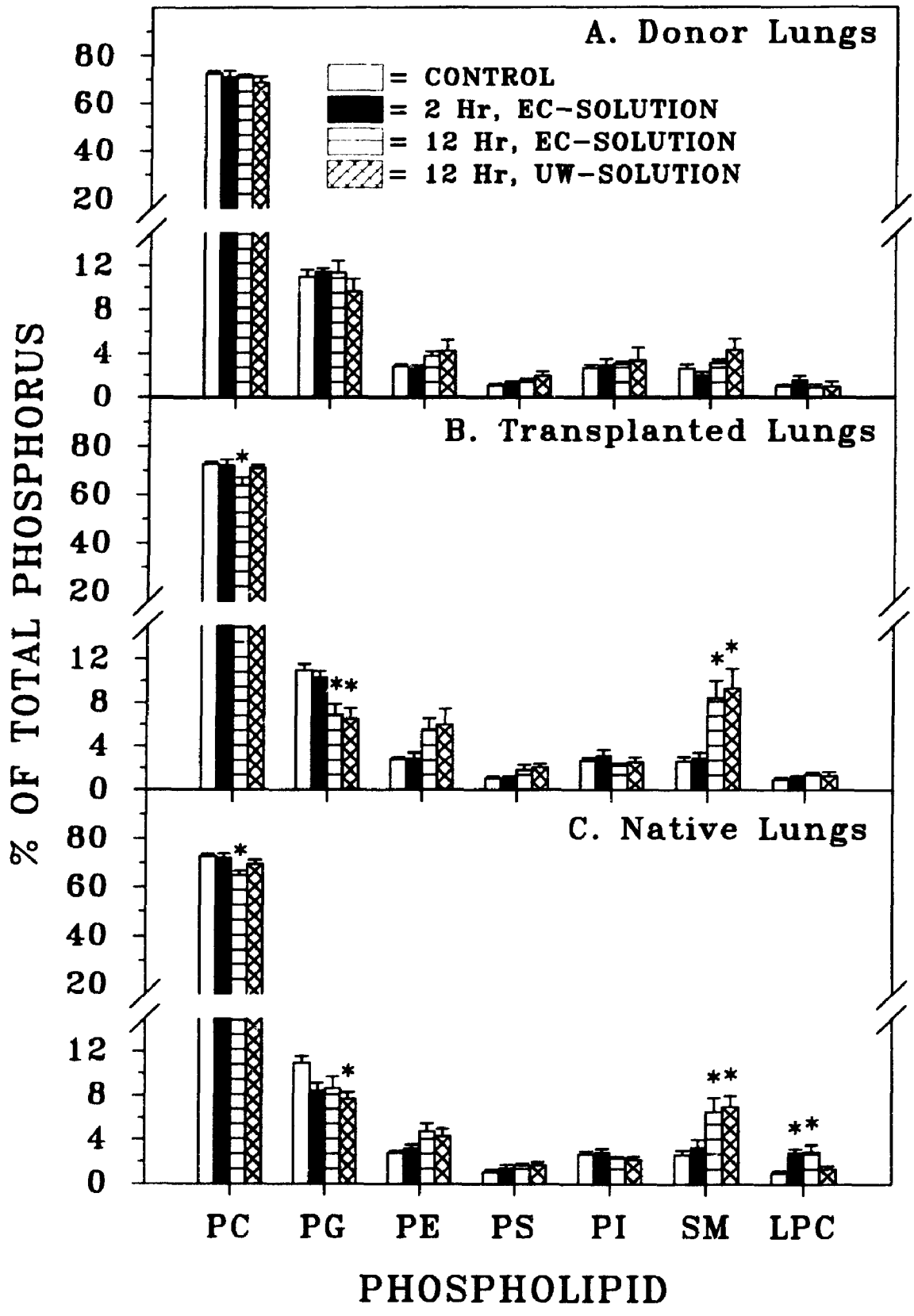

A. Transplanted lungs
(RPA snared)
□ = 2 Hr, EC-solution
△ = 12 Hr, EC-solution
◇ = 12 Hr, UW-solution
B. Native lungs
(LPA snared)
PO2 (mm Hg)
500
400
300
200
100
0
PRE-TX
0 HRS
2 HRS
4 HRS
6 HRS
Time

Figure 4.3. Phospholipid composition of large surfactant aggregates. (mean ± SEM, * = $p<0.05$ versus control values).

A) Donor lungs

B) Transplanted lungs

C) Native lungs


A. Donor Lungs
= CONTROL
= 2 Hr, EC-SOLUTION
= 12 Hr, EC-SOLUTION
= 12 Hr, UW-SOLUTION
B. Transplanted Lungs
C. Native Lungs
% OF TOTAL PHOSPHORUS
80
60
40
20
12
8
4
0
*
**
PC
PG
PE
PS
PI
SM
LPC
PHOSPHOLIPID

The phospholipid compositions of the large surfactant aggregates are shown in figure 4.3. Surfactant obtained from donor lungs lavaged immediately after 2 or 12 hours of storage had a phospholipid composition similar to the controls (figure 4.3A). The composition of the large aggregates from transplanted and native lungs after 2 hour of storage and 6 hours of reperfusion were also similar to controls. After 12 hours of storage the large aggregates from transplanted lungs showed a significant increase in sphingomyelin (SM) and a significant decrease in phosphatidylglycerol (PG) (figure 4.3B). There was also an increase in phosphatidylethanolamine (PE) and, in the EC 12 hour storage group, a slight decrease in phosphatidylcholine (PC) content but the latter changes were not significant. Native lungs of dogs receiving a 12 hour stored lung showed similar, but less pronounced, changes in large aggregate composition as the transplanted lung (figure 4.3C). However, the changes were less pronounced in the native lung (figure 4.3C). Interestingly, there was a small increase in lysophosphatidylcholine (LPC) in the native lung after EC-solution flushing, a change not observed with transplanted lungs. There was no significant difference in total large aggregate yield between the different experimental groups.

The biophysical activity of the lipid extracts of the large surfactant aggregates was tested by examining their ability to reduce surface tension on a pulsating bubble

surfactometer. These extracts contain only the phospholipids and the hydrophobic surfactant proteins SP-B and SP-C. The surface tensions of lipid extracts dispersed at a concentration of 2.5 mg/ml in saline/1.5 mM $CaCl_2$ were plotted at minimum bubble size (figure 4.4). The results clearly demonstrate that the lipid extracts of large aggregates from the different experimental groups are all equally capable of reducing surface tension at R_{min}. No significant differences were observed at R_{max} (data not shown).

The content of SP-B in the large aggregates is shown in Table 4.1. The values obtained with the 12 hour storage experiments are not significantly different from the control values (Table 4.1). The levels of SP-B were also examined in the 2 hr storage group. Unfortunately, because of alterations in antibodies it was necessary to alter the ELISA assay during the course of these studies. As a result the data for the 2 hour storage group could not be directly compared with the values for the 12 hr storage groups. Nevertheless, the results obtained with the two hour storage experiments did not reveal any significant difference in SP-B level between the donor, native and transplanted group (results not shown). Antibodies recognizing SP-C are not available, and it was thus not possible to determine SP-C levels.

Figure 4.4. Surface tension reducing ability of large aggregates. All samples were measured at 2.5 mg phospholipid/ml and 20 pulsations per min. Surface tensions at minimum bubble size are expressed. (Mean ± SEM)

A) Donor lungs

B) Transplanted lungs

C) Native lungs

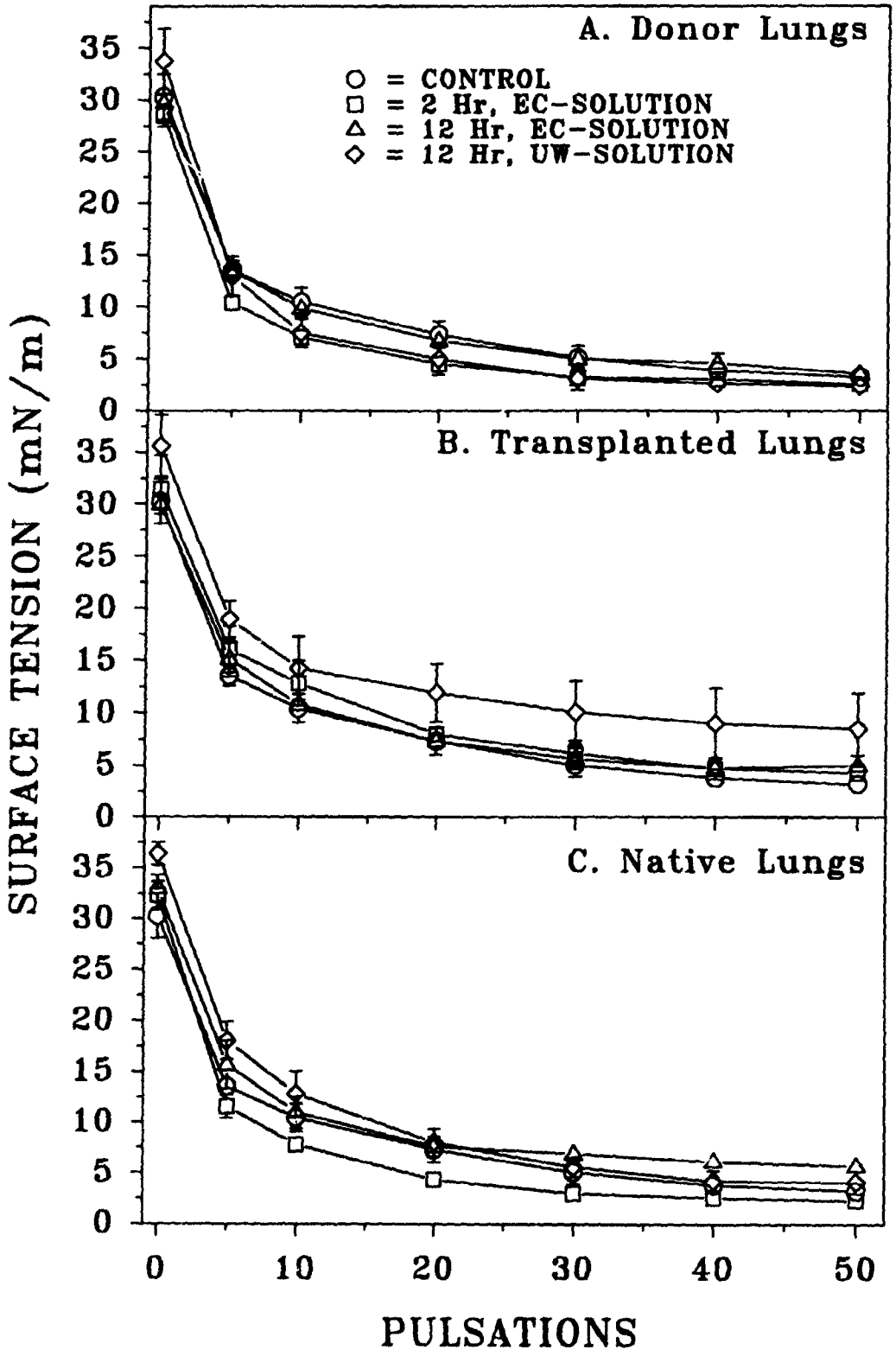

A. Donor Lungs
○ = CONTROL
□ = 2 Hr, EC-SOLUTION
△ = 12 Hr, EC-SOLUTION
◇ = 12 Hr, UW-SOLUTION
B. Transplanted Lungs
C. Native Lungs
SURFACE TENSION (mN/m)
PULSATIONS
0
5
10
15
20
25
30
35
40
50

Table 4.1. SP-B content of large aggregates as a percent of phospholipid. (SP-B ELISA assays were performed by Bill Hull in Dr Jeffrey Whitsetts laboratory at the University of Cincinnati)

		SP-B % of phospholipid % ± SEM
Control		0.28 ± 0.05
12 Hr, EC-solution	Donor	0.19 ± 0.07
	Transplanted	0.25 ± 0.15
	Native	0.24 ± 0.09
12 Hr, UW-solution	Donor	0.36 ± 0.10
	Transplanted	0.14 ± 0.03
	Native	0.21 ± 0.08

Since surfactant associated protein-A has been implicated in the reversal of surfactant inhibition by blood proteins and in host defence mechanisms (Cockshutt et al 1990, Van Iwaarden et al 1990) therefore, SP-A could be critical for compromised lungs. No differences were observed in SP-A levels of the large surfactant aggregates fractions from donor, transplanted or native lungs of the two hour storage group (figure 4.5). However, after 12 hours of storage the SP-A content of the large aggregates obtained from donor lungs decreased significantly to approximately half the control values. A similar low level of SP-A was also found after 6 hours reperfusion of the transplanted lungs. Surprisingly, the native lungs also showed a decrease in SP-A content after transplantation. No significant difference was observed between EC-solution and UW-solution.

Surfactant small aggregates appear to be the metabolic product of the large aggregates and presumably represents the form taken up by Type-II cells for recycling (Magoon et al 1983). Changes in the small to large surfactant aggregate ratio have recently been reported with several animal models of lung injury (Lewis et al 1990, Gross 1991a). As shown in figure 4.6 the ratio of small (48,000g supernatant phospholipid) to large (48,000g pellet) aggregates was significantly increased in both the transplanted and native lung after transplantation of a twelve hour-stored lung.

Figure 4.5. SP-A content of large aggregates as an percent of phospholipid. (mean ± SEM, * = $p<0.05$ versus control values). (SP-A ELISA assays were performed by Bill Hull in Dr Jeffrey Whitsetts laboratory at the University of Cincinnati)

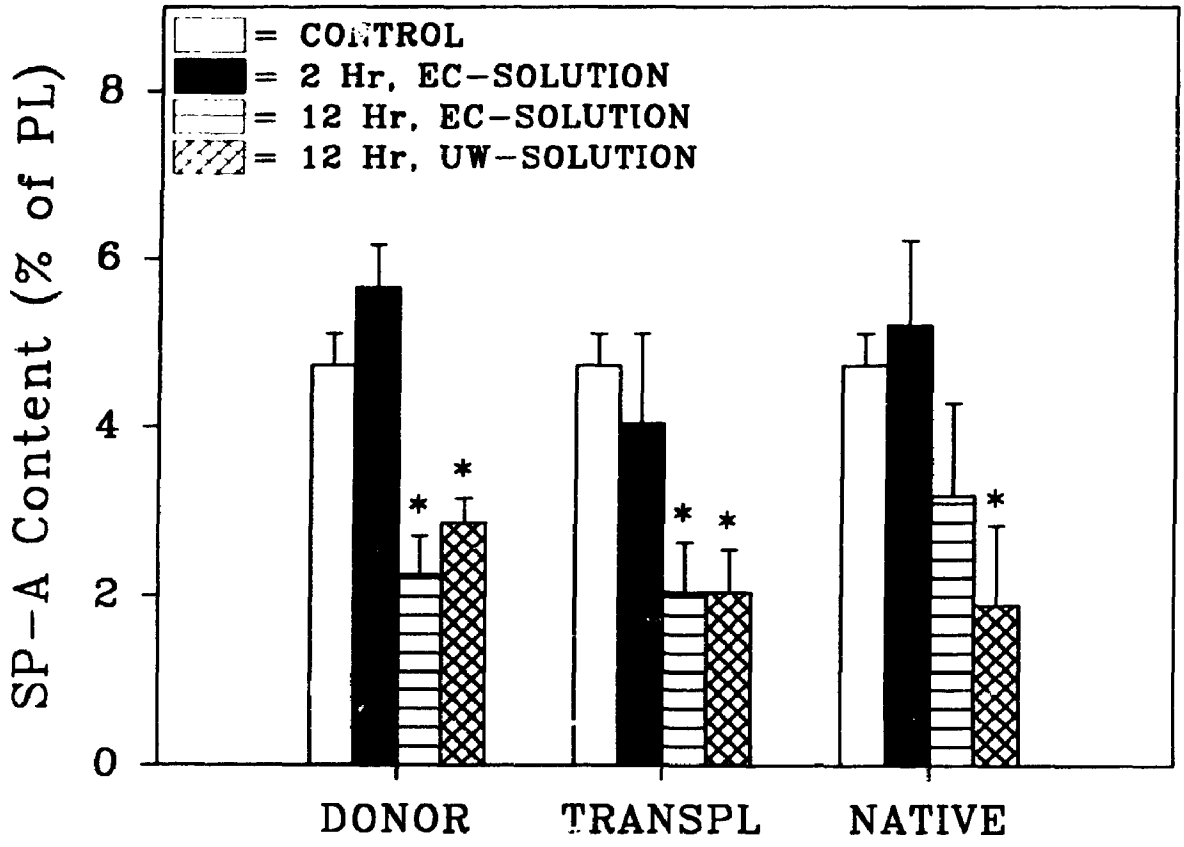
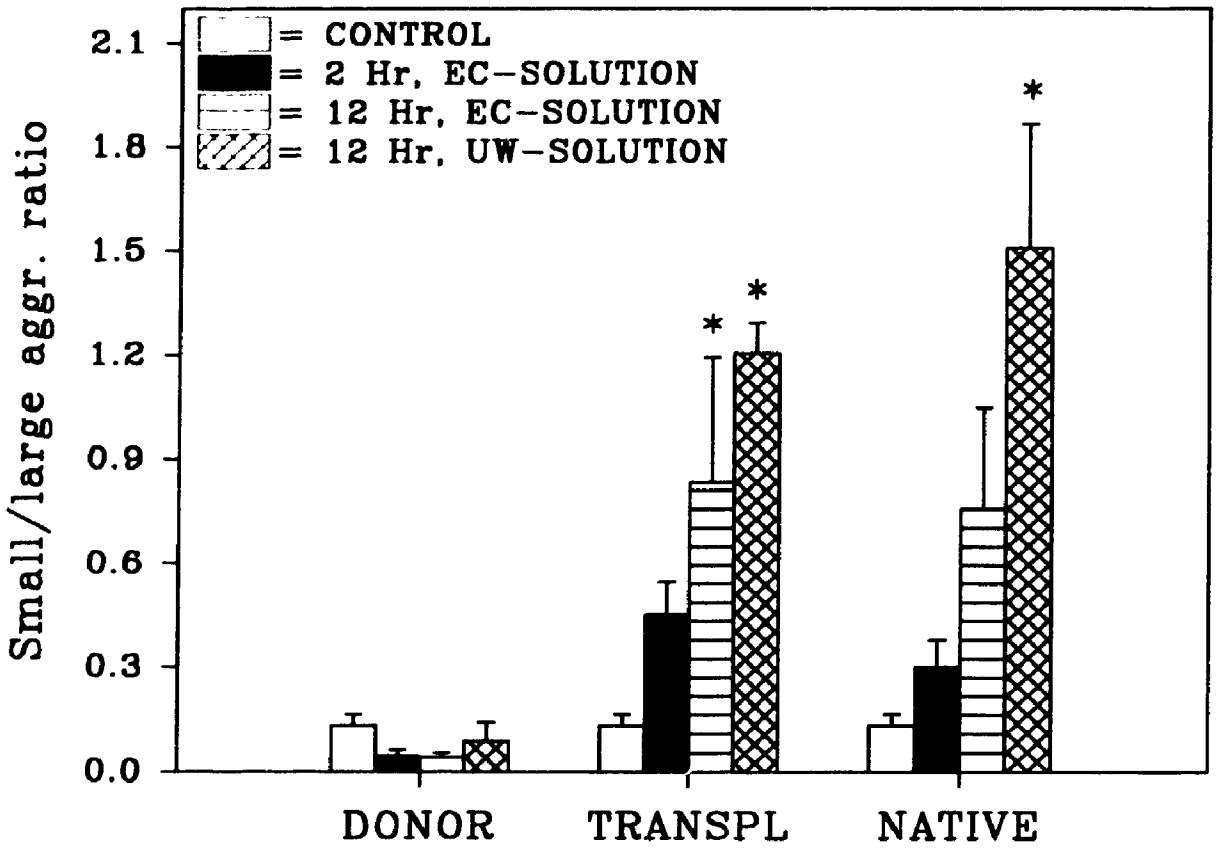

= CONTROL
= 2 Hr. EC-SOLUTION
= 12 Hr. EC-SOLUTION
= 12 Hr. UW-SOLUTION
SP-A Content (% of PL)
0
2
4
6
8
*
*
*
*
*
DONOR
TRANSPL
NATIVE

Figure 4.6. Small/large surfactant aggregate ratios. (mean ± SEM, * = $p<0.05$ versus control values).

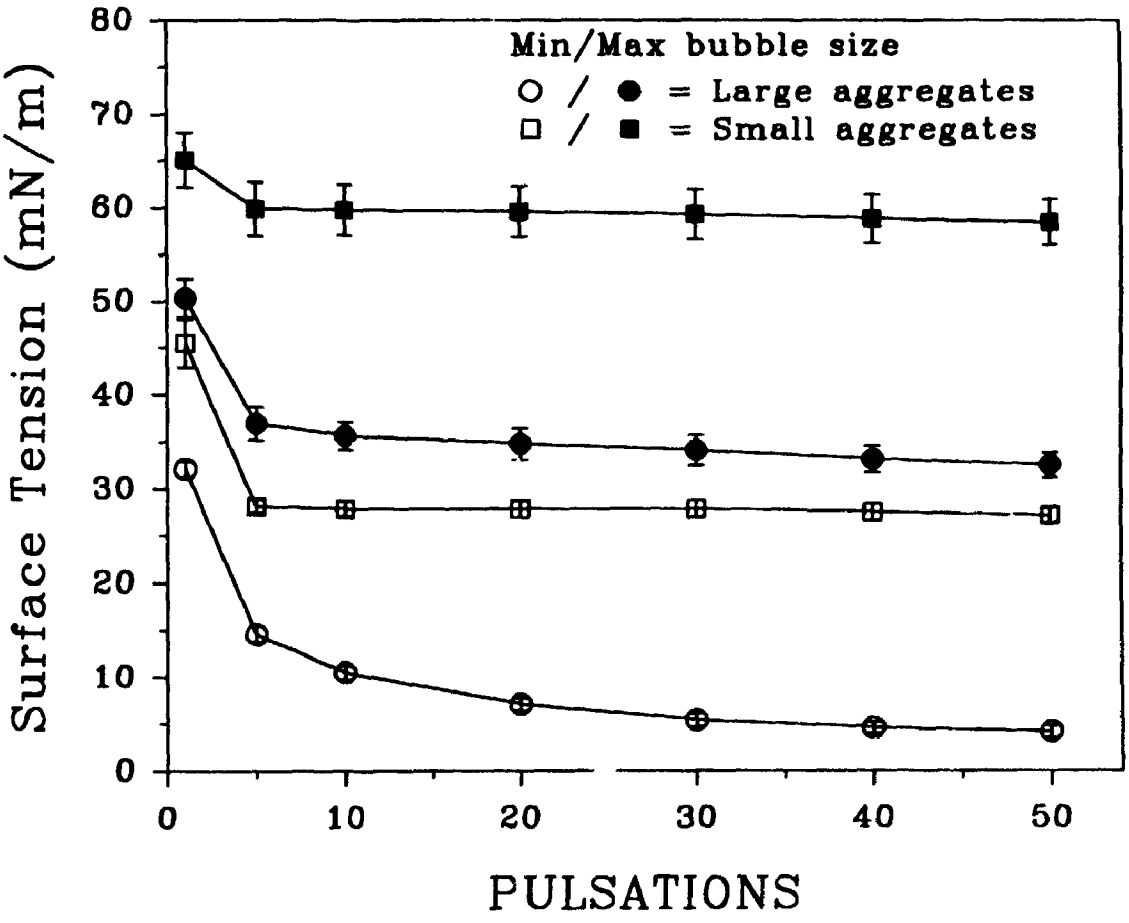

= CONTROL
= 2 Hr, EC-SOLUTION
= 12 Hr, EC-SOLUTION
= 12 Hr, UW-SOLUTION
Small/large aggr. ratio
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
*
*
*
DONOR
TRANSPL
NATIVE

Higher ratios were observed with UW solution than EC-solution. The ratio was lower in donor lungs than in control lungs but this was not significant.

Lipid extracts of large and small aggregates were analyzed for their ability to reduce surface tension on a pulsating bubble surfactometer. Whereas extracts from large aggregates reduced surface tension to low values within 50 pulsations, small aggregate extracts were unable to reach low surface tensions (figure 4.7). No differences were observed between the experimental groups. Examination of a representative number of small aggregate lipid extracts on TLC with the Dittmer-Lester phosphorus spray revealed slightly higher LPC and SM levels in the phospholipid profile of small aggregates compared to the large surfactant aggregates (results not shown). SP-B was undetectable in small aggregates by immunoblot analysis (results not shown).

It is well established that serum proteins can inhibit the surface tension reducing activity of surfactant (Cockshutt 1990, Jobe 1989). Total protein yields of the lung lavages are shown in figure 4.8. The protein yields obtained from donor lungs was not different from control values, regardless of the storage interval. After transplantation an increased level of protein was observed in lung lavages from both the native and transplanted lungs. This increase was significant with the transplanted EC 12 hr

Figure 4.7. Surface tension reduction by lipid extracts of large and small surfactant aggregates. R_{min} is expressed in the open symbols, R_{max} in filled symbols. Samples were measured at 2.5 mg phospholipid/ml and 20 pulsations per min. (mean ± SEM)


Min/Max bubble size
○ / ● = Large aggregates
□ / ■ = Small aggregates
Surface Tension (mN/m)
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
PULSATIONS

and with the native EC 12 hr. samples compared to control values. No significant difference was observed between UW and EC sclution.

Equal amounts of lavage protein and serum from the recipient animals were electrophoresed on SDS-PAGE gels. After the proteins were stained with Coomassie blue, the protein profile of the lavage proteins and serum appeared similar (figure 4.9). The protein profile of other experimental groups and of donor and control lungs also appeared similar to serum proteins (not shown).

4.5. Discussion

Long term lung preservation remains a major obstacle in clinical lung transplantations (Novick et al 1992, Haverich et al 1985). Many mediators have been implicated for this pulmonary ischemia/reperfusion injury. However, the relative importance and the hierarchy of each of these mediators remains controversial. Changes in surfactant lipids and serum protein levels in bronchoalveolar lavages after transplantation have been reported with rejection and infection (Jurmann et al 1989). However, the total phospholipid yield compared to protein and neutral lipids and the lipid composition of the material isolated with the bronchoalveolar lavage procedure used in that study (Jurmann et al 1989) appears different from the composition found in

Figure 4.8. Protein yields in the small surfactant aggregates samples. (mean ± SEM, * = $p<0.05$ versus control values).

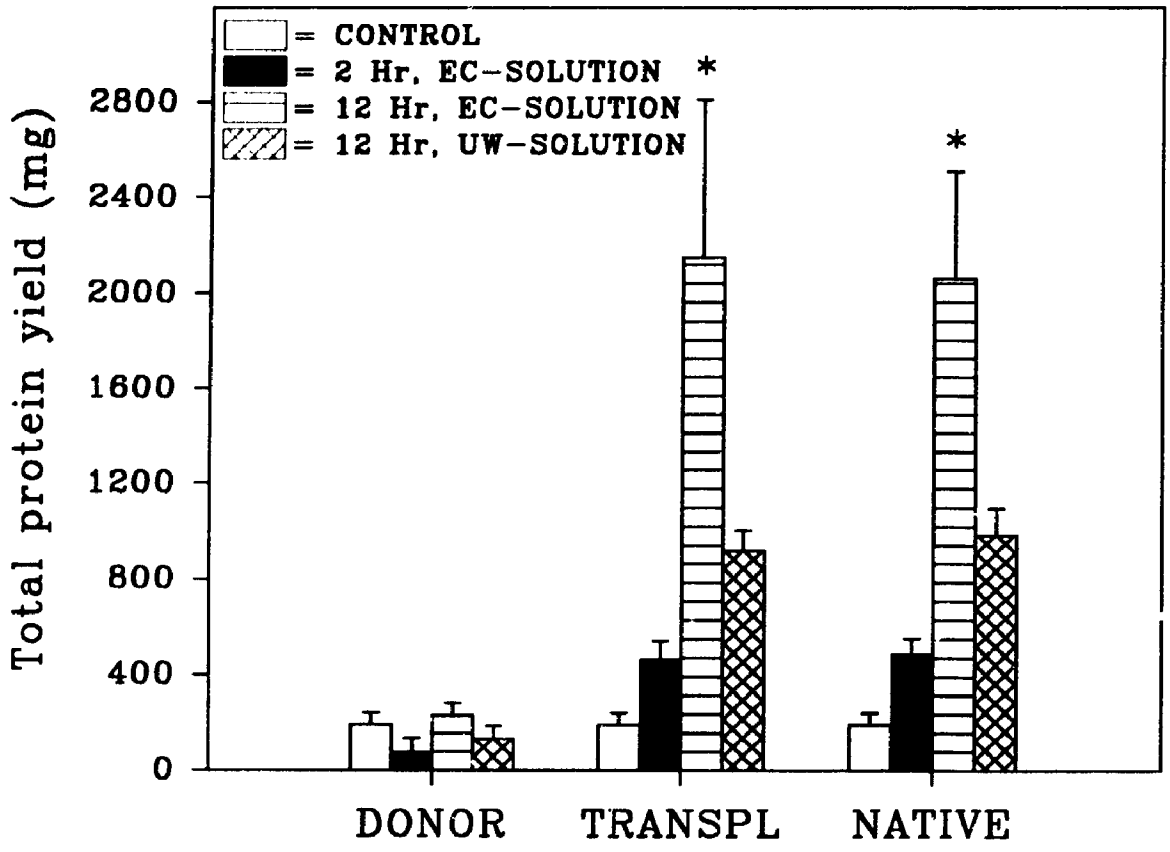
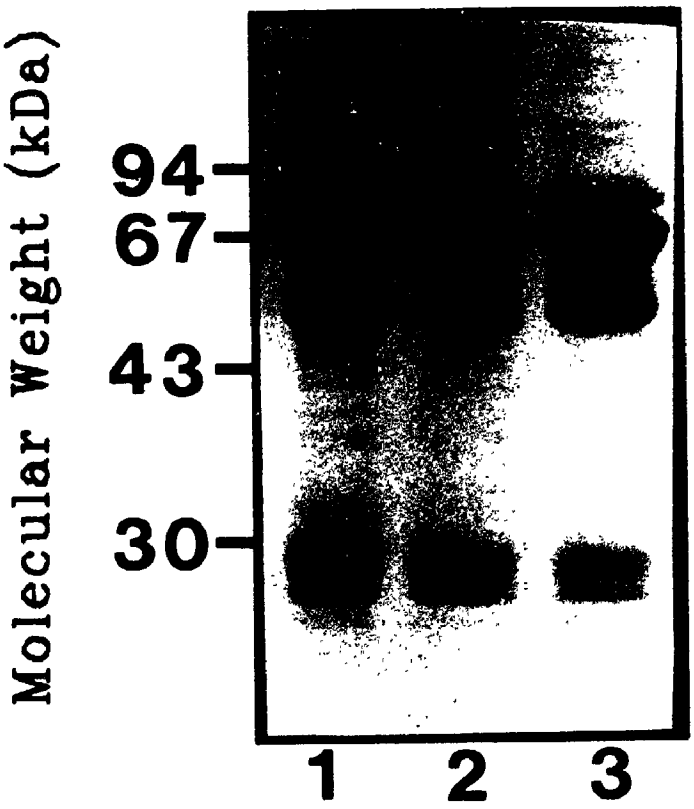

= CONTROL
= 2 Hr, EC-SOLUTION
= 12 Hr, EC-SOLUTION
= 12 Hr, UW-SOLUTION
*
*
Total protein yield (mg)
2800
2400
2000
1600
1200
800
400
0
DONOR
TRANSPL
NATIVE

Figure 4.9. SDS (12%) gel of 15 μg protein of different lung lavages and serum stained with Coomassie blue.

Lane 1) Lavage protein of a native lung from the 12 hours EC-solution group.

Lane 2) Lavage protein of a transplanted lung from the 12 hours EC-solution group.

Lane 3) Serum protein from the recipient animal of the same experiment as the lavage samples.


Molecular Weight (kDa)
94
67
43
30
1
2
3

the present and in previous studies on dog surfactant (King 1984). Klepetko and associates have reported a decrease in the percentage of DPPC with lung ischemic damage related to transplantation (Klepetko et al 1990).

Our study presents the first detailed analysis of surfactant after experimental lung transplantation. Our results show similar alterations in surfactant after experimental lung transplantation as seen in ARDS (Gregory et al 1991) and in many experimental models of ARDS (Holm and Matalon 1989, Robertson 1991).

Little difference in surfactant composition was observed in lungs stored for 2 or 12 hours. The small/large surfactant aggregate ratio was slightly lower in stored lungs compared to controls. These lower values might arise through the uptake of small aggregates during storage. SP-A content was significantly reduced after 12 but not 2 hours of storage. In view of the proposed function of SP-A in host defence mechanisms (Van Iwaarden et al 1990) and in counteracting protein inhibition (Cockshutt et al 1990), this reduced level of SP-A could have a severe impact after transplantation.

Satisfactory gas exchange and no significant alterations in the surfactant system was observed after transplantation of lungs stored for two hours. Twelve hours of storage before transplantation resulted in a marked

reduction in gas exchange, altered phospholipid composition, decreased SP-A content, increased small/large surfactant aggregate ratios and increased serum protein yield. Similar changes have been reported in experimental models of ARDS (Holm and Matalon 1989, Robertson 1991). Serum proteins inhibit surfactant function in vitro and in vivo (Cockshutt et al 1990, Jobe 1989). Cockshutt and associates showed that SP-A has the potential to counteract serum protein inhibition on the pulsating bubble surfactometer (Cockshutt et al 1990). After transplantation, the SP-A level is approximately half the control values while the serum proteins are markedly increased suggesting surfactant could be severely inhibited. Moreover, since SP-A has been implicated in host defence mechanisms (Van Iwaarden et al 1990), the lower SP-A values could result in an increased chance of infection.

Recent reports show that alterations in the small to large surfactant aggregates ratio can occur after lung injury. In radiation pneumonitis, the small to large aggregate ratio is decreased compared to normal (Gross 1991a). On the other hand, acute lung injury induced by N-nitroso-N-methylurethane resulted in an increased small/large aggregate ratio (Lewis et al 1990). Studies using an *in vitro* surfactant conversion system have implicated a serine protease in the formation of small aggregates (Gross and Schultz 1990). The low small/large

aggregate ratio in radiation pneumonitis has been attributed to an increase in protease inhibitor in the lung (Gross 1991b). While an increase in serine protease(s) is thought to be responsible for the increased ratio in the acute lung injury model (Higushi et al 1992).

The phospholipid composition of the small aggregate surfactant fraction appeared to have a slightly higher LPC and SM content than large aggregates on a thin layer chromatogram. Small surfactant aggregates were markedly increased in transplanted lungs, resulting in a higher small to large aggregate ratio. This higher ratio could result from an increase in serine proteases resulting in an increased formation of small aggregates. Neutrophil elastase would be a likely candidate for this role as a protease since it can be increased in ARDS. Alternatively, a decrease in the level of protease inhibitors, such as α-antitrypsin could also be responsible for the changed ratios. Another explanation may be that the lower SP-A values found in the large aggregates of lungs with higher small/large aggregate ratios could permit a more rapid conversion of large to small aggregates during ventilation. Higuchi and associates (Higuchi et al 1992) have demonstrated that the addition of SP-A to large aggregates could block the conversion to small aggregates *in vitro*. Finally, a reduced uptake of small aggregates may also contribute to the increased small to

large aggregate ratios. Further studies are necessary to investigate these possibilities.

Lipid extracts of the large aggregate but not the small aggregate fraction can reduce surface tension to near 0 mN/m in the pulsating bubble surfactometer. This observation agrees with the Wilhelmy balance measurements reported by Gross and Narine (Gross and Narine 1989b) and the pressure-volume curves of Lewis and associates after instilling small aggregates in premature rabbits (Lewis et al 1990). The biophysical assays and the immunoblot analysis of SP-B indicate that the functional impairment of the small aggregate fraction is not due to protein inhibition but is related to a lack of the hydrophobic protein SP-B. Whether an increased amount of small aggregates in the lung impairs the function of the large surfactant aggregates must still be investigated. It is clear, however, that the ratio of surfactant subtypes can change with lung injury.

The alterations in surfactant noted in transplanted lungs after 12 hours of storage were also observed in the native lungs. The PO_2, although initially better in the native lung, decreased to the same level as with the transplanted lungs within 6 hours of reperfusion. These findings imply that the alterations result not only from local effects in the transplanted lung, but are also due to the effects of mediators. Considerable research has focused

on these mediators in transplantation models (Novick et al 1992) but the hierarchy of these mediators is still unknown and may be species specific.

Some studies have observed superior lung preservation after flushing with UW solution (Aeba et al 1992, Hirt et al 1992, Naka et al 1990, 1991) but other have shown only a slight (LoCicero et al 1989, 1990, Semik et al 1990) or no significant benefit (Miyoshi 1992, Oka et al 1991). In our transplant model, UW solution did not appear to have any significant beneficial effect on lung preservation. The efficacy of UW solution in organ preservation has been shown to be clearly related to its shelf-life (Wicomb and Collins 1989) possibly related to conversion of reduced to oxidized glutathione (Astier and Paul 1989). Since none of the studies using UW solution for experimental lung preservation specified the shelf-life of the flush solution, the efficacy of UW solution in lung transplantation remains an open question.

Transplantation after a prolonged ischemic interval appears to alter pulmonary surfactant in several different ways: 1) a change in the phospholipid composition of large aggregates, 2) a decrease in SP-A levels in the large aggregates, and 3) an increase in small/large aggregate ratio. Furthermore, the level of serum proteins in the lung

lavage was increased after transplantation. These changes could inhibit surfactant function and therefore contribute to the impaired gas exchange. Similar changes in surfactant have been noted in animal models of ARDS (Holm and Matalon 1989) and in patients with ARDS (Seeger et al 1990, Gregory et al 1991). In several animal models of ARDS, surfactant supplementation has been successful in restoring gas exchange to normal levels (Robertson 1991). Based on these results we conclude that surfactant supplementation should be investigated in lung transplantation. Furthermore, the reduced levels of SP-A found in our experiments suggest a potential beneficial effect of including SP-A in surfactant for supplementation.

CHAPTER 5 DEGRADATION OF SURFACTANT-ASSOCIATED PROTEIN B (SP-B) OCCURS DURING *IN VITRO* CONVERSION OF LARGE TO SMALL SURFACTANT AGGREGATES.

5.1. Summary

Pulmonary surfactant obtained from lung lavages can be separated by differential centrifugation into two distinct subfractions known as large surfactant aggregates and small surfactant aggregates. The large aggregate fraction is the precursor of the small aggregate fraction. The ratio of the small not surface-active to large surface-active surfactant aggregates increases after birth and in several types of lung injury. We have utilized an *in vitro* system, surface area cycling, to study the conversion of large to small aggregates. Small aggregates generated by surface area cycling were separated from large aggregates by centrifugation at 40,000g for 15 min rather than by the normal sucrose gradient centrifugation. This new separation method was validated by morphological studies.

Surface tension reducing activity of total surfactant extracts, as measured on a pulsating bubble surfactometer, was impaired after surface area cycling. This impairment was related to the generated small aggregates. Immunoblot analysis of large and small aggregates separated by sucrose gradient revealed the presence of detectable amounts of SP-B

in large aggregates but not in small aggregates. SP-A was detectable in both large and small aggregates. We conclude that SP-B is degraded during the formation of small aggregates *in vitro* and that a change in surface area appears to be necessary for exposing SP-B to the protease activity.

5.2. Introduction

Pulmonary surfactant is a mixture of approximately 90% lipid and 10% protein that reduces the surface tension across the air-liquid interface of the alveoli. Surfactant contains at least three surfactant associated proteins, surfactant-associated protein A (SP-A), SP-B and SP-C (Possmayer 1988, Hawgood and Clements 1990). SP-A is a watersoluble glycoprotein while SP-B and SP-C are two small hydrophobic proteins. Another lung specific glycoprotein has been identified and named SP-D, however the association of this protein with surfactant is still controversial.

Differential centrifugation or sucrose gradient centrifugation can separate alveolar surfactant, obtained by lung lavages, into different subfractions (Baritussio et al 1984). The larger or denser subtype contains tubular myelin, lamellar bodies and large vesicles (Magoon et al 1983). These large aggregates contain all three surfactant associated proteins and are capable of reducing surface

tension to low values. Pulse chase studies have demonstrated that the large aggregate subtype is the precursor of smaller, lighter subfractions (Magoon et al 1983, Baritussio et al 1985). The small surfactant aggregate fraction consist mainly of small vesicles, contains less surfactant associated proteins and is functionally inferior to the large aggregates (Yamada et al 1990).

Rapid changes in phospholipid distribution in the different subfractions have been observed at the onset of air breathing in rats (Spain et al 1987). Prior to birth surfactant consists mainly of the heavy subtype, while after birth an increased small aggregate fraction is observed. Changes in surfactant subtype pool sizes also occur in a number of lung injury models. In N-nitroso-N-methylurethane induced lung injury in adult rabbits, the ratio of small to large aggregates was increased several fold over control rabbits (Lewis et al 1990). Similar findings were reported for an ischemic/reperfusion injury related to experimental lung transplantation (chapter 4). On the other hand, radiation pneumonitis in rats resulted in a decreased ratio of small to large aggregates compared to control animals (Gross 1991a). Gross and Narine have developed an *in vitro* system, surface area cycling, to study the mechanisms involved in the conversion of large to small surfactant aggregates (Gross and Narine 1989a). In this procedure a tube containing resuspended large surfactant aggregates is

rotated end over end changing the surface area resulting in the conversion of large aggregates to small aggregates. The large and small aggregates are separated using a sucrose gradient centrifugation. Gross and Schultz have used several protease inhibitors to demonstrate the involvement of a serine protease, which they have named convertase, in the conversion of surfactant aggregates *in vitro* (Gross and Schultz 1990). This protease has recently been tentatively identified as a 75 kDa protein closely associated with surfactant phospholipid (Gross and Schultz 1992).

Surface area cycling has also been employed to study the altered surfactant subfraction ratios after lung injury. In N-nitroso-N-methylurethane induced lung injury the increased ratio of small to large surfactant aggregates was correlated with an increased conversion of large to small aggregates *in vitro*. The authors suggested that an increase in protease may be responsible for the increased conversion rate (Higuchi et al 1992). The decreased ratio of small to large aggregates in radiation pneumonitis has been explained by an increase in protease inhibitor (Gross 1991b).

In this report we describe the development of a more rapid method of separating large and small aggregates after surface area cycling. In addition, evidence for the degradation of surfactant-associated protein B during surface area cycling is presented.

5.3. Materials and Methods

5.3.1. Preparation of large aggregates

Normal dog lungs were lavaged twice via the trachea using approximately 1 L 0.15 M NaCl, 1.5 mM $CaCl_2$. The lung lavage was centrifuged at 150g for 10 min at 4°C to remove cells and cellular debris. A large surfactant aggregate pellet was obtained by centrifugation at 40,000g for 15 min at 4°C. The large aggregates were resuspended in conversion buffer (0.15 M NaCl, 10 mM Tris, 1 mm $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4) and immediately frozen at - 20°C.

5.3.2. Surface area cycling

Large surfactant aggregates were resuspended in conversion buffer at a concentration of 0.25 mg phospholipid/ml. Two ml aliquots were placed in plastic tubes (Falcon 2058), capped, and attached to a rotator (Roto-torque rotator, Cole-Parmer Instruments). The tubes were cycled at 40 RPM at 37°C so that the surface area changed from 1.1 cm^2 to 9.0 cm^2 twice each cycle (Gross and Narine 1989a, Higuchi et al 1992). Unless otherwise specified, samples were cycled for 180 min. Identical, non-cycled control samples were kept at 37°C for the same duration as the cycled samples.

Some experiments utilized the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The PMSF was dissolved in ethanol (500 μM) and added to the samples to a final concentration of 10 μM. An equal amount of ethanol was added to samples not receiving PMSF.

5.3.3. Separation of large and small aggregates

Sucrose density gradient centrifugation was used to separate the large and small surfactant aggregates (Higuchi et al 1992). Samples were loaded on a linear sucrose gradient from 0.1 M to 0.75 M sucrose in conversion buffer. The gradients were centrifuged for 60 hr at 74,000g at 6°C in a SW-28 swinging-bucket rotor. Each gradient was fractionated into 1 ml fractions. Aliquots were taken for refractive index determination (50 μl), phospholipid measurement (0.8 ml) and immunoblot analysis for SP-A and SP-B (50 μl). The refractive index was measured at room temperature and used to calculate the sucrose density of the fractions. The lipids were extracted into chloroform (Bligh and Dyer 1959) and phospholipid phosphorus measurements were performed (Duck-Chong 1979).

As an alternative to sucrose density gradient centrifugation large and small aggregates were separated by centrifugation at 40,000g for 15 min at 4°C. Total phospholipid in the large aggregates pellet and in the small

surfactant aggregates in the remaining supernatant were determined by lipid extraction (Bligh and Dyer 1959) and phosphorus analysis (Rouser et al 1970). A validation of this procedure is given in the result and discussion section of this chapter.

5.3.4. Morphological studies

Surfactant suspensions were fixed in glutaraldehyde (2.5% final concentration) in the conversion buffer. After the addition of fixative samples were incubated at 37°C for 4 hours, and then centrifuged for 10 min at 7,000g. After this primary fixation the pellets were incubated in 1% OsO_4, 1.5% $K_4Fe(Cn)_6$ for 1 hour. Samples were dehydrated in a graded series of alcohol and rinsed in two changes of absolute acetone. Polybed 812 (Polysiences, Warrington, PA) was used to embed the samples. Thin sections were counterstained with uranyl acetate and lead citrate. Representative areas were photographed and printed at a final magnification of 17,940x. Post-fixation and electron microscopy were kindly performed by Dr Stephen Hearn (Dept of Pathology, The University of Western Ontario).

5.3.5. Dot blot and western blot analysis

Samples were spotted on nitrocellulose using a Bio-Dot microfiltration apparatus. The nitrocellulose was blocked for 30 min with 3% gelatin in Tris-buffered saline (TBS, 20 mM Tris 500 mM NaCl pH 7.5), washed with 0.05% Tween 20 in TBS and incubated with the primary antibody in 1% gelatin, 0.05 Tween 20 in TBS for 1 hr.. The blot was washed twice and incubated for 1 hr with the secondary antibody, alkaline phosphatase conjugated goat anti-mouse (1 in 3000 dilution). After washing, the colour was developed with 20 mM Tris, 20 mM NaCl, 1 mM $MgCl_2$, 0.04 μM p-nitroblue tetrazolium chloride, 3.8 μM 5-bromo-4-chloro-3-indoylphosphate, pH 9.5 for 5 to 10 min The reaction was stopped by washing with water.

For western blots, 12% SDS gels were developed according to Laemmli (Laemmli 1970). After electrophoresis the proteins were transferred to nitrocellulose using a Bio-Rad Trans-Blot Cell. Transfer was carried out at 4°C, 100 V for 4 hours using 25 mM Tris 192 mM glycine 20% methanol as a transfer buffer. SP-A was detected on the nitrocellulose as described for the dot blot analysis.

5.3.6. Biophysical assay

Lipid extracts of cycled or noncycled, large and small surfactant aggregates were obtained by chloroform extraction by the method of Bligh and Dyer and subsequent acetone

precipitation (Bligh and Dyer 1959, Weber and Possmayer 1984). Dried extracts were resuspended in 0.15 M NaCl, 1.5 mM $CaCl_2$ to a final concentration of 5 mg phospholipid/ml. Samples were incubated for at least 90 min at 37°C before being analyzed with a pulsating bubble surfactometer (Electronetics Corporation) as described by Enhorning (Enhorning 1977). With this technique a bubble is created in a surfactant suspension. After 10 seconds, the bubble is pulsated between the maximum bubble radius (R_{max}) of 0.55mm and a the minimum bubble radius (R_{min}) of 0.4mm at 37°C. The pressure across the air liquid interface is measured by a pressure transducer. Surface tension was calculated by the law of Young and Laplace which states that the pressure across a sphere is directly proportional to twice the surface tension and indirectly proportional to the radius. Surface tensions at R_{max} and R_{min} were expressed.

5.4. Results

Separation of small and large aggregates from lung lavages is frequently done by high speed centrifugation (Lewis et al 1990, chapter 4, Baritussio et al 1984). However, for surface area cycling aggregate separation has normally been performed by a time consuming density centrifugation (Higuchi et al 1992, Gross and Narine 1989a). To test whether centrifugation at 40,000g could be used to

separate the small and large aggregates after surface area cycling, samples of large surfactant aggregates were cycled for various periods of time. After cycling, the surfactant suspensions were centrifuged for 15 min at 40,000g and the phospholipid content of the supernatants and pellets were determined by phosphorus analysis. The results (figure 5.1) show a steady increase in the amount of phospholipid-phosphorus in the supernatant with increasing time of cycling and a corresponding steady decrease in phospholipid in the pellet.

To validate the centrifugation method for aggregate separation after surface area cycling, the results obtained by this method were compared with those of the density centrifugation method used in previous studies. Large surfactant aggregates were cycled for 180 min. Noncycled, cycled, as well as the supernatant obtained after a 40,000g spin of cycled surfactant were centrifuged on a sucrose gradient. Figure 5.2 shows the phosphorus content in the different fractions of these gradients. Surfactant that has not been cycled consists mainly of large aggregates (density 1.07-1.06) while after cycling there is a marked increase in small aggregates (density 1.064-1.04). The supernatant obtained by 40,000g centrifugation after cycling contains phosphorus in the same density range as the small aggregates peak of cycled surfactant. The percentage of phosphorus in small aggregates after cycling was similar for the two

Figure 5.1. The effect of surface area cycling on the amounts of phosphorus recovered in a 40,000g supernatant and pellet. All samples were kept at 37°C for the 180 min period.

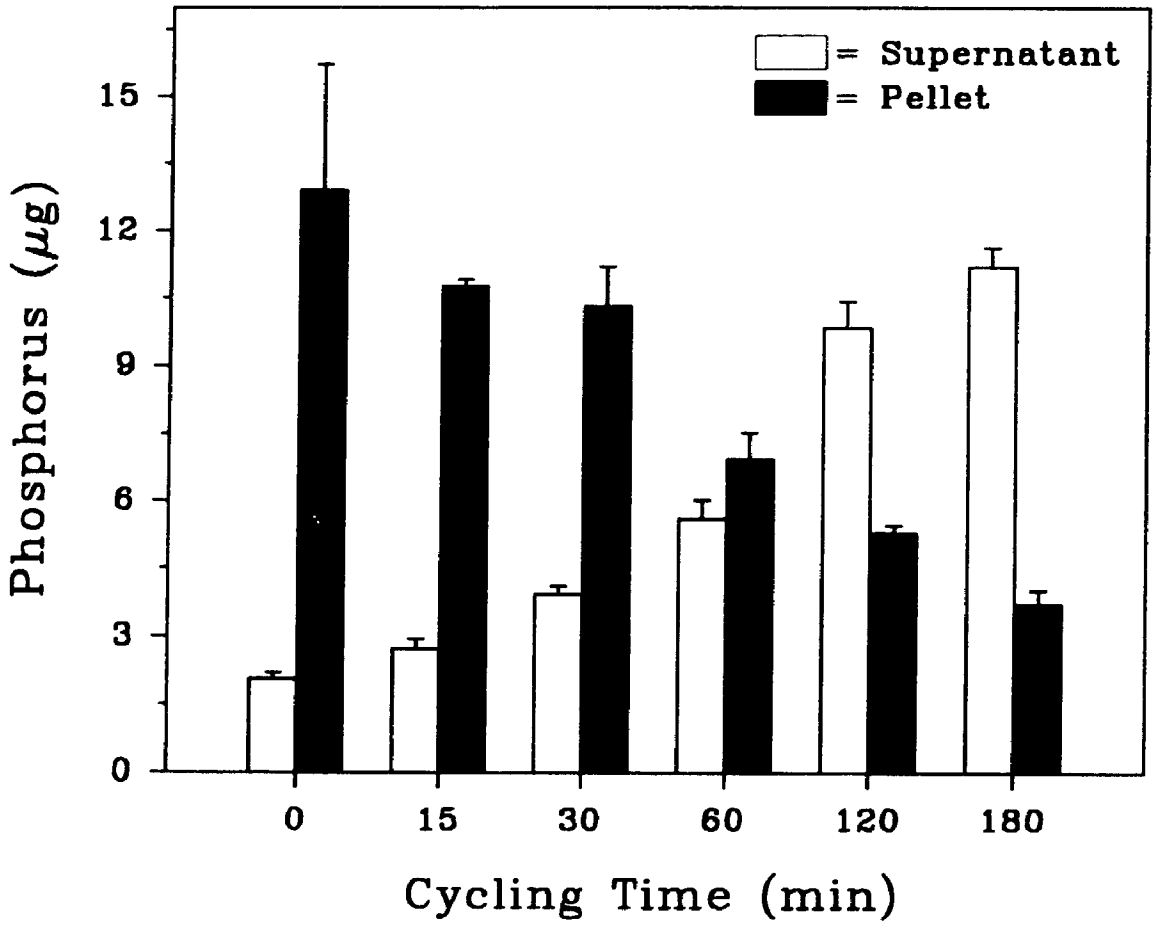
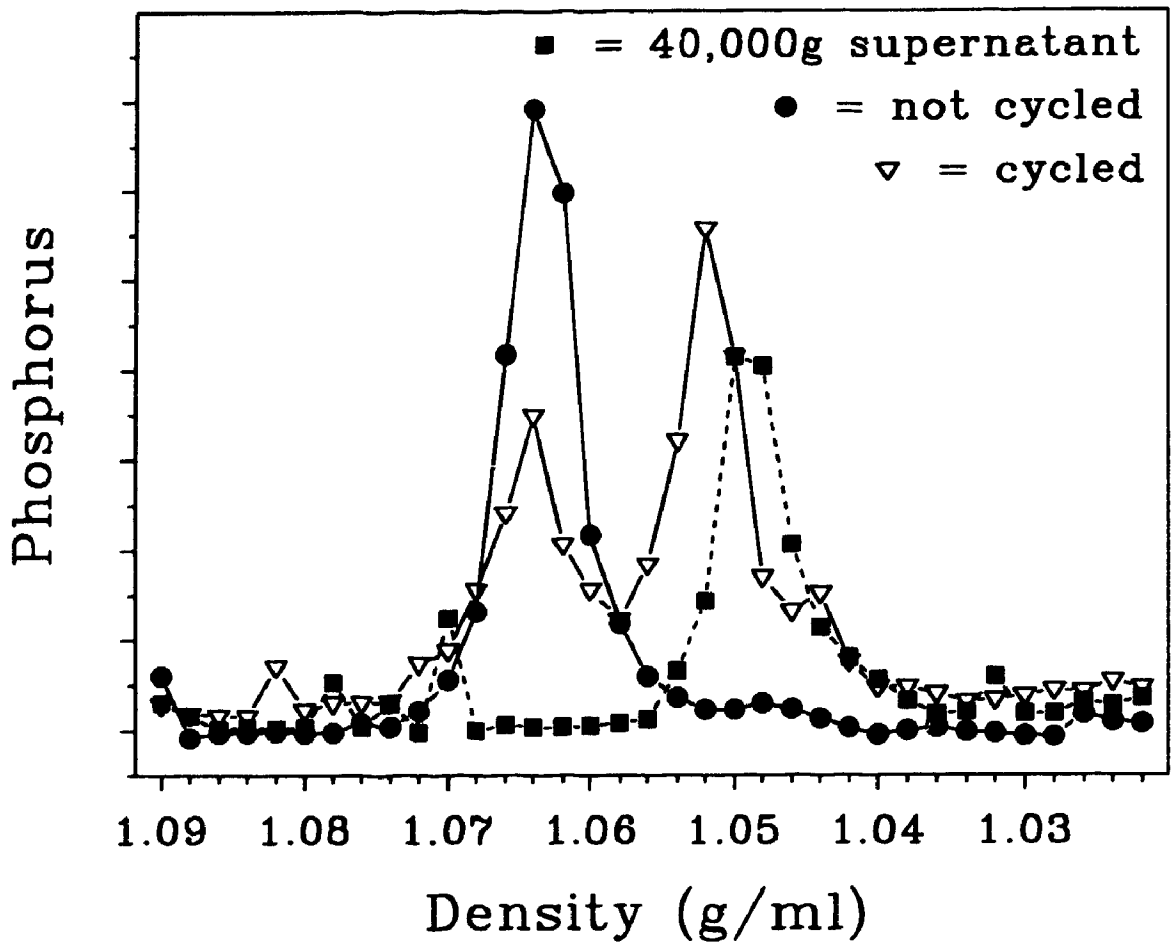

= Supernatant
= Pellet
Phosphorus (μg)
0
3
6
9
12
15
0
15
30
60
120
180
Cycling Time (min)

Figure 5.2. Sucrose gradient density centrifugation of 40,000g supernatant, not cycled and cycled large aggregates. The 40,000g supernatant analyzed by sucrose gradient was obtained from the centrifugation of a similar amount of phosphorus as the cycled and the noncycled preparation.


■ = 40,000g supernatant
● = not cycled
▽ = cycled
Phosphorus
1.09
1.08
1.07
1.06
1.05
1.04
1.03
Density (g/ml)

methods, 59% for the gradient method and 58% for the centrifugation method.

The 40,000g centrifugation procedure for separating large and small aggregates was further validated by electron microscopy. Figure 5.3 shows the morphological difference between large and small surfactant aggregates. Dense lipid structures, tubular myelin and large vesicles are observed in the large aggregates (figure 5.3A), while the small aggregate fraction obtained after cycling contains only small vesicles and appear relatively uniform (figure 5.3B).

The effect of incubation and cycling with protease inhibitor PMSF on surfactant conversion is shown in Table 1. Large aggregates were incubated for 3 hr at 37°C, and then cycled for 3 hr at 37°C. PMSF was added either before the incubation, before the cycling or after cycling. Small and large aggregates were separated by centrifugation at 40,000g and measured by phosphorus analysis. The results (table 1) show inhibition of conversion by PMSF when the protease inhibitor is present during cycling. Preincubation in the presence or absence of PMSF does not affect this result. Addition of the protease inhibitor after cycling does not influence the amount of small aggregates generated.

The biophysical activities of lipid extracts of cycled and control surfactant examined on the pulsating bubble surfactometer are shown in figure 5.4. Noncycled surfactant contains about 98% large surfactant aggregates whereas

Figure 5.3. Electron micrographs of large and small surfactant aggregates.

A) Large aggregates, obtained from a noncycled sample.

B) Small aggregates, obtained from a 40,000g supernatant after surface area cycling of large surfactant aggregates.


B
A

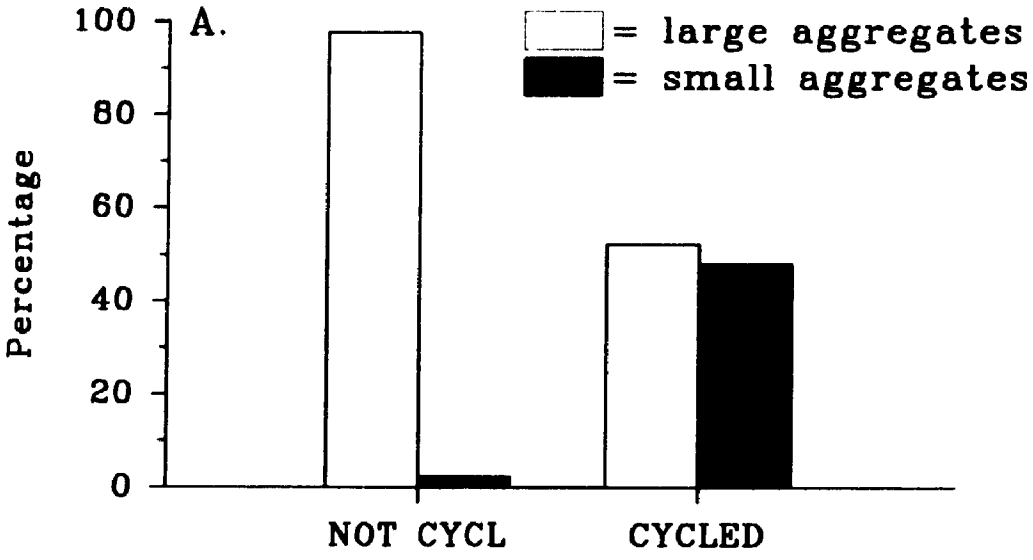
Table 5.1. The effect of incubation at 37°C and subsequent surface area cycling in the presence (+) and absence (-) of 10 μM PMSF on the formation of small aggregates as determined by centrifugation at 40,000g.

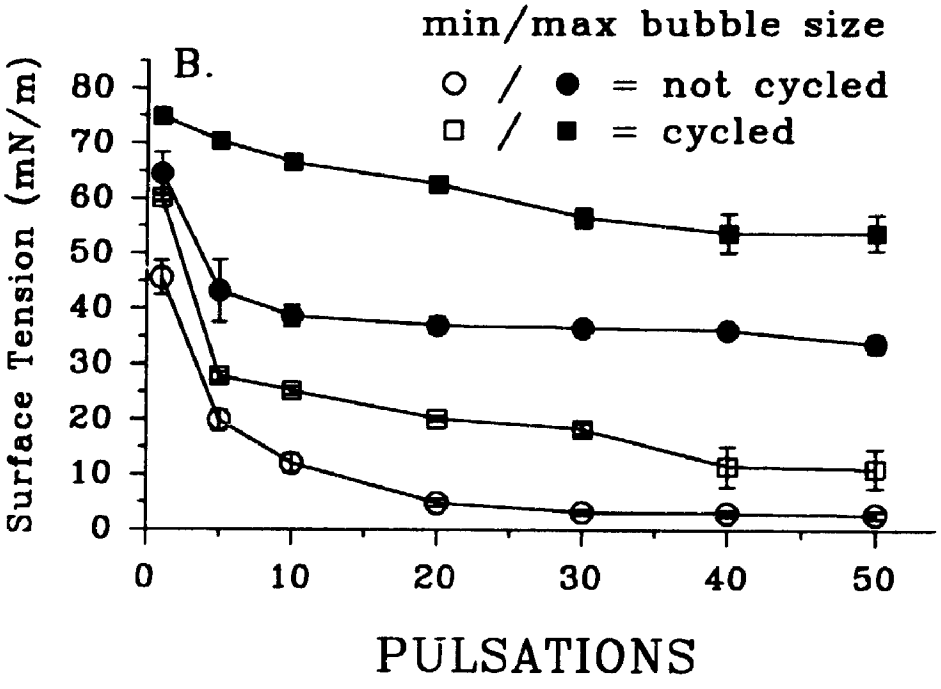
Time		Presence of PMSF			Phospholipid in small aggregates
Incubation	Cycling	Incubation	Cycling	40000g	% of total
6 hr	0 hr	-	-	-	7%
3 hr	3 hr	-	-	-	63%
3 hr	3 hr	-	-	+	64%
3 hr	3 hr	-	+	+	15%
3 hr	3 hr	+	+	+	12%

Figure 5.4. The effect of surface area cycling on the biophysical activities of lipid extracts.

A) Percentages of total phospholipid in small and large aggregates before and after surface area cycling.

B) Biophysical activity of lipid extracts of noncycled and cycled surfactant on the pulsating bubble surfactometer. Samples were analyzed at 5 mg phospholipid/ml at 20 pulsations per minute. (R_{min} is expressed in the open symbols, R_{max} is expressed in the closed symbols).


A.
100
80
60
40
20
0
Percentage
= large aggregates
= small aggregates
NOT CYCL
CYCLED

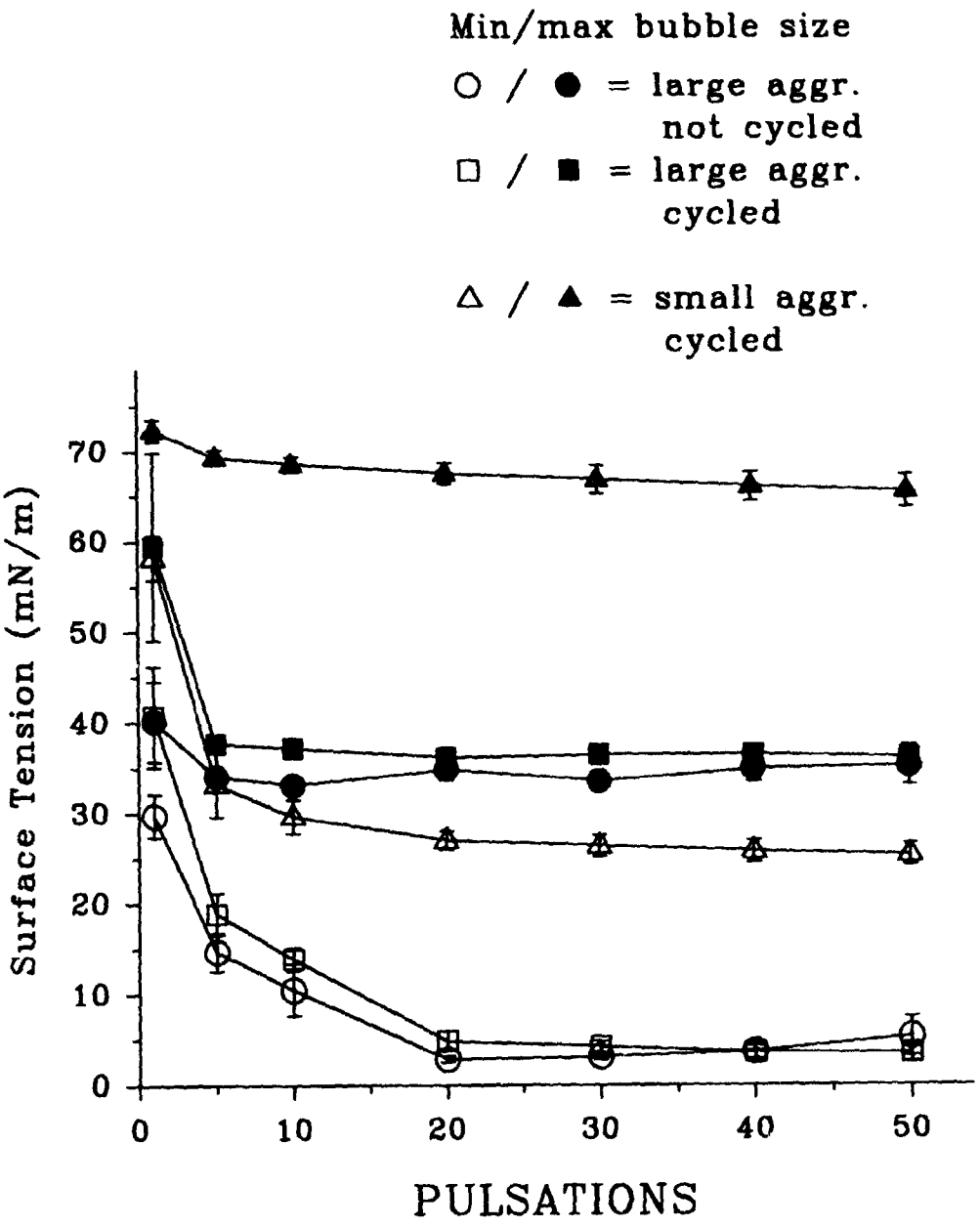

B.
min/max bubble size
○ / ● = not cycled
□ / ■ = cycled
Surface Tension (mN/m)
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
PULSATIONS

cycled surfactant contains approximately 50% large and 50% small aggregates (figure 5.4A). Low surface tension values were obtained with lipid extracts of noncycled surfactant within 50 pulsations (figure 5.4B) whereas lipid extracts of cycled surfactant do not reach near zero values within 50 pulsations.

To observe whether the difference between the biophysical activity of noncycled and cycled surfactant is related to the generation of small aggregates, small and large aggregates were assayed on the pulsating bubble surfactometer. Large and small aggregates were isolated by 40,000g centrifugation. Lipid extracts of large aggregates from both noncycled and cycled surfactant rapidly reduce the surface tension to near zero values (figure 5.5). Small aggregates exhibit very poor surface tension reducing activity.

Lipid extracts of surfactant contain the surfactant lipids and SP-B and SP-C but do not contain surfactant-associated protein A. The lipid profile of noncycled and cycled, large aggregates and small aggregates appeared similar by thin layer chromatography (not shown). Immunoblots detected the presence of SP-A in both large and small aggregates isolated by 40,000g centrifugation. However, SP-B was only detected in large aggregates (not shown).

Figure 5.5. Biophysical activity of lipid extracts of large and small surfactant aggregates. Large aggregates were obtained as a 40,000g pellet from noncyled and cycled samples, small aggregates were obtained from the 40,000g supernatant of the cycled sample. Samples were analyzed at 5 mg phospholipid/ml at 20 pulsations per minute. (R_{min} = open symbols, R_{max} = closed symbols).


Min/max bubble size
○ / ● = large aggr. not cycled
□ / ■ = large aggr. cycled
△ / ▲ = small aggr. cycled
Surface Tension (mN/m)
0
10
20
30
40
50
60
70
0
10
20
30
40
50
PULSATIONS

A more detailed analysis of the effect of surface area cycling on SP-A and SP-B using sucrose gradients is shown in figure 5.6. Small and large aggregates from noncycled and cycled surfactant were separated by centrifugation on a sucrose gradient. After fractionation, aliquots of each fraction were used for phosphorus analysis. Immunoblot analysis for SP-A and SP-B were performed on every second fraction. As shown in figure 5.6A, noncycled surfactant consist mainly of large aggregates in which both SP-A and SP-B are detected. After cycling the surfactant mainly consists of small aggregates (figure 5.6B). Immunoblots detected little or no SP-B in these fractions, SP-A on the other hand was detectable in the small aggregate fractions (figure 5.6B).

Figure 5.7 shows a Western blot analysis of SP-A before and after cycling as well as from large and small aggregates. In all fractions the apparent molecular weight of the immunoreactive products is similar to purified SP-A.

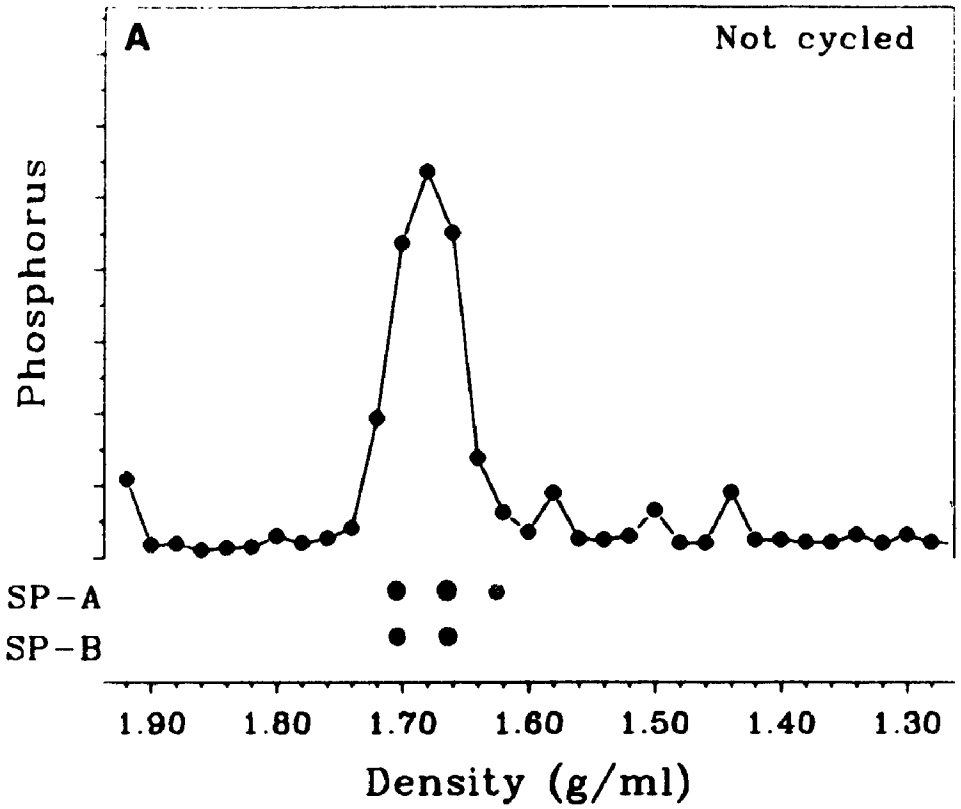
5.5. Discussion

Pulmonary alveolar surfactant has several different physical forms, such as lamellar bodies, tubular myelin and vesicles (Magoon et al 1983). Some of these different forms of surfactant can be separated according to their buoyant densities (Magoon et al 1983, Baritussio 1984, Wright et al

Figure 5.6. Sucrose gradient density separation and dot blot analysis before and after surface area cycling. Surfactant small and large aggregates were separated on a sucrose gradient, the gradient was fractionated and analyzed for phosphorus and the presence of SP-A and SP-B.

A) Noncycled surfactant

B) Cycled surfactant


A
Not cycled
Phosphorus
SP-A
SP-B
1.90
1.80
1.70
1.60
1.50
1.40
1.30
Density (g/ml)

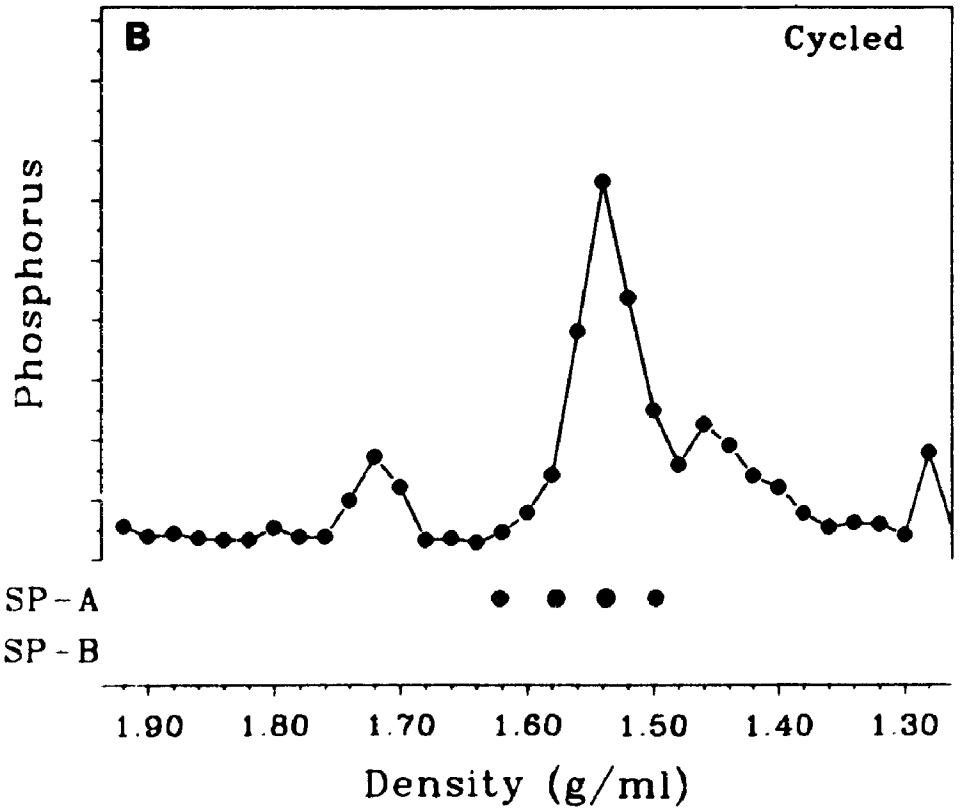

B
Cycled
Phosphorus
SP-A
SP-B
1.90
1.80
1.70
1.60
1.50
1.40
1.30
Density (g/ml)

Figure 5.7. Western blot analysis for SP-A before and after surface area cycling. Both the glycosilated and unglycosilated form of SP-A are detected.

Lane 1. SP-A standard

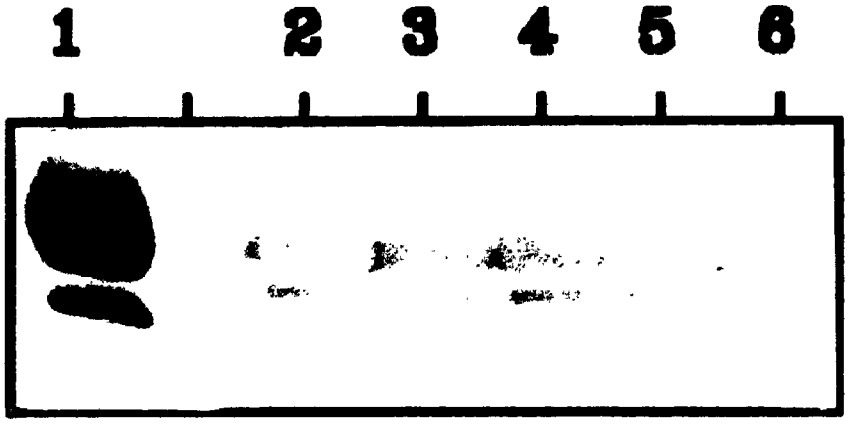
Lane 2. noncycled surfactant

Lane 3. cycled surfactant

Lane 4. large aggregates from noncycled surfactant

Lane 5. large aggregates from cycled surfactant

Lane 6 small aggregates from cycled surfactant.


1
2
3
4
5
6

1984). Surface area cycling represents an *in vitro* system with which the conversion from large to small surfactant subtypes can be studied (Gross and Narine 1989a).

We have modified the time consuming sucrose gradient separation for a single 40,000g centrifugation for separating large and small aggregates. This method has been validated by morphology and by the density of 40,000g isolated small aggregates on a sucrose gradient. The density observed for small aggregates in this study is slightly higher then in previous reports (Higuchi et al 1992, Gross and Narine 1989a, Gross and Schultz 1990). Whether the difference between our observations and those in other studies arises from species specificity or a difference in procedures is not known. However, the morphology of the small aggregate fraction clearly shows the presence of small vesicles and a lack of tubular myelin or myelin like structures.

Advantages of the 40,000g centrifugation method include a dramatic decrease in centrifugation time as well as the ability to analyze a greater number of samples as one phosphorus analysis will determine the amount of small aggregates generated by cycling. However, in contrast to sucrose gradient centrifugation, this method of separation does not differentiate between the heavy and ultra-heavy surfactant subtypes. Therefore, although 40,000g centrifugation can be used for rapid analysis of large to

small surfactant aggregate conversion, sucrose density centrifugation remains the method of choice for a more detailed analysis and studies on ultra-heavy surfactant subtypes.

A serine-protease, named convertase, has been reported to be involved in the conversion of large to small surfactant aggregates *in vitro* (Gross and Schultz 1990). We have tested whether convertase activity is dependent on the change in surface area. If convertase cleaves its target protein only during surface area cycling, cycling in the presence of inhibitor would result in inhibition of conversion regardless of the preincubation. Alternatively, if convertase cleaves its target and in doing so produces a surfactant that can convert to small aggregates, incubation in the absence of inhibitor followed by cycling in the presence of inhibitor would still lead to the formation of small aggregates. Preincubation in the presence or absence of PMSF did not affect the extend inhibition of aggregate conversion by PMSF during cycling. This suggests that for aggregate conversion to occur the protease activity was necessary during surface area cycling.

Small surfactant subtypes isolated from lung lavages of normal lungs have been reported to have poor surface activity both *in vitro* and *in vivo* (Yamada et al 1990, chapter 4). Our biophysical studies using lipid extracts show that large aggregates subjected to surface area cycling

lose some of their surface tension reducing ability. This effect was presumably related to the generation of small aggregates since both cycled and noncycled large aggregates reduce surface tension rapidly. Similar to small aggregates isolated from lung lavages, small aggregates generated *in vitro* do not reduce surface tension to low values. Lipid extracts contain surfactant lipids and the two hydrophobic surfactant-associated proteins B and C. These hydrophobic proteins appear to be important for the surface tension reduction by increasing adsorption and lipid squeeze-out of the monolayers (Yu and Possmayer 1990, 1992). Since lipid extract surfactant was used in the biophysical assays, the differences observed cannot be attributed to protein inhibition or to changes in SP-A content. Since no changes in lipid composition were observed after cycling and a serine protease has been implicated in conversion we hypothesized that the change in biophysical activity could be due to degradation of one of the hydrophobic proteins. This was investigated by dot blot analysis after sucrose gradient separation.

Both the water soluble SP-A and hydrophobic protein SP-B were analyzed. Whereas SP-A was detectable in both large and small aggregates, immunoreactive SP-B was only present in large aggregates. We assumed that SP-B was degraded during surface area cycling. SP-B was not degraded during incubation at 37°C without surface area cycling,

suggesting that the change in surface area was necessary for exposing SP-B to convertase activity.

Interestingly, after surface area cycling SP-A remains associated with small aggregates. This SP-A could be important for the reuptake of these lipids by Type II cells (Wright et al 1987). Studies on the relative reuptake of small aggregates compared to other forms of surfactant such as large aggregates or large vesicles would be informative.

The observation that SP-B was degraded was somewhat surprising in view of the studies by Higuchi et al who were able to block aggregate conversion by adding SP-A to large aggregates (Higuchi et al 1992). However, this blocked conversion could be accounted for by the formation of ultra-heavy surfactant aggregates. SP-A degradation was also suggested by earlier studies that showed small aggregates obtained from lung lavages contain relatively less SP-A than large surfactant aggregates (Wright et al 1984) however, SP-B was not measured.

From the results reported here we hypothesize that during the increase in surface area surfactant adsorbs to the air liquid interface. The process of adsorption exposes SP-B to convertase, however in large aggregates SP-B is inaccessibly packed in the lipid structures. During the subsequent decrease in surface area the altered, SP-B depleted surfactant would form small aggregates. When convertase is either absent or inhibited, the surfactant

adsorbed to the air liquid interface can flow back into large aggregates upon the decrease in surface area. Conversely, when convertase is increased, or its inhibitors decreased, the formation of small aggregates might occur faster. This hypothesis could be extended to the *in vivo* situation in which the respiration would cause the change in surface area. This would explain the results by Spain et al who observed mainly large aggregates before, and the generation of small aggregate after, the onset of air-breathing in rats (Spain et al 1987).

In several lung injury models the ratio of small to large surfactant aggregates is increased (Lewis et al 1990, chapter 4). Although not measured in those studies an increase in neutrophil elastase has been observed in Adult Respiratory Distress Syndrome (Lee et al 1981, McGuire et al 1982). Pison et al reported that incubation of surfactant preparations with neutrophil elastase can affect the biophysical activity of lipid extracts, presumably by cleavage of one or both of the hydrophobic proteins (Pison et al 1989). This raises the possibility that in lung injury other proteases, such as neutrophil elastase, can contribute to an increased conversion of large to small aggregates. Reconstitution of purified surfactant with pure proteases in combination with surface area cycling could be used to investigate this possibility further.

CHAPTER 6 SURFACE AREA CYCLING OF DIFFERENT SURFACTANT PREPARATIONS; SP-A AND SP-B ARE ESSENTIAL FOR LARGE AGGREGATE INTEGRITY.

6.1. Summary

Surface area cycling is an *in vitro* procedure for the conversion of large to small surfactant aggregates. In this procedure a tube containing a surfactant suspension is rotated at 37°C so that the surface area of the suspension changes twice each cycle. We have utilized this method to study the mechanisms involved in aggregate conversion. Several different surfactant preparations were analyzed; 1) Bovine natural surfactant, a sucrose gradient purified material containing surfactant phospholipid and surfactant associated proteins (SP-) SP-A, SP-B and SP-C. 2) Lipid extract surfactant which contains the surfactant phospholipids and SP-B and SP-C. 3) Mixtures of DPPC:PG 7:3 reconstituted with one or more surfactant proteins. Aggregate conversion was measured by phosphorus analysis of a 40,000g supernatant (small aggregates) and pellet (large aggregates) before and after surface area cycling. Surface area cycling of lipid extract surfactant or lipids plus SP-B or SP-C resulted in rapid aggregate conversion. Lipids alone did not convert. Only a small percentage of

natural surfactant was converted to small aggregates. Addition of SP-A to lipid extract surfactant could inhibit aggregate conversion of this material but this was only observed when an additional 1% (w/w) of SP-B was added to the lipid extract. Addition of trypsin or collagenase to natural surfactant and subsequent surface area cycling resulted in an increased formation of small aggregates.

It is concluded that SP-A is important for large aggregate integrity. It appears that SP-A acts in conjunction with SP-B. The presence of SP-B and/or SP-C is required for aggregate conversion; it is proposed that this reflects the necessity for lipid adsorption in aggregate conversion.

6.2. Introduction

Pulmonary surfactant prevents alveolar collapse by reducing the surface tension across the air-liquid interface of the alveoli. Surfactant is a mixture of approximately 90% lipid and 10% protein (Yu et al 1983). The protein component of surfactant consists of at least three proteins, surfactant-associated protein A (SP-A), SP-B and SP-C (Possmayer 1988). As described earlier (Chapter 1), SP-A is a collagen-like glycoprotein with a large number of ascribed functions; the formation of tubular myelin (Suzuki et al 1989), the enhancement lipid adsorption (Chung et al 1989,

Schürch et al 1992) the counteraction of blood protein inhibition (Cockshutt et al 1990), the regulation of secretion and reuptake of surfactant (Wright and Clements 1987) and an involvement in host defence mechanisms (Van Iwaarden 1991). SP-B and SP-C are two small hydrophobic proteins important for the formation and maintenance of the surface active monolayer (Yu and Possmayer 1992). A fourth lung specific protein from alveolar lavage has been isolated and named SP-D (Persson et al 1988), however the bulk of this protein in lavage is not associated with the surface-active large surfactant aggregates (Hawgood and Shiffer 1991, Kuroki et al 1991).

Pulmonary surfactant obtained from lung lavages consists of different subfractions. These subfraction differ in morphological appearance, buoyant density and protein composition (Magoon et al 1983, Baritussio et al 1984). The relationship between the different subfractions has been studied by pulse chase experiments. These studies showed that the larger, heavier subfraction acts as the metabolic precursors of the smaller, lighter surfactant subtype (Magoon et al 1983, Baritussio et al 1984). The conversion of large to small surfactant aggregates can be reproduced *in vitro* by a technique known as surface area cycling (Gross and Narine 1989a). In surface area cycling, aggregate conversion is promoted by rotating a tube containing a suspension of large aggregates end over end so that the

surface area of the suspension changes from 1.1 cm^2 to 9.0 cm^2 twice each cycle (Gross and Narine 1989a). The conversion of large to small aggregates in this method is dependent on a change in surface area and serine protease activity (Gross and Schultz 1990, 1992). Conversion is blocked at low temperatures (Gross and Schultz 1990, 1992).

Although the change in surface area during cycling is much larger than the area change during respiration, results obtained with surface area cycling have correlated well with *in vivo* findings. In N-nitroso-N-methylurethane-induced lung injury in rabbits an increased ratio of small to large surfactant aggregates in lung lavage was associated with an increased rate of conversion of large to small aggregates *in vitro* (Higuchi et al 1992). The authors suggest that an increase in serine protease(s) might be responsible for this increased conversion (Higuchi et al 1992). A decreased ratio of small to large surfactant aggregates in lung lavage has been observed in radiation pneumonitis in rats (Gross 1991a). Surface area cycling of large aggregates obtained from lung lavages of these injured rats showed a decreased aggregate conversion compared to normal (Gross 1991b). An increase in serine protease inhibitor was suggested to be responsible for this decreased rate of aggregate conversion (Gross 1991b). Furthermore, as was observed with small aggregates obtained from lavage, the small aggregates

obtained by surface area cycling do not contain SP-B and are impaired in their biophysical activity (chapter 5).

Previous studies on surface area cycling have used relatively crude preparations of surfactant. To investigate the mechanisms involved and the roles of individual surfactant components in aggregate conversion, experiments described in this chapter were performed with purified surfactant proteins, lipids, lipid extract surfactant (LES) and sucrose gradient purified, natural surfactant. It is concluded that lipid adsorption is essential for small aggregate formation. Furthermore SP-A and SP-B appear necessary for large aggregate integrity.

6.3. Materials

Bovine lipid extract surfactant (LES) was a kind gift from bLES Biochemicals. Purified bovine SP-B and SP-C were kindly provided by Riad Qanbar and Dr. Dave Bjarneson of this laboratory. Bovine natural surfactant and purified bovine SP-A were generously provided by Kevin Inchley of this laboratory.

6.4. Methods

6.4.1. Surfactant preparation and reconstitution

Natural surfactant was prepared as described previously (Yu et al 1983). The lyophilized powder was resuspended in saline 1.5 mM $CaCl_2$ and centrifuged at 40,000g for 15 min at 4°C. The pellet was resuspended in conversion buffer (0.15 M NaCl, 10 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4) and frozen at -20°C until used. LES was prepared by chloroform extraction and subsequent acetone precipitation of natural surfactant as described by Cockshutt et al (Cockshutt et al 1991). Bovine SP-A was purified using a mannose-affinity column (Cockshutt et al 1991).

For the reconstitution of SP-B and SP-C with lipid, the protein was combined with the lipids (DPPC:PG 7:3) and extracted by the method of Bligh and Dyer (Bligh and Dyer 1959). The lipid/protein mixtures in the chloroform layer were dried under nitrogen. The samples were resuspended in conversion buffer by vortexing for 15 minutes at room temperature. An identical approach was used for the reconstitution of SP-B with LES. LES reconstituted with SP-A was prepared by resuspending dried LES with buffer containing SP-A. Calcium was added to a final concentration of 5 mM and the suspension was incubated at 37°C overnight.

6.4.2. Surface area cycling

Different surfactant preparations were resuspended in conversion buffer at a concentration of 0.25 mg

phospholipid/ml. Two ml aliquots were placed in plastic tubes (Falcon 2058), capped, and attached to a rotator (Roto-torque rotator, Cole-Parmer Instruments). The tubes were cycled at 40 RPM at 37°C so that the surface area changed from 1.1 cm^2 to 9.0 cm^2 twice each cycle (Gross and Narine 1989a, Higuchi et al 1992). Unless otherwise specified, samples were cycled for 180 min. Identical, non-cycled control samples were kept at 37°C for the same duration as the cycled samples.

6.4.3. Separation of large and small aggregates

Large and small aggregates were separated by centrifugation at 40,000g for 15 min at 4°C (chapter 5). Total phospholipid in the large aggregates pellet and in the small surfactant aggregates remaining in the supernatant were determined by lipid extraction (Bligh and Dyer 1959) and subsequent phosphorus analysis (Rouser et al 1970).

6.4.4. Biophysical assays

For the surface tension reducing activity assays, lipid extracts were obtained by chloroform extraction by the method of Bligh and Dyer (Bligh and Dyer 1959). Dried extracts were resuspended in 0.15 M NaCl, 1.5 mM $CaCl_2$ to a final concentration of 5 mg phospholipid/ml. Samples were

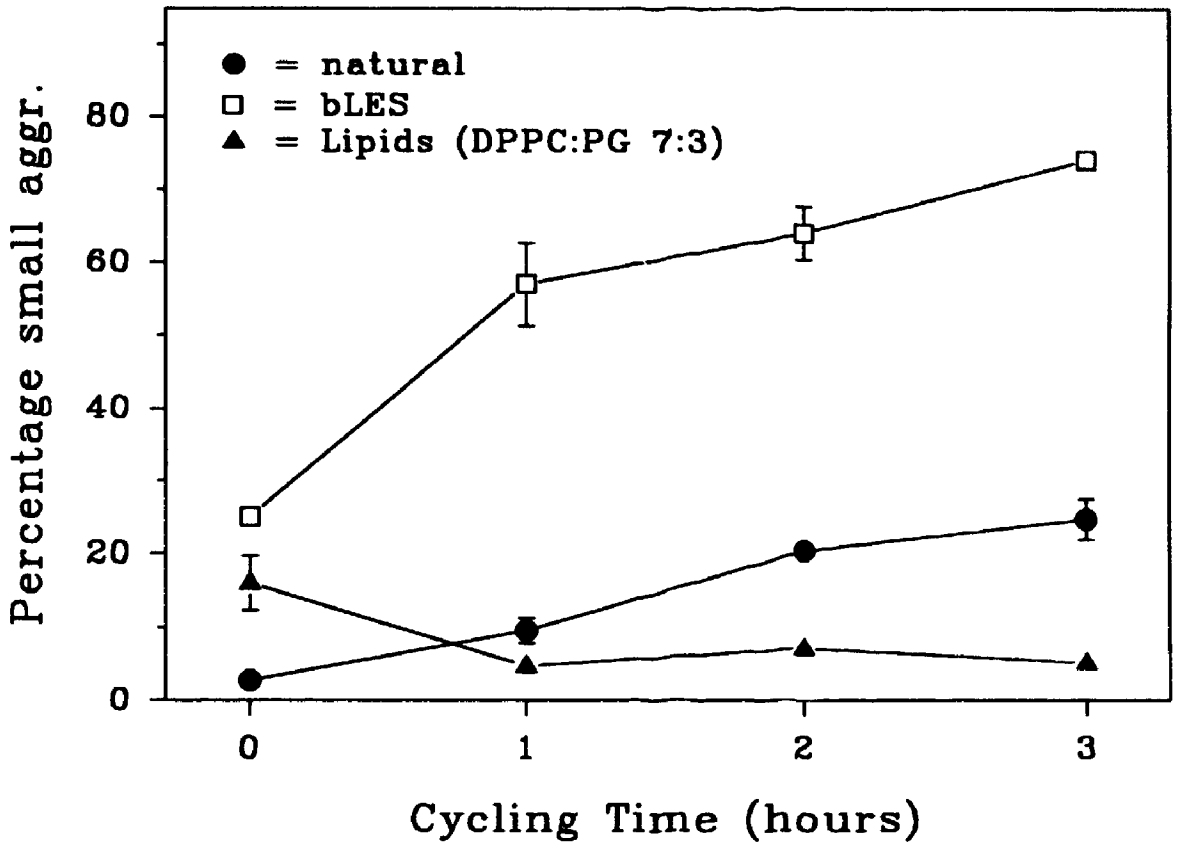
incubated for at least 90 min at 37°C before being analyzed with a pulsating bubble surfactometer (Electronetics Corporation) as described by Enhorning (Enhorning 1977). With this technique a bubble is created in a surfactant suspension. After 10 seconds the bubble is pulsated at 20 pulsations per minute between the maximum bubble radius (R_{max}) of 0.55mm and a the minimum bubble radius (R_{min}) of 0.4mm at 37°C. The pressure across the air liquid interface is measured by a pressure transducer. Surface tension was calculated by the law of Young and Laplace which states that the pressure across a sphere is directly proportional to twice the surface tension and indirectly proportional to the radius. Surface tensions at R_{min} were expressed.

For the adsorption measurements, samples were analyzed at 5 mg/ml in conversion buffer. Samples were incubated at 37°C for at least 90 min before being analyzed. Adsorption measurements were conducted on the pulsating bubble surfactometer by following the change in pressure across the air liquid interface during the first 10 seconds after the formation of the bubble. Surface tension was calculated as described above.

6.5. Results

The formation of small aggregates during surface area cycling was investigated with three different surfactant

Figure 6.1. The formation of small aggregates by three different surfactant preparations; natural surfactant, lipid extract surfactant (LES) and lipids (DPPC:PG 7:3).


● = natural
□ = bLES
▲ = Lipids (DPPC:PG 7:3)
Percentage small aggr.
0
20
40
60
80
0
1
2
3
Cycling Time (hours)

preparations; 1) Natural surfactant, a preparation containing SP-A, SP-B, SP-C and presumably a small amount of contaminating proteins, 2) LES containing the surfactant phospholipids and the two hydrophobic proteins SP-B and SP-C and 3) lipids, a preparation containing only DPPC:PG (7:3). A small percentage of natural surfactant converts to small aggregates during 3 hours cycling (figure 6.1). Surface area cycling of LES leads to a rapid formation of small aggregates, whereas lipids alone do not convert to small aggregates(figure 6.1).

The effect of the surfactant-associated proteins (1% w/w) on the conversion of DPPC:PG mixtures is shown in figure 6.2. Both hydrophobic proteins, SP-B and SP-C can promote the formation of small aggregates during cycling. Lipids alone or in the presence of SP-A do not form small aggregates.

Figure 6.3 shows the adsorption of lipid protein mixtures as assayed on the pulsating bubble surfactometer. High surface tensions of approximately 70 mN/m were observed with either lipids alone or with lipids to which SP-A had been added. The surface tension of these samples was not significantly different from the surface tension of conversion buffer alone. This indicates that the lipids in these samples did not rapidly adsorb at the air-liquid interface. When SP-B or SP-C was added to DPPC:PG there was

Figure 6.2. The effects of the addition of 1% SP-A, SP-B or SP-C to lipid (DPPC:PG 7:3) on the formation of small aggregates during surface area cycling.

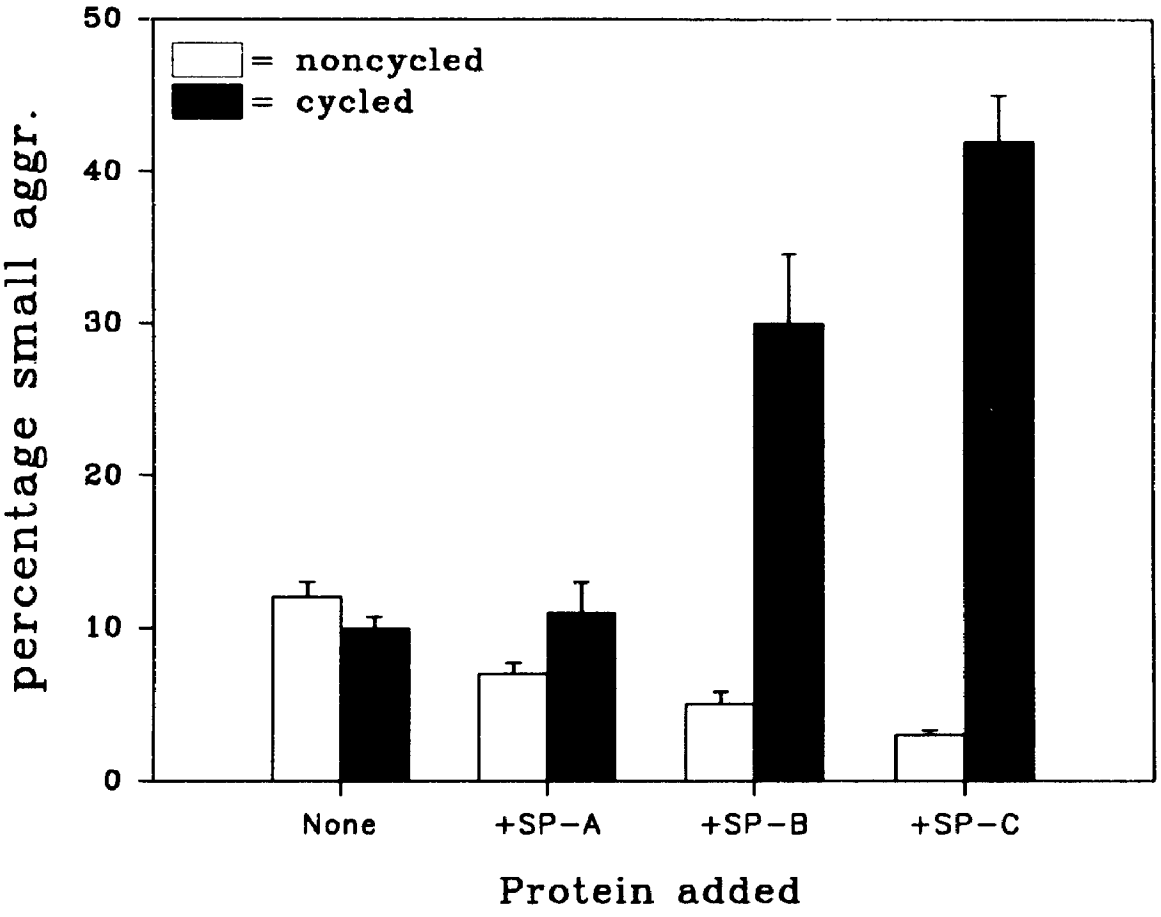
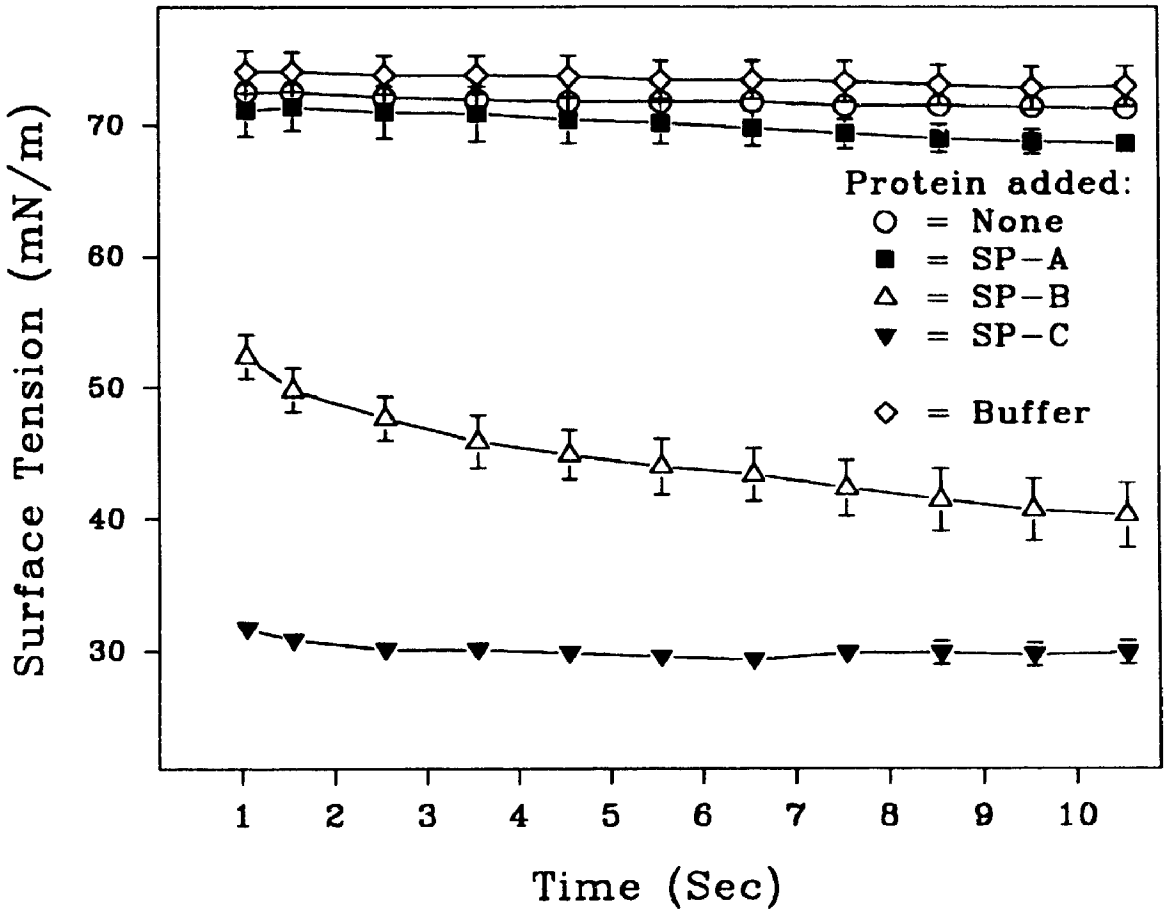

50
40
30
20
10
0
= noncycled
= cycled
percentage small aggr.
None
+SP-A
+SP-B
+SP-C
Protein added

Figure 6.3. The effects of the addition of 1% SP-A, SP-B or SP-C to lipid (DPPC:PG 7:3) on lipid adsorption.


Surface Tension (mN/m)
70
60
50
40
30
1
2
3
4
5
6
7
8
9
10
Time (Sec)
Protein added:
○ = None
■ = SP-A
△ = SP-B
▼ = SP-C
◇ = Buffer

a the rapid adsorption of lipids to the interface (figure 6.3).

The difference between the rapid conversion of large to small aggregates observed with LES compared to the slow conversion observed with natural surfactant (figure 6.1) could be due to the presence of SP-A in natural surfactant. Addition of 5 or 10% (w/w) SP-A to LES did not affect the rapid aggregate conversion of LES (not shown). Neither incubation at 37°C overnight nor increasing the calcium concentration to 5 mM had any effect (not shown). Surface area cycling of LES plus 20% (w/w) SP-A in 5 mM Ca^{2+} after incubation at 37°C overnight resulted in a slight decrease in the amount of small aggregates generated (figure 6.4). However, when the LES sample was supplemented with an additional 1% (w/w) SP-B a marked decrease in small aggregate formation was observed in the presence of 20% (w/w) SP-A (figure 6.4).

The effects of different concentrations of SP-A on the conversion of LES or LES plus 1% (w/w) SP-B is shown in figure 6.5. Whereas the addition of SP-A to LES does not result in a significant inhibition of surfactant aggregate conversion, LES plus SP-B shows a reduced conversion even when only 1% (w/w) SP-A is added (figure 6.5). At 5 or 10% (w/w) SP-A added to LES plus SP-B the small aggregate formation was at its lowest level (figure 6.5).

Figure 6.4. Surface area cycling of LES in the presence or absence of 1% SP-B and/or 20% SP-A.

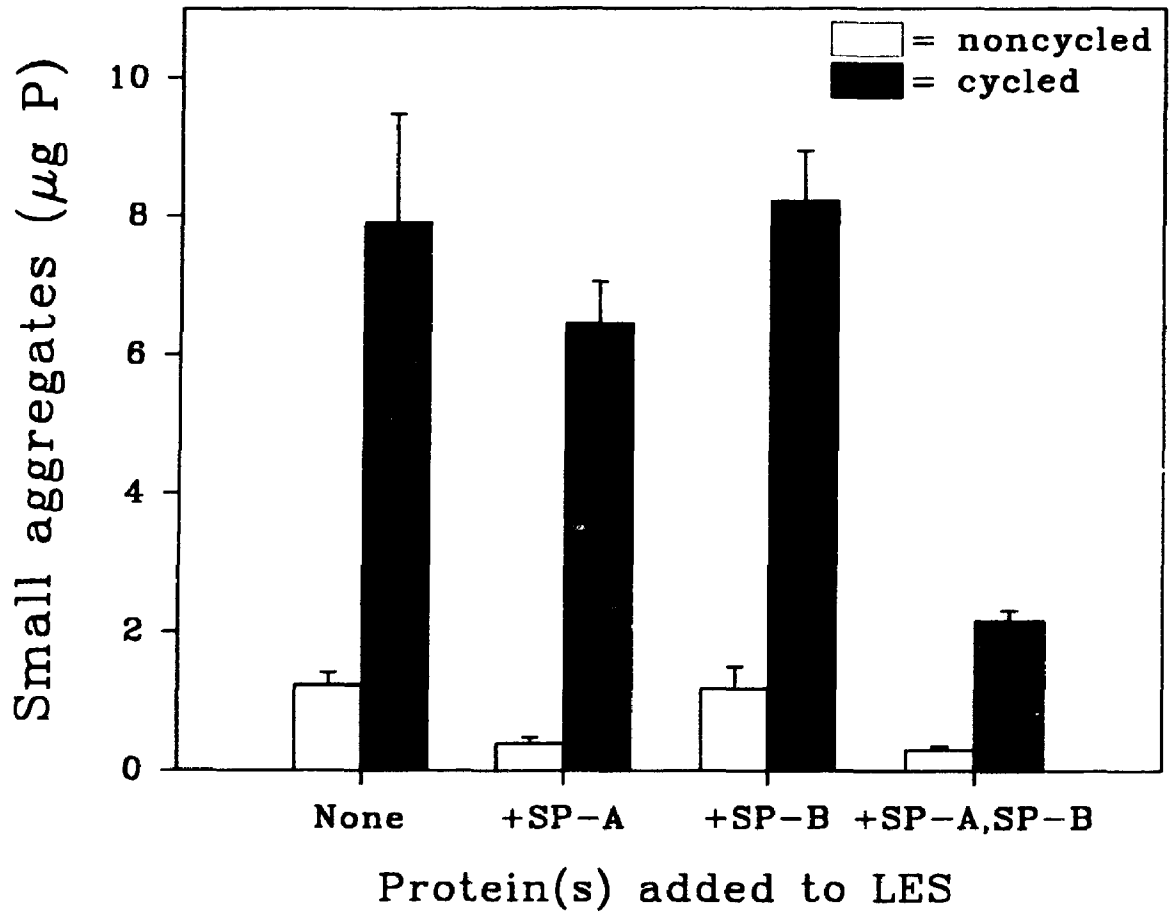
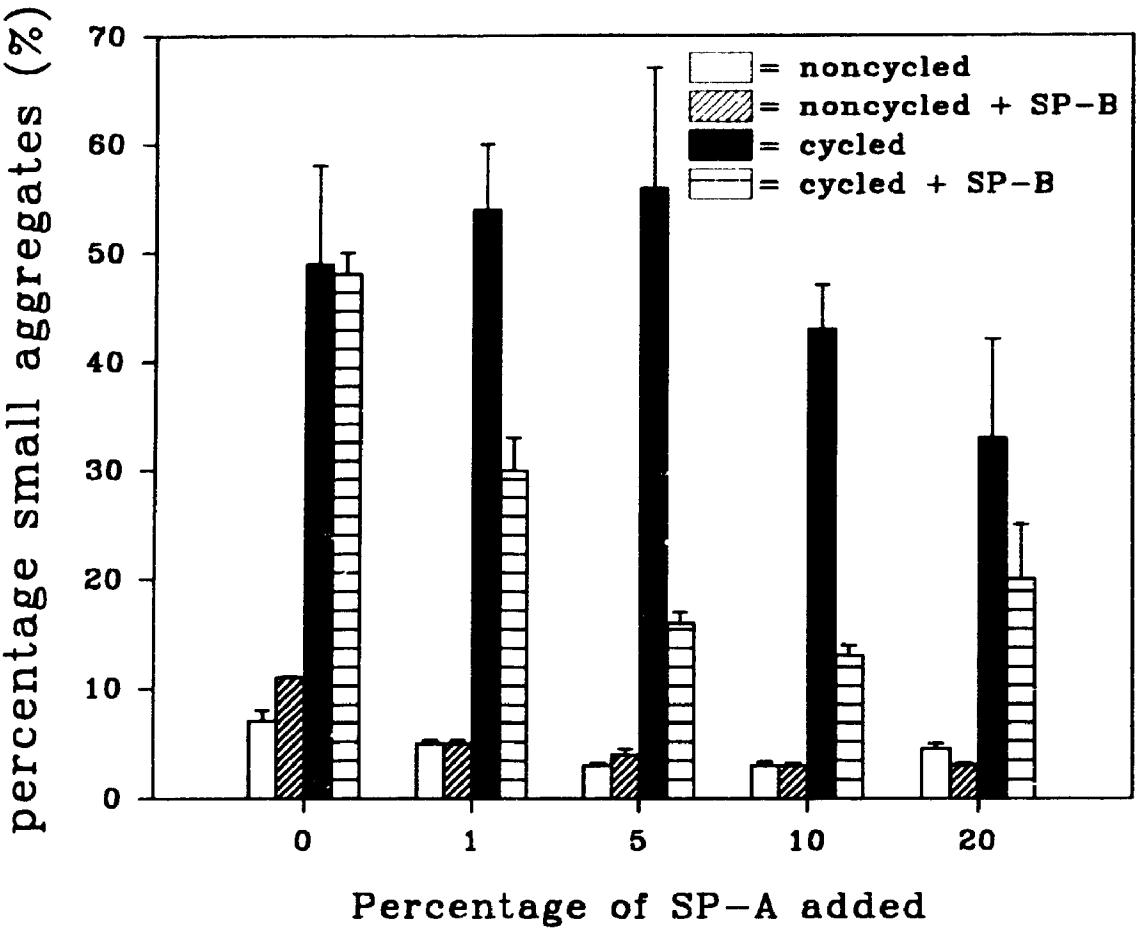

= noncycled
= cycled
Small aggregates (μg P)
10
8
6
4
2
0
None
+SP-A
+SP-B
+SP-A,SP-B
Protein(s) added to LES

Figure 6.5. The effects of adding different concentrations of SP-A to LES or LES plus 1% SP-B before surface area cycling.


percentage small aggregates (%)
70
60
50
40
30
20
10
0
= noncycled
= noncycled + SP-B
= cycled
= cycled + SP-B
0
1
5
10
20
Percentage of SP-A added

We have previously observed that small aggregates recovered after surface area cycling of canine large surfactant aggregates have poor surface tension reducing properties (chapter 5). The biophysical activities of large and small aggregates obtained after cycling of bovine natural surfactant was analyzed on the pulsating bubble surfactometer. Lipid extracts of large surfactant aggregates from non-cycled and cycled natural surfactant reduced surface tension to near zero values within 50 pulsations. Lipid extracts of small aggregates did not reach near zero values within 100 pulsations (figure 6.6).

The effect of two proteases, trypsin and collagenase, on the formation of small aggregates by surface area cycling is shown in figure 6.7. Compared to natural surfactant alone trypsin markedly increased the amount of small aggregates generated. Addition of collagenase to natural surfactant also resulted in an increased amount of small aggregates after surface area cycling (figure 6.7). Immunoblot analysis of the samples incubated with and without trypsin showed detectable but clearly decreased levels of SP-A in the trypsin-treated sample (not shown). Incubation of pure SP-A and pure SP-B with trypsin led to a complete loss of immuno-reactive SP-A while the immuno-reactivity of SP-B remained unchanged (not shown).

Figure 6.6. The surface tension reducing ability of lipid extracts of large and small aggregates isolated from natural surfactant.
Large aggregates were obtained as a 40,000g pellet from cycled and noncycled large natural surfactant. Small aggregates were obtained from the supernatant of a 40,000g centrifugation of cycled natural surfactant. (Values at minimum bubble size are expressed)

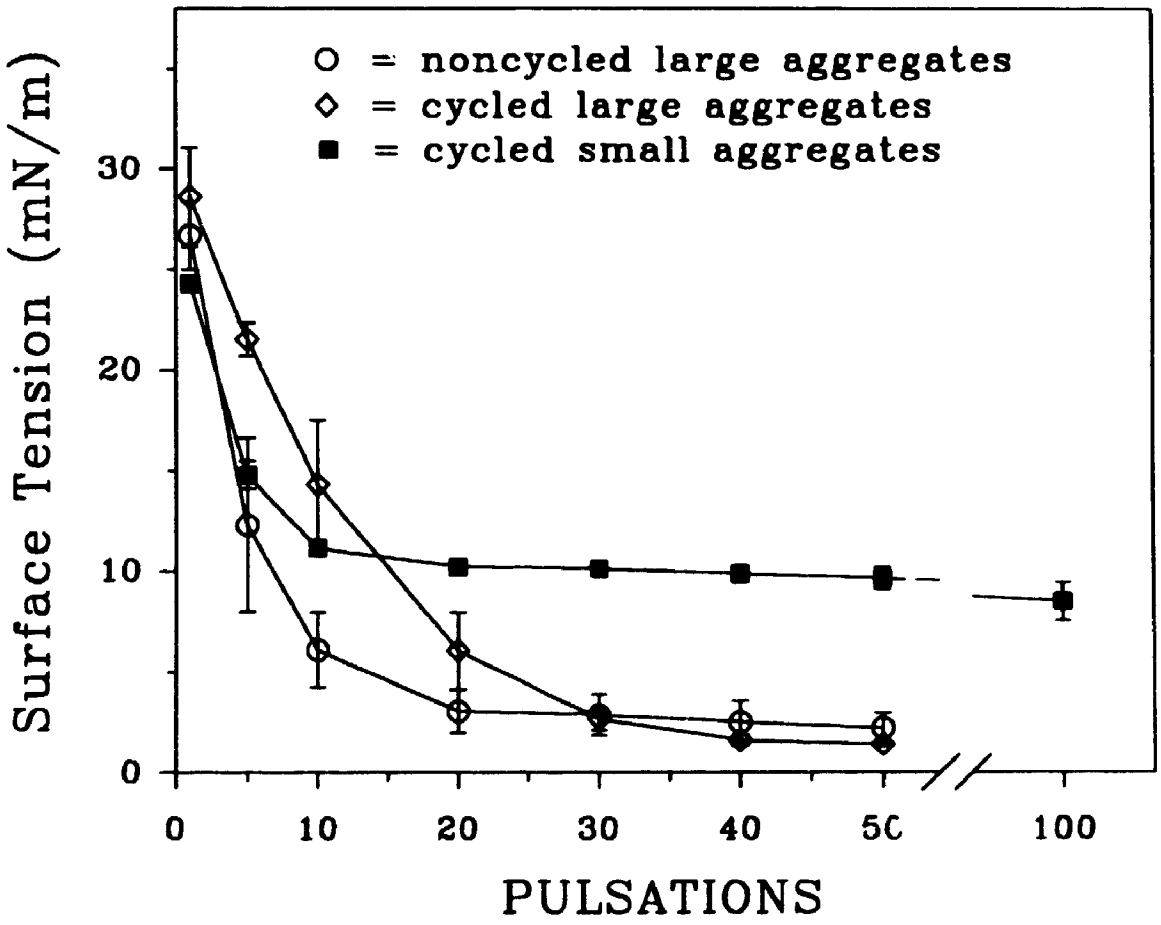
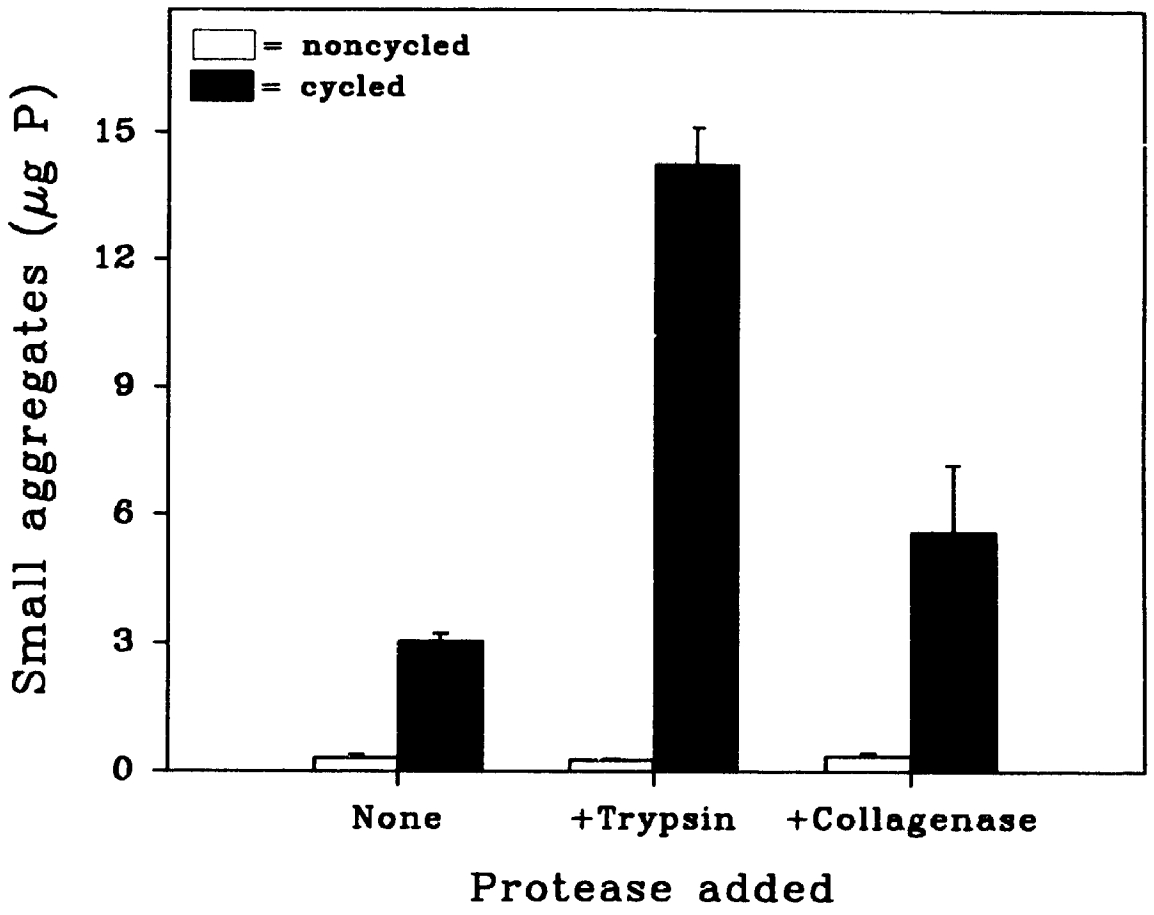

○ = noncycled large aggregates
◇ = cycled large aggregates
■ = cycled small aggregates
Surface Tension (mN/m)
30
20
10
0
0
10
20
30
40
50
100
PULSATIONS

Figure 6.7. The effects of trypsin and collagenase on the surfactant aggregate conversion of natural surfactant.


= noncycled
= cycled
Small aggregates (μg P)
15
12
9
6
3
0
None
+Trypsin
+Collagenase
Protease added

5.6. Discussion

It has been reported that conversion of large to small surfactant aggregates *in vitro* is dependent on a change in surface area and protease activity (Gross and Narine 1989a, chapter 5). This has led to the following hypothesis; an increase in surface area leads to the adsorption of lipid to the air-liquid interface. This adsorption process exposes SP-B to the protease activity (chapter 5).

The results presented here indicate that only those samples that adsorb rapidly to an air liquid interface, such as LES and lipids plus SP-B or SP-C, can convert to small aggregates during surface area cycling. This conversion is independent of protease activity. When LES samples were cycled in tubes filled to a level where an air liquid interface was still present but where rotation did not cause a change in surface area, there was no formation of small aggregates (not shown). These results are consistent with the hypothesis that adsorption is necessary for aggregate conversion. Furthermore it suggests that phospholipids at the air liquid interface form small vesicles upon a rapid decrease in surface area.

Although surfactant preparations containing all three surfactant associated proteins rapidly adsorbs to the air liquid interface (Chung et al 1989, Cockshutt et al 1990, Schürch et al 1992) it does not convert to small aggregates.

This indicates that SP-A is important for the integrity of large surfactant aggregates. This hypothesis was supported by the following observations; 1) Highly purified natural surfactant converts slowly and only forms small amounts of small aggregates. 2) Cleavage of SP-A in natural surfactant by either trypsin or collagenase resulted in marked increase in small aggregate formation. Cleavage of SP-B or SP-C by these proteases seems unlikely since these proteins are resistant to a large number of proteases (Curstedt et al 1988, Johansson et al 1988). 3) Addition of SP-A to LES blocks the conversion to small aggregates. Unexpectedly, this inhibition of conversion was only observed after an additional 1% (w/w) SP-B was added to the lipid extract surfactant.

The requirement for an additional 1% SP-B to block the conversion of LES by SP-A is an interesting observation. This result could imply a some kind of stoichiometric relationship between SP-A and SP-B. At 10% SP-A (w/w) and 1% SP-B (w/w) the ratio of the SP-A monomer to SP-B dimer would be approximately 1:1. Since SP-A forms oligomeric structures (18'mers) and LES already contains approximately 0.5-1.0% SP-B (Yu et al 1987) the actual molar ratio of SP-A:SP-B is a lot lower. A more likely explanation for the requirement of additional SP-B is that a considerable proportion of SP-B is inaccessibly packed in the lipids and cannot interact with exogenously added SP-A. This suggestion is supported by

an observation made in this laboratory during the development of an ELISA assay for measurements of SP-B. When identical amounts of SP-B were analyzed in the presence and absence of surfactant lipids it was found that presence of lipid significantly reduced the amount of detectable SP-B (Kevin Inchley, personal communication). Regardless of the mechanism involved, these results support an interaction between SP-A and SP-B in large aggregates.

Both SP-A and SP-B are essential for the formation of tubular myelin *in vitro* (Suzuki et al 1989, Williams et al 1992). Immunogold labelling of SP-A in tubular myelin has suggested that SP-A is located in the corners of this lattice like structure (Voorhout et al 1991). It is thought that lipid and/or SP-B binding occurs in these corners, while self aggregation of the head groups of SP-A stabilizes the structure (Haagsman et al 1991). It has been suggested that tubular myelin can rapidly adsorb to the air liquid interface (Goerke and Clements 1985). (See figure 1.3. for tubular myelin structure and possible localization of SP-A)

Based on this proposed structure of tubular myelin, the following hypothesis for the mechanisms involved in surface area cycling of natural surfactant can be made. Tubular myelin or a tubular myelin-like structure adsorbs rapidly to the air liquid interface during an increase in surface area. This rapid adsorption might be accomplished by a detachment of SP-A from either the lipids and/or SP-B or from another

SP-A molecule. During the decrease in surface area relinking of the SP-A leads to the reformation of tubular myelin or tubular myelin-like structures which behave as large aggregates. Cleavage of SP-B during the adsorption process, as suggested in the preceding chapter, would result in the loss of an attachment site for SP-A and therefore results in small aggregate formation. In the absence of SP-A, large aggregates adsorb to the air liquid interface. However, without SP-A this adsorbed lipid cannot reform into large aggregates thus leading to the formation of small aggregates.

Large to small aggregate conversion is part of the extracellular metabolic pathway of surfactant (Magoon et al 1983, Baritussio et al 1984, Wright and Clements 1987). In chapter 5 the degradation of SP-B during this process was demonstrated. The results described in the current chapter suggest that degradation of SP-A can also lead to the formation of small aggregates. Aggregate conversion based on the degradation of SP-A might be prominent in lung injury. Increases in proteolytic activity in lung lavages from ARDS patients has been observed (Lee et al 1981, Mcguire 1982). A recent report by Gregory et al showed a decreased level of SP-A in large aggregates from ARDS patients (Gregory et al 1991). SP-A was also decreased in large aggregates observed after experimental lung transplantation (chapter 4). The

lavage obtained from this latter study showed an increased small to large surfactant aggregates ratio (chapter 4).

CHAPTER 7 DISCUSSION AND FUTURE DIRECTIONS.

7.1. The GPC-dependent pathway

Chapters 2 and 3 of this thesis indicate that the synthesis of PC by the acylation of glycerol-3-phosphocholine does not occur to a significant extent in lung or liver. The previously published experimental evidence in favour of this pathway was based on the formation of radioactive GPC from labelled glycerol-3-phosphate and CDP-choline. In chapter 3 it was shown that this evidence might be the result of an inaccurate identification of the reaction products of the hypothetical GPC synthetase reaction. Enzymatic reactions were used to demonstrate that in liver, the main products formed were glucose and glycerol. The formation of glucose and glycerol was also reported by an independent study by Thompson and Belina (Thompson and Belina 1991). In lung another, unidentified, product was formed. This product could be separated from GPC by TLC and could not be labelled by radioactive CDP-choline. It is therefore clear that the product formed in lung is not GPC. Preliminary experiments showed that the unidentified lung product reacted with phenylhydrazine and could possibly be pyruvate. This was not investigated further.

In addition to the GPC dependent formation of PC, the synthesis of other phospholipids have been proposed by similar pathways (Infante 1984, 1986b). Baranska reported on the formation of phosphatidylserine from glycerol-3-phosphoserine (Baranska 1988), however the evidence presented was inconclusive. With the possible exception of this latter study, the pathways for phospholipid biosynthesis proposed by Infante are strictly hypothetical. The evidence against the occurrence of PC synthesis via this pathway (Veldhuizen et al 1989, 1991, Thompson and Belina 1991) makes it less likely that other phospholipids are produced via this general pathway.

7.2. Surfactant in lung transplantation

The main conclusion from chapter 4 of this thesis is that surfactant supplementation might be useful in lung transplantation. In contrast to surfactant supplementation in response to ARDS, surfactant supplementation in lung transplantation has unique potential since the surfactant can be delivered before the reperfusion period. Under these circumstances, surfactant might have a protective effect which could prevent the severe lung injury rather then being used as treatment for an established lung injury. General considerations for surfactant supplementation experiments include the choice of the surfactant preparation to be used,

the timing of surfactant delivery, the dose and delivery method.

Supplementation experiments in lung transplantation are currently in progress in collaboration with Dr Richard Novick (Division of Cardiovascular-Thoracic Surgery, University Hospital, London Ont.) and Dr Jim Lewis (Dept. of Medicine, Lawson Research Institute, UWO). Modifications in the experimental procedure involving the recipient animal resulted in improved gas-exchange after 12 hours of storage without surfactant supplementation. These modifications include the use of positive end expiratory pressure (PEEP) and the avoidance of snaring the pulmonary arteries for blood gas measurements during the reperfusion period. Therefore, for the present surfactant supplementation experiments the ischemic time has been extended to 36 hours.

The surfactant used in our studies is a bovine lipid extract surfactant (bLES). This preparation contains the hydrophobic proteins SP-B and SP-C which have reported to be important constituents of exogenous surfactant (Hall et al 1992). In a recent study, Cummings et al used surfactant supplementation of surfactant-deficient premature lamb to compare the effect of different surfactant preparations (Cummings et al 1992). The authors found that a surfactant extract is a more effective surfactant than protein-free artificial surfactant or surfactant derived from minced lung extracts (Cummings et al 1992).

Possible clinical use of surfactant was the deciding factor in the timing of surfactant supplementation. Immediately before transplantation appeared to be the most realistic time since in the clinical situation the surfactant expertise will most likely be at the site of the transplantation. In these experiments, surfactant is administered as a 50 mg phospholipid/ml suspension in saline 1.5mM $CaCl_2$ at a dose of 50 mg/kg. The bLES is labelled with radioactive DPPC which allows us to estimate the recovery, the uptake and any spill-over to the native lung.

Preliminary results show a rapid drop in gas-exchange in non-treated animals. This impaired lung function is associated with an increase in serum-protein and an increased ratio of small to large surfactant aggregates in the lung lavages. This confirms our findings of chapter 4 in this improved experimental design. In four surfactant supplemented animals, two animals showed little improvement over the untreated group and two animals showed a marked improved gaseous-exchange. The two animals with improved gaseous-exchange had lower serum protein levels and normal small to large aggregate ratios. The fact that there appear to be responders and non-responders to surfactant treatment could be related to the severity of the lung injury.

In addition to the clinical significance of these surfactant supplementation experiments, these studies could provide useful information on the metabolism of exogenous

surfactant. The preliminary results suggest, that even when a lung is supplemented with a large dose of exogenous surfactant, the ratio of small to large surfactant aggregates is increased under conditions of impaired gaseous-exchange and normal in the case of normal gaseous-exchange.

Depending on the outcome of the current supplementation experiments, future supplementation experiments could be performed with a SP-A containing surfactant preparation. SP-A has been reported to counteract protein inhibition (Cockshutt et al 1990). Furthermore, large aggregates recovered after lung transplantation showed a decrease in SP-A content (chapter 4). Since cyclosporine is given after clinical transplantation, the immunological consequences of bovine SP-A supplementation can be avoided.

Other experiments with surfactant in lung transplantation could include changing the method of surfactant delivery. Lewis et al have found that a nebulized surfactant results in superior distribution of surfactant compared to instillation (Lewis et al 1992). Supplementation of the lung before the storage period should also be investigated. Surfactant might have a protective effect during the storage period. Furthermore, surfactant could be used as a drug delivery system to distribute other protective agents in the lung.

7.3. Surface area cycling

Surface area cycling is an *in vitro* procedure with which the conversion of large surfactant aggregates to small aggregates can be studied. In this procedure aggregate conversion is achieved by continuously changing the surface area of a large aggregate suspension at 37°C. Gross and his associates, who developed this method, have shown that aggregate conversion is dependent on a change in surface area and the activity of a serine protease. Aggregate conversion can be blocked by low temperatures (Gross and Narine 1989a, Gross and Schultz 1990, 1992). Chapters 5 and 6 have used this method to study several aspects of surfactant aggregate conversion.

In chapter 5 of this thesis, evidence was presented for the degradation of SP-B during the conversion of large to small surfactant aggregates *in vitro*. Several questions regarding this process remain to be answered; 1) What is (are) the cleavage site(s) of the serine protease? 2) Where do the generated peptides go after cleavage of SP-B? and 3) Is there a change in the oligomeric structure of SP-A during surface area cycling?

The SP-B antibody used in the experiments described in chapter 5 is a monoclonal antibody that recognizes the active SP-B homodimer. Experiments with antibodies against synthetic peptides of SP-B could detect the generated SP-B

fragments and potentially help determine the cleavage site of this protease. Emrie et al reported a homology of SP-B (Gly-43 to Tyr-53) with the active site of certain protease inhibitors based on the cDNA sequence of rat SP-B (Emrie et al 1989). This makes it possible that this sequence (Gly-43 to Tyr 53) is the cleavage site for the serine protease. The oligomeric structure of SP-A before and after cycling could be examined by electrophoresis of SP-A samples on a nonreducing gel followed by western blotting.

In chapter 6, surface area cycling of different surfactant preparations was used to investigate the role of the individual surfactant associated proteins in the conversion of large to small surfactant aggregates *in vitro*. SP-A and SP-B were found to be essential for the integrity of large aggregates (chapter 6). The results were consistent with the involvement of the following steps in surfactant aggregate conversion; 1) Adsorption of lipids to the air-liquid interface. 2) Degradation of SP-B in the surface monolayer during the adsorption process or, alternatively, degradation of SP-A before or during the adsorption process. 3) A decrease in surface area leading to the generation of small aggregates from the SP-A or SP-B depleted surfactant lipids.

Since the surfactant proteins affect surfactant structure, morphological studies should be used to correlate the changes in structure with the biochemical observations.

Based on some preliminary morphological studies and the reported requirement for SP-A and SP-B in the *in vitro* formation of tubular myelin (Suzuki et al 1989, Williams et al 1991), a proposal for the morphological structures involved in surface area cycling can be made. Figure 7.1 shows a schematic representation of these proposed structures and the mechanisms involved in the conversion to small aggregates. Most of these proposed structures must still be confirmed by electron microscopy.

Surface area cycling has been used to analyze the properties of large aggregates obtained after experimental lung injury (Gross 1991b, Higuchi et al 1992). In these experimental models an altered ratio of small to large aggregates in these experimental models was correlated with an altered aggregate conversion rate *in vitro* (see chapter 6 for a more detailed discussion). It will be interesting to conduct such an analysis with other injury models where the aggregate ratio is altered.

These studies should include measurements of the levels of surfactant proteins before surface area cycling, and determination of elastase and other proteolytic enzymes activities in the lavage. Furthermore, it will be very interesting to analyze which surfactant associated protein is cleaved during cycling of the large aggregates obtained after lung injury. The ischemic reperfusion injury described in chapter 4 would be a candidate for such studies.

Figure 7.1. Proposed structures and mechanisms involved in surface area cycling of different surfactant preparations.

Abbreviations used:

L.V. = large vesicles, either unilamellar or multilamellar.

T.M. = Tubular myelin or tubular myelin-like structures.

M.L. Monolayer.

S.A = small aggregates.

A) Lipids alone do not adsorb to the air liquid interface.

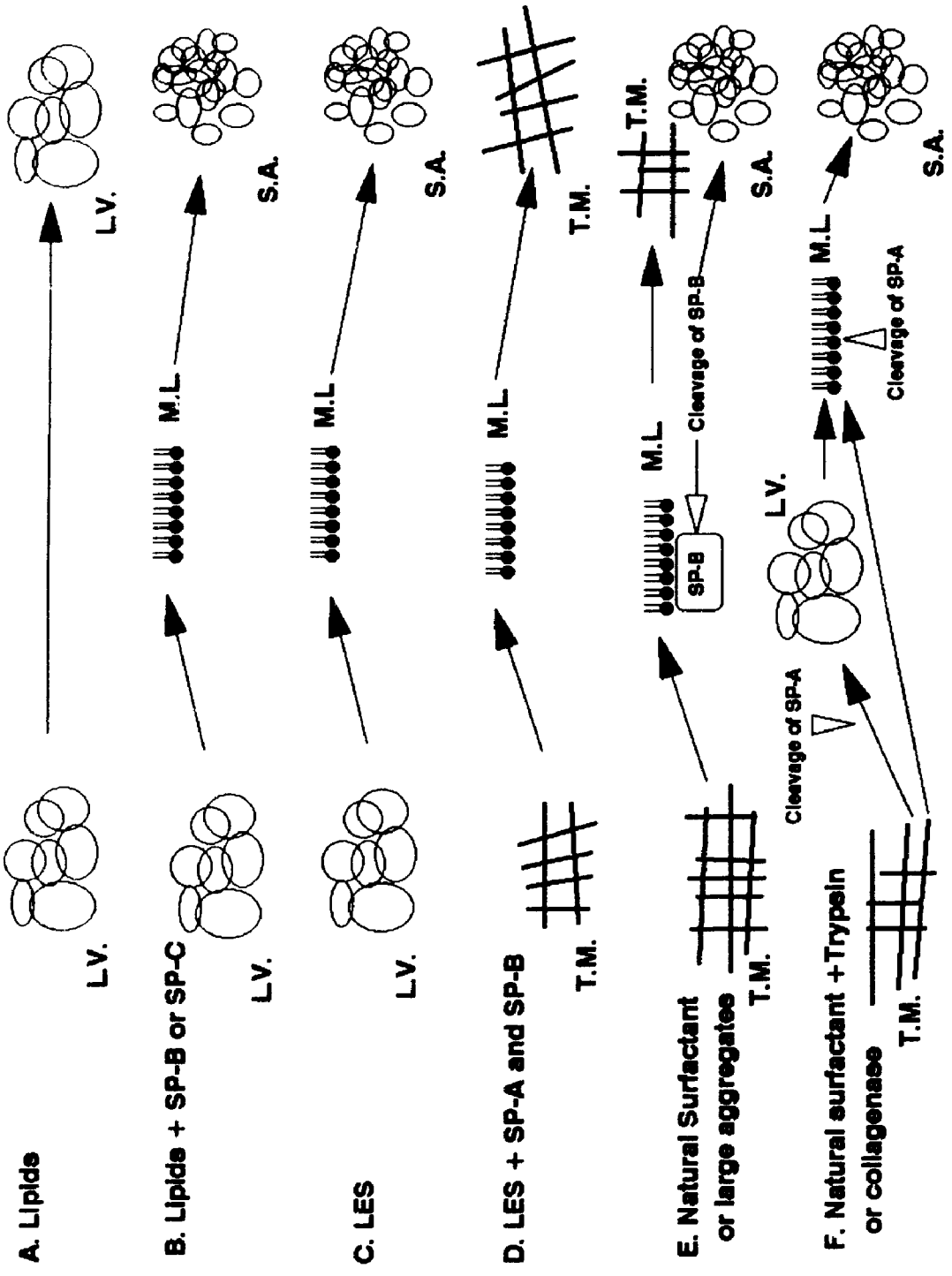
B) Lipids plus SP-B or SP-C adsorb and convert to small aggregates.

C) LES adsorbs and converts to small aggregates.

D) LES plus SP-A and SP-B forms a tubular myelin-like structure which adsorbs and reforms into a large aggregate structure.

E) Natural surfactant or large aggregates contain tubular myelin-like structures. These adsorb to the air liquid interface, subsequent cleavage of SP-B leads to small aggregate formation. In the absence of protease activity the tubular myelin like structure is reformed.

F) Natural surfactant plus trypsin or collagenase forms small aggregates through the cleavage of SP-A either before or during surface area cycling.


A. Lipids
LV.
LV.
B. Lipids + SP-B or SP-C
LV.
M.L
S.A.
C. LES
LV.
M.L
S.A.
D. LES + SP-A and SP-B
T.M.
M.L
T.M.
E. Natural Surfactant
or large aggregates
T.M.
M.L
SP-B
Cleavage of SP-B
T.M.
S.A.
F. Natural surfactant +Trypsin
or collagenase
T.M.
Cleavage of SP-A
LV.
M.L
Cleavage of SP-A
S.A.

Accumulation of pulmonary surfactant large aggregates is observed in alveolar proteinosis and silicosis (Hook 1991). Increased surfactant synthesis and decreased catabolism can only explain part of this large increase in alveolar surfactant (Hook 1991). Measurements of the small aggregates in lavages obtained from these types of injury has not been reported. Inhibition of surfactant conversion could contribute to the accumulation of large aggregates in these circumstances. This possibility could be tested by surface area cycling studies.

Tentative identification of the protease involved in surface area cycling, named convertase, has recently been reported (Gross and Schultz 1992). Purification of convertase would greatly enhance studies on the mechanisms of aggregate conversion. Furthermore, sequencing and cloning would provide useful tools for studying many aspects of convertase expression and extracellular surfactant metabolism.

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