To Lilá and Décio, my parents

Evaluation of Lung Surfactant Composition and Function: an Integrated Approach

A thesis presented for the degree of
Master of Philosophy

by

Marta Maciel Lyra Cabral

Child Health
Faculty of Medicine
University of Southampton

1992

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE
CHILD HEALTH

Master of Philosophy

Evaluation of Lung Surfactant Composition and Function: an Integrated Approach

by Marta Maciel Lyra Cabral

Several experimental techniques were integrated to establish an approach for evaluation of lung surfactant composition and function. Bronchoalveolar lavage (BAL) from term and preterm (day 65) guinea pigs were analysed at one hour after birth and after 3 day exposure either to room air (21% O_2) or to hyperoxia (95% O_2). After 3 days of hyperoxia exposure, a group of preterm guinea pig was left at room environment for 4 days to investigate recovery from the hyperoxia-induced inflammation.

An enzymatic assay to determine phosphatidylcholine (PC) concentration, based on hydrolysis by phospholipase D (PLD), was modified to a microscale. When preterm guineas pig were exposed to 21% O_2 for 3 days, BAL PC concentration increased, while BAL PC in term guinea pigs did not change with similar treatment. BAL PC content of term pups tended to increase over three days of hyperoxia, while for preterm guinea pigs this increase was significant. When transfered to room air, PC concentration of hyperoxia-exposed preterm pups decreased to initial values by 7 days of age.

Surfactant function was analysed using the Wilhelmy balance, at a constant temperature of 37°C. Guinea pig BAL was used either at constant concentration (85nmols PC) or constant volume (5ml). No significant difference was found between 1 hour old or 3 day old pups exposed to air or hyperoxia.

Total protein concentration was analysed using bicinchoninic acid. BAL protein increased in preterm pups exposed to air, and this increase was significant for both term and preterm pups exposed to hyperoxia. After ceasing hyperoxia, total protein in preterm pups decreased to initial values by 7 days of age. An antibody anti guinea pig SP-A (surfactant associated protein A) was raised in rabbit to identify the 36 kDa band. Results are compared with PE-10, a monoclonal antibody against human SP-A. Both the anti guinea pig SP-A and PE-10 detected SP-A in homogenized lung from guinea pig fetus after 55 days of gestation.

Contents

Acknowledgements

I wish to thank all those who contributed to the completion of this work.

I am grateful to Dr. A.D.Postle for his guidance, criticisms, encouragement, and friendship demonstrated at all stages of this research.

I acknowledge Prof. J.Warner for use of facilities in the department of Child Health.

Thanks are also due to Prof. I.C.Normand for his kind reception and discussions in the early stages of this research, despite his busy schedule.

I am also grateful to Dr. Graham Burdge for his suggestions and many fruitful discussions.

I am indebted to Sally Little and Dr. Alan Hunt for their assistance with some of the techniques used in this study and also to Gary Phillips for providing guinea pig BAL samples.

To Dr. Tara Assudulahi, Liz Milles, Debbie Smith and Mike Byford thanks for their friendship.

Thanks are also due to the staff of the Department of Child Health, in particular to Carol, Tina and Brenda.

To Jaime, my husband, for his help in typing and drawing some of the figures of this thesis, and for his constant encouragement, cooperation and support.

Thanks to my parents, brothers and sister for their constant support despite the distance.

To Marisa Lyra, Beatriz and Henry, Celina and Paulo, Paula and Jeferson, Aparecida and Aloísio, Letícia and Fred, Cecília and José Paulo my sincere thanks for their friendship and help on several occasions.

Finally, I acknowledge the financial support from the Brazilian Scientific and Technologic Research Council (CNPq) during my stay in Britain.

Abbreviations

AF - Amniotic fluid
AOE - Anti oxidant enzyme
BAL - Broncho alveolar lavage
BCA - Bicinchoninic acid
BPD - Bronchopulmonary dysplasia
BSA - Bovine serum albumin
CAT - Catalase
CLD - Chronic lung disease
CPAP - Continuous positive airways pressure
DPPC - Dipalmitoyl phosphatidylcholine
FRC - Functional residual capacity
GLC - Gas-liquid chromatograph
GSH-Px - Glutathione peroxidase
HPLC - High performance liquid chromatography
HPV - High frequency oscillation (pressure ventilation)
i.p. - Intraperitoneal
IPPV - Prolonged inspiratory time
NS - Nonsignificant
PC - Phosphatidylcholine
PE - Phosphatidylethanolamine
PI - Phosphatidylinositol
PG -Phosphatidylglycerol
PL - Phospholipid
PS - Phosphatidylserine
PLD - Phospholipase D
RTP - Room temperature and pressure
RDS - Respiratory Distress syndrome
S - Sphingomyelin
SOD - Superoxide dismutase
SP-A, SP-B, SP-C and SP-D - Surfactant apoprotein
TLC - Thin layer chromatography

Chapter One
Introduction

1.1 Introduction

In the last few decades, much research effort has been focused on conditions that cause respiratory difficulties after birth, in the first days of life. Reports in different countries have shown that a large percentage of early neonatal pathology ocurred due to respiratory disorders; for example, about 1% infants worldwide suffer Respiratory Distress Syndrome [Jobe and Ikegami, 1987]).

The adaptations required by the lung to establish normal breathing at birth are considerable, and include rapid liquid absorption through the alveolar wall, a dramatic increase in blood flow through the pulmonary capillary bed, and establishment of a functional surfactant monolayer to prevent alveolar collapse.

Lung surfactant plays an important role in the respiratory process in human beings and other mammalian species. It is recognised that deficiency of surfactant is the main contributory factor to neonatal respiratory distress syndrome (RDS) especially in preterm newborn infants. Diminished surfactant function is also known to be a factor in the pathogenesis of adult RDS and related lung diseases. It has been suggested that surfactant may be involved in several defense mechanisms of the lung [Wright and Clements - 1987], for example prevention of development of bronchiolar epithelial necrosis and hyaline membranes during artificial ventilation, and reduction of protein leakage [Van Golde *et al.*, 1988]. A mechanical explanation for this is given by O'Brodovich and Mellins [1985]. Surfactant deficiency or dysfunction increases surface tension and consequently promotes closure of alveoli with dilatation of alveolar ducts [Weibel, 1984] and this may lead to necrosis or severe damage to the distal bronchiolar epithelium.

Furthermore, the capillary pressure of the lung becomes even more negative than usual relative to atmospheric pressure when the surface tension characteristics of the lungs are increased [Beck and Lai-Fook,1983]. This raises the possibility that the transvascular pressure gradient in infants with RDS may be significantly increased, promoting pulmonary edema [Ikegami *et al.*, 1983]. Increased surface tension may mechanically produce damage to the alveolar - capillary membrane, which then abnormally leak water and solutes [Nilson *et al.*, 1978].

Since the 1960s, much surfactant-related research has been oriented towards an understanding of surfactant function for clinical application in the therapy of neonatal RDS. A number of papers have been written describing

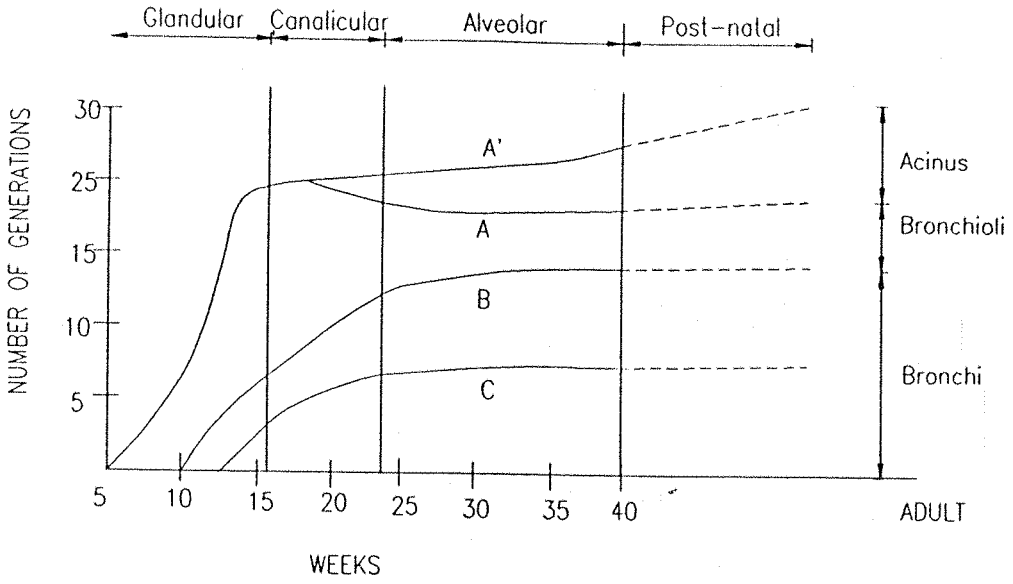

Figure 1.1: Development of the bronchial tree. The upper line shows that intrasegmental development starts at about the 6th. week of intrauterine life and that the bronchial tree is developed by the 16th. week (from Reid, 1967

biochemical characteristics, physiological aspects, clinical applications and techniques for obtaining natural and artificial surfactant. For instance Possmayer [1984] has reviewed the biochemistry of pulmonary surfactant during fetal development and in the perinatal period, Jobe and Ikegami [1987] have presented a review about use of surfactant for treatment of RDS, Wright and Clements [1987] reviewed metabolism and turnover of lung surfactant, and Van Golde *et al*, [1988] reviewed in detail biochemical aspects and functional significance of pulmonary surfactant system.

The present study has combined a number of these findings and experimental techniques in an attempt to establish an integrated approach for evaluation of lung surfactant, composition and function.

1.2 Lung development

Lung development starts a few weeks after conception and continues for several years after birth. It starts with an ectodermal diverticulum of the foregut which develops during the fourth week of embryonic life; from this the larynx and trachea are formed. Bronchial buds then appear

which proceed to form the primary bronchi. By 16 weeks of gestation, the growth of secondary, segmental and subsegmental bronchi accounts for the formation of some 70% of the full term bronchial tree [Cockburn and Drillieu, 1974]. Figure 1.1 shows the development of the bronchial tree for different ages.

The morphological development of the lung has been divided into 5 phases as reviewed in Farrell [1982]. The first 5 weeks constitute the embryonic period of lung development when the lung first appears as an outgrowth of the foregut endoderm. During the second stage which lasts from 5 to 16 weeks gestation, the conducting airways are formed. The third, canalicular stage, from 17 to 28 weeks is characterized by initiation and vascularization of prospective respiratory zone. The capillaries grow towards the respiratory bronchioles and saccules, which are originated from respiratory bronchioles and are distinguished as narrow walled structures with a characteristic epithelium containing both thin type I cells and type II pneumonocytes, and eventually penetrate between epithelial cells. The terminal sac period (28 to 36 weeks gestation) is characterized by subdivision of the saccules into shallow alveoli. At the end of the saccular period, the acinus consists of one or two generations of prospective respiratory bronchioles. Finally, at 36 weeks gestation, mature alveoli line the sacculus. By term, three generations of both respiratory bronchioles and alveolar ducts can be identified [Hislop *et al.*, 1986]. After birth the lungs continue to develop up to puberty.

1.3 Respiratory distress syndrome

RDS, also called Hyaline Membrane Disease, usually affects preterm infants particularly those of short gestational age and low birth weight. RDS is an acute lung disease of preterm infants caused by a primary deficiency of lung surfactant, associated with structural immaturity. Once the disease process is initiated, it is aggravated by leakage of serum proteins into the air spaces. Because of decreased surfactant, pulmonary capillary pressure exceeds interstitial pressure in the lung parenchyma, proteins from the plasma enter the alveolar space, and this leads to further surfactant inactivation [Ikegami *et al.*, 1983b].

Although mortality from RDS among preterm newborns has decreased in recent years because of improvements in intensive care and ventilatory techniques in neonatal units, RDS is still a major cause of morbidity and mortality. Jobe and Ikegami [1987] have estimated that approximately 1%

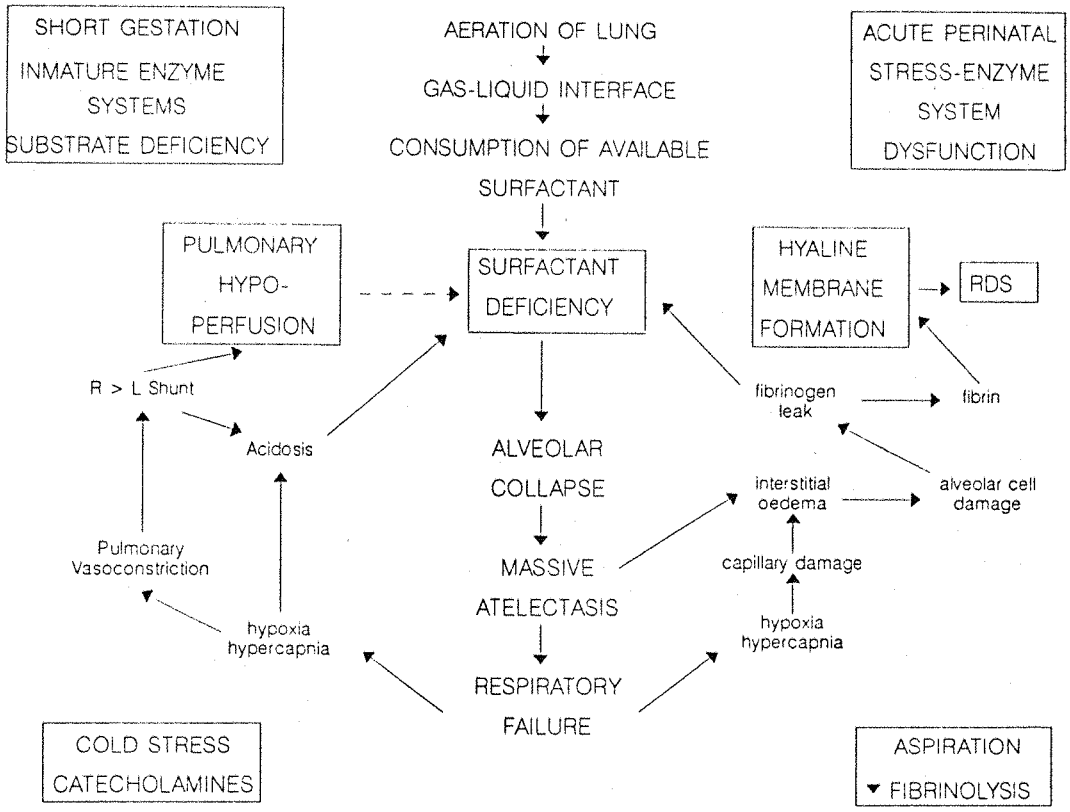

Figure 1.2: Major pathophysiological mechanisms thought to be implicated in respiratory distress syndrome (after Campbell, 1976)

of infants worldwide develop RDS, and Lindroth [1988] reported that this disease accounts for 15% - 20% of all neonatal deaths in Scandinavia and U.S.A.. The high level of mortality is due to the association of RDS with prematurity. Most of the babies with RDS also suffer from inadequate regulation of cerebral blood flow, increased susceptibility to infections and apneic episodes [Van Golde *et al.*, 1988].

In Southampton, around 30% of all pre-term infants less than 37 weeks gestation suffer from RDS, which represents approximately 70 babies per year and is about 1% of the total number of live births [Princess Anne Hospital, Southampton, 1989].

RDS is a consequence of the failure of newborn infants' lungs to inflate efficiently and to maintain alveolar integrity following birth. Initially, RDS may be clinically similar to congenital pneumonia or transient tachypnea. It starts shortly after birth, progresses over the first 4 hours and can be confirmed by chest X-ray. Figure 1.2 summarizes the major pathophysiological mechanisms implicated in RDS, in which deficiency of surfactant is shown to be crucial. Many abnormalities of metabolism may be present but what are 'causes' and what are 'effects' is difficult to establish [Campbell, 1976].

The characteristic symptoms of RDS are grunting during expiration, tachypnea (over 60 breaths per minute), retractions of sternum and intercostal spaces, cyanosis in room air and chest X-ray with typical reticulogranular pattern and air bronchogram.

Usual therapy for RDS involves increased oxygen supply with ventilator treatment, sometimes with high pressures and prolonged inspiration times. Unfortunately these therapies have some harmful side effects. Continuous exposure of the lung to high concentrations of oxygen in animal models produces a well recognized pathology. Even short exposure times to high concentrations of oxygen has been shown to be associated with alveolar-capillary leak in humans [Davis *et al.*, 1983]. Artificial ventilation causes abnormal alveolar expansion, leading to the development of epithelial necrosis. In many cases, with newborn infants this treatment contributes to additional complications such as pneumothorax and chronic lung disease(CLD).

1.3.1 Surfactant deficiency

In 1959, Avery and Mead discovered that neonatal RDS was associated with the absence or delayed appearance of pulmonary surfactant. This finding is considered by many to be the basis for neonatal intensive care because, although there are other problems, treatment of RDS has always been the major focus of neonatology units [Sinkin and Shapiro, 1988]. Avery and Mead's discovery led to extensive studies on the ontogeny of the surfactant systems, and surfactant has come to be considered as the most important indicator of pulmonary maturity.

Animal models have been extensively used to study pulmonary development in the perinatal period. Some animals provide useful models for human lung prematurity studies [Hunt *et al.*, 1991]. However in clinical practice, one has to rely on indirect methods. Gluck and his coworkers in 1971 reported that lung surfactant accumulates in amniotic fluid and that its content and composition could provide indicative values for the prediction of fetal lung maturity and consequently diagnose the risk of RDS. They have used the ratio of lecithin to sphingomyelin (L/S) as a quantitative measure of amniotic fluid surfactant (see section 1.8 for more detail).

Since then numerous other methods have been developed to measure amniotic fluid phospholipids in attempts to simplify the techniques and increase the accuracy of the assessment of fetal lung maturity. Chemical tests have focused on measuring surfactant-specific substances in amniotic fluid,

e.g. phosphatidylcholine, phosphatidylglicerol and fatty acids, while the physical tests have taken advantage of the surface tension lowering property of surfactant, *e.g.* shake tests and the Wilhelmy surface tension balance. Some of these methods will be described in section 1.8.

Fujiwara and his colleagues published in 1980 their study of treatment of preterm newborn infants suffering severe RDS with an exogenous surfactant isolated from bovine lungs. Subsequently, other clinical trials have been performed using surfactant from lung of other mammalian sources. More recently artificial surfactant with satisfactory properties has been reconstituted in laboratories.

Sinkin and Shapiro [1988] reviewed a range of clinical trials of a variety of surfactant preparation in different countries; They showed a reduction in the incidence or severity of RDS with surfactant therapy. However, a number of questions remain to be answered,including the optimum timing for first giving the surfactant, the amount of surfactant to be administered, and the number and frequency of doses.

1.3.2 Current practice in treatment

The major aim of RDS therapy is the maintenance of adequate gas exchange to provide oxygen to the tissues and avoid the consequences of hypoxemia or hypercapnia. Any therapeutic programme for RDS should correct the deficiency of surfactant and must provide a satisfactory supporting environmental as well.Consistent observation and monitoring, control of environment and body temperature, provision of fluid, electrolytes and calories are all important basic considerations.

Sufficient oxygen must be supplied to maintain the Pa O_2 above the critical minimum for aerobic cellular respiration and the maintenance of cellular integrity (30 mm Hg or 4 KPa). Although there is no agreement on optimal values for arterial blood gases, for practical purposes Pa O_2 should be maintained between 50 and 70 mm Hg (6.7 and 9.3 KPa) and Pa CO_2 in the range of 40 to 55 mm Hg (5.4 to 7.4 KPa). Perhaps of greater importance than Pa O_2 is the oxygen saturation, which should be maintained at 90% or greater, and pH, which should be maintained no lower than 7.2 to 7.25.

The observation that alveolar patency could be maintained in surfactant deficient lung by the use of continuous positive airway pressure (CPAP) by

Gregory *et al.*, [1971], resulted in a greater advance in survival in preterm infants. CPAP acts in the same way as when the patient is grunting. Physiological effects are decreased mucosal swelling, dilatation of airways, increased compliance and decreased edema. This reduces airway resistance, increases functional residual capacity (FRC) and decreases formation of hyaline membranes.

In the past 20 years, ventilatory techniques have improved. The work of Herman and Reynolds [1973] resulted in the use of prolonged inspiratory time (IPPV). The indications for IPPV in RDS are clinical deterioration with recurrent apneas and deteriorating blood gases. In practice, usually IPPV is applied if Pa O_2 is below 40 mm Hg (5.5 KPa) and/or Pa CO_2 is above 65 mm Hg (9.0 KPa). IPPV has been used in many centres, although there is some evidence correlating IPPV with Chronic Lung Disease(CLD) (see section 1.4). Recently, ventilation by high frequence oscillation (HPV) has been used. Using this technique, gas exchange can occur with pressure fluctuations much lower than required for conventional ventilation. The HPV has been effective in the treatment of RDS [Boynton *et al.*, 1984], [Butler *et al.*, 1980], [Frantz *et al.*, 1983], [Marchak *et al.*, 1981] and reduction of CLD incidence.

Diuresis of infants with RDS has been correlated with improvement in pulmonary function. Heaf *et al.* [1982] demonstrated that FRC and dynamic lung compliance rapidly improved when diuresis began. Several studies have examined the effect of administering a diuretic to facilitate the spontaneously ocurring diuresis and perhaps ameliorate the course of RDS. Yeh *et al.* [1984] used furosemide in a randomized clinical trial and found some beneficial effects, such as reduction in mean airway pressure and extubation earlier than in the control group.

The use of muscle relaxation with pancuronium in infants mechanically ventilated for RDS remains controversial. Stark *et al.* [1979] and Crone and Favorito [1980] demonstrated improvement in gas exchange and low incidence of pneumothorax. Greennough *et al.* [1984] found reduction in air leak, and other reported benefits include decrease of oxygen therapy, reduction of barotrauma [Pollitzer *et al.*, 1981], and reduction of intracranial pressure elevation [Finer and Tommey, 1981].

The most exciting recent advance in RDS therapy has been the administration of exogenous surfactant to infants early in the course of their disease. Several clinical trials of the use of surfactant for infants with RDS have been reported: Hallman *et al*, 1985, Enhorning *et al.*, 1985, Kwong *et al.*, 1985, Shapiro *et al.*, 1985, Merritt *et al.*, 1986, Gitlin *et al*, 1987. Some of these

trials reported a significantly decreased incidence of air leak and pulmonary interstitial emphysema. Three of the trials demonstrated that use of surfactant results in lower Fi O_2, less barotrauma and shortened periods of ventilation, and consequently a diminished incidence of CLD.

1.4 Neonatal chronic lung disease

Infants under 28 weeks of age or who weigh less than 1000g are increasing likely to require oxygen and ventilatory support for several weeks. This can lead to the appearance of chronic pulmonary lesions. This pathological condition was originally called bronchopulmonary dysplasia (BPD) and was characterised by: 1)a respiratory disorder that began with acute lung injury (whether primary or induced by assisted ventilation) during the first 2 weeks of life, 2) oxygen dependence at 28 days of postnatal age and 3) significant clinical (tachypnea, retractions, etc), radiologic (hyperinflation or obvious cystic areas) and blood gas tension abnormalities (Pa $O_2 < 60$ Torr or Pa $CO_2 > 45$ Torr while inhaling ambient air at sea level) [O'Brodovich and Mellins, 1985]. BPD was initially reported by Northway and colleagues [1967] and the disease has been associated with the use of supplemental oxygen and mechanical ventilation for RDS therapy. Because infants can develop chronic respiratory problems without passing through all the steps described by Northway, chronic lung disease (CLD) is now the preferred alternative term.

There are some controversies about the relative role of oxygen and positive pressure in the production of CLD, but it is accepted that the therapy for RDS is strongly implicated in its pathogenesis, and any therapy that reduces the severity of respiratory distress and the need for oxygen or mechanical ventilation should decrease the incidence and severity of CLD.

The factors with influence on development of CLD are gestational age and birth weight of the infants that are ventilated. At the University of Miami, it was found in 1981/1982 that the incidence of CLD varied from 85% in infants with birth weight between 500-699g to 5% in infants above 1300g birth weight. Smaller infants are more prone to develop CLD for several reasons. These include higher incidence of RDS, incomplete development of distal air spaces, lack of antioxidant enzymes (AOE) such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (see subsection 1.4.2), nutritional deficiencies of vitamins E and A and of other substances such as selenium and sulphur-containing amino acids.

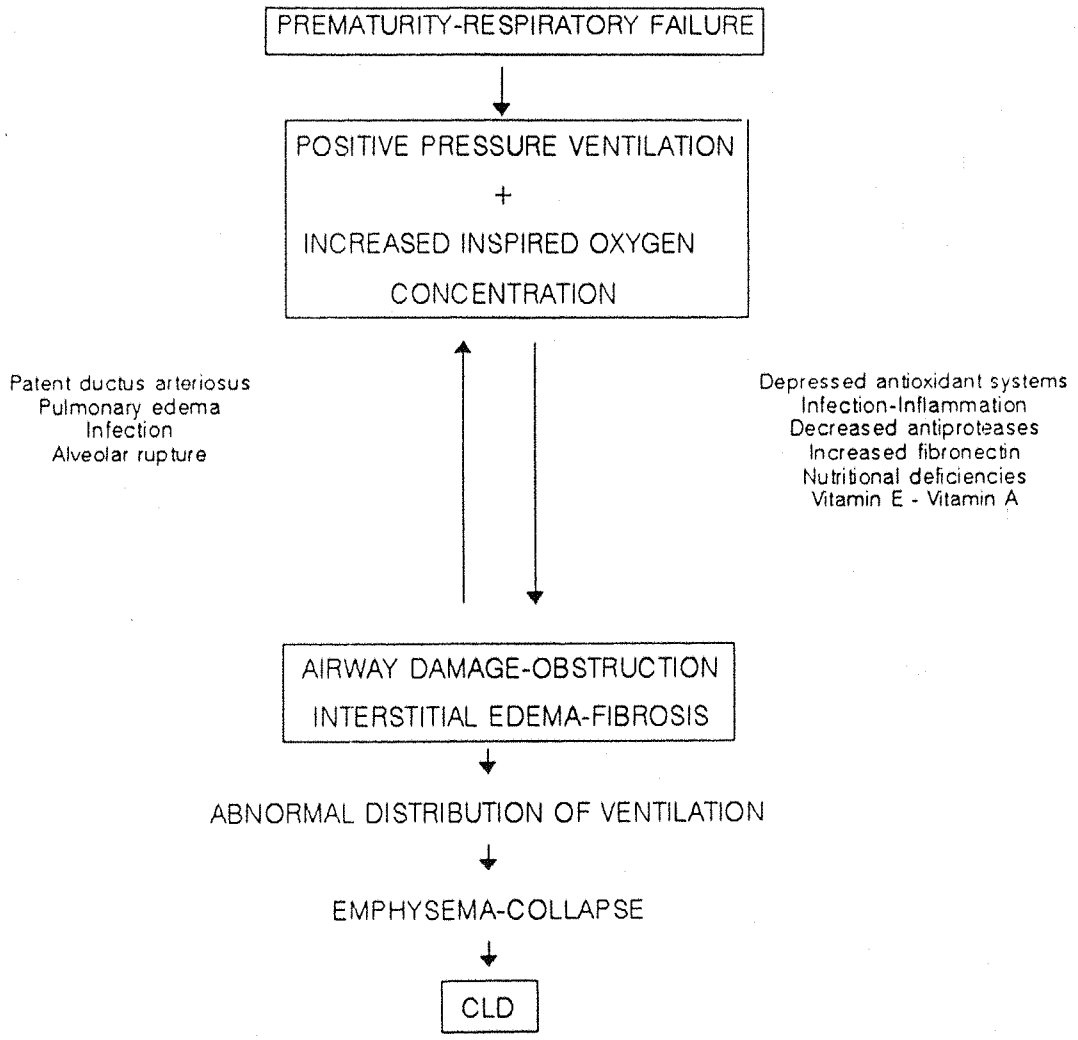

Figure 1.3: Factors implicated in pathogenesis of CLD (after Bancalari, 1988)

The clinical presentation of CLD has changed over the last years, making the definition of risk factors more difficult [Bancalari, 1988]. The aetiology of CLD is not yet completely defined but some factors have been associated with its development, such as the structural or functional immaturity of the lungs due to low gestational age of the infant, barotrauma, oxygen toxicity, pulmonary inflammation, pulmonary edema and increased airway resistance. Figure 1.3 shows a diagram with the factors implicated in the pathogenesis of CLD and these factors are discussed below.

1.4.1 Barotrauma

Most cases of CLD occur in infants who receive intermittent positive pressure and in only a few cases of CLD in infants ventilated with intermittent negative pressure [Monin *et al.*, 1976]. Reynolds and Taghizadeh [1974, 1976] reported that more severe lesions of CLD were correlated with the use of high airways pressures. While positive pressure ventilation is associated with CLD, there is not enough clinical or experimental evidence to point one particular characteristic of ventilation as the main factor in the pathogenesis of CLD [Bancalari, 1988]. Generally, it is difficult to separate the effect of pressure from other causes detailed below.

1.4.2 Oxygen toxicity

Northway *et al.* [1967] reported that the ocurrence of CLD was related to the high oxygen inspired concentration and long duration of ventilation. Later, other studies have confirmed that hyperoxia may be harmful to pulmonary surfactant. This can occur through suppression of Type II alveolar epithelial cell growth, or surfactant synthesis, or by surfactant inactivation by plasma protein components that permeate the alveolar-capillary barrier [Haagsman and Van Golde, 1985].

Although 60% O_2 has been noted to be a threshold for exaggerated pulmonary O_2 toxicity in adults, this level of hyperoxia may exceed the detoxification capabilities of the preterm infants' lungs. Multiple mechanisms exist by which oxygen metabolites can injure the lung. They can damage proteins such as those important for energy production, membrane transport, or other metabolic functions of the lung [Black and Fisher, 1977]. They can damage membrane lipids through peroxidative reactions [Tappel, 1973] and they can react with DNA, either with the bases or with the ribose

phosphate backbone [Waitberg *et al.*, 1985], [Pepine *et al.*, 1981].

Some lung enzymes have an important role in the antioxidant defense mechanism. Superoxide dismutase is held to be of primary importance in the defense of the lung against toxic oxygen metabolites. It is distributed throughout cells, associated either with manganese or copper-zinc [White and Peppine, 1985]. Two other enzymes exist in the lung for detoxifing hydrogen peroxide: Catalase which functions maximally at high concentrations of H_2O_2 and glutathione peroxidase which is widely distributed within cells and is effective at low concentration of H_2O_2 [Chance *et al.*, 1979].

Elevated lung antioxidant enzyme (AOE) activities may be associated with tolerance to hyperoxia. In a variety of different species the activities and amounts of lung AOEs increase during the final 10-15% of gestation [reviewed in Allen & Balin, 1989]. So the AOE activities of the lungs of premature animals are quite low [White, 1988] and unrestricted hyperoxia can apparently worsen CLD and convert it into a lethal or more morbid lesion.

AOE activities have been observed to rise before term in the relatively precocious guinea pig. CAT activity increases by 100-300%, SOD activity increases by 30-66% and GSH-Px activity increases by 57-160% units/mg DNA [Frank & Sosenko, 1987], [Rickett & Kelly, 1990].

Data on AOE activities during human lung development is much less complete. Strange and colleagues found little change in either SOD or GSH-Px activities during human fetal and neonatal lung development [Strange *et al.*, 1988]. McElroy [1991] confirmed that both SOD and GSH-Px activities remained relatively constant between 11-52 weeks post conceptual age, but the development of CAT activity (μ units/ mg protein) increased by greater than 3 fold between 11 weeks gestation and term.

In addition to the enzymatic systems,vitamins and trace metals may also play an important supplementary role. Vitamin E functions as an important antioxidant in membranes by terminating lipid chain peroxidation reactions [Tappel, 1982]. Vitamin A is another fat soluble vitamin, with an antioxidant role, although less well defined. Concerning metals, a dietary deficiency of selenium worsens pulmonary oxygen toxicity [Cross *et al*, 1977] and deficiency of sulfur containing amino-acids can result in depletion of antioxidant systems [Deneke *et al.*, 1983],[Deneke *et al.*, 1985].

1.4.3 Inflammation and infection

CLD occurs generally in infants who have endotracheal intubation for a long time which generally is associated with bacterial infection of the lower respiratory tract. This fact leads to the suggestion that infection is a possible contributing cause of CLD, although the correlation has not been clearly established.

The combination of increased microvascular permeability and polymorphonuclear recruitment to the lung is another aspect of the inflammatory response that has been implicated in the pathogenesis of CLD. Merritt and co-workers [1981,1983] demonstrated that neutrophils and elastase of neutrophil origin are present within airway fluids during the later part of the first week of life in infants destined to develop CLD. Premature infants appear to be more vulnerable to neutrophil protease-induced lung injury. Several studies have demonstrated an association between the development of CLD and deficient serum α_1-proteinase inhibitor, the major inhibitor of elastase released during inflammation. McCarthy *et al.* [1984], Rosenfeld *et al.* [1986] found decreased trypsin inhibitory capacity in serum of patients who developed CLD.

The influx of inflammatory cells into the lung results in the release of a variety of humoral factors (leukotrienes, platelet activating factor (PAF) and prostaglandins). The exact mechanisms whereby neutrophils induce pulmonary damage are still incompletely understood but are likely to involve protease release and generation of toxic radicals.

Concerning infection, the normal colonization of the upper airways and gastrointestinal tracts occurs within the first week [Smith, 1950], [Rotini and Duerden, 1981] but it is altered by antibiotic treatment, intubation, and aspiration around the endotracheal tubes in the intensive care setting [Goldman *et al.*, 1978], [Spruntk *et al.*, 1978], [Borderon *et al.*, 1981]. Neutrophilic bactericidal activity in preterm infants is impaired; the lung's normal upper respiratory antimicrobial defences are bypassed via intubation and ventilation; and the infant is born with low levels of antioxidants [Chirico *et al.*, 1985]. Alveolar macrophages are sparse in number, further accentuating the susceptibility to infection [Alenghat and Esterly, 1984] which is an important determinant of mortality in the neonatal intensive care and is likely to be an important additional injury to lungs.

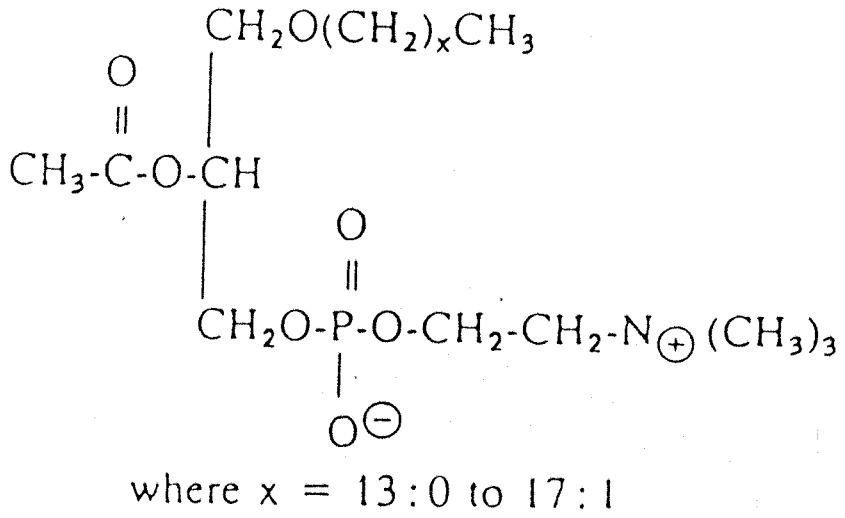

Figure 1.4: Structural formula of platelet activating factor (1-O-alkyl-2acyl-sn-glycero-3-phosphocholine)

1.5 Platelet - activating factor (PAF)

Platelet activating factor (PAF) is the general used name for a group of potent biologically active, ether-linked phosphocholine, (alkylacetylglycerol phosphocholines) [Synder, 1987]. There are two pathways described for the synthesis of PAF: the *remodelling* pathway and the *de novo* synthesis. The remodelling pathway involves the deacylation of membrane bound 1-O-alkyl-2acyl-sn-glycero-3-phosphocholine by phospholipase A_2 (PLA_2), and acetylation by a specific acetytransferase (figure 1.4). Both PLA_2 and acetyltransferase can be stimulated in platelets [Albert, 1982], [Henson, 1992]. The *de novo* pathway involves the synthesis of 1-alkyl-2-lysoglycero-3-phosphate, acetylation and dephosphorylation to 1-O-alkyl-2-acetyl-glycerol and its conversion to PAF by choline-phosphotransferase; this seems to be involved in generating basal levels of PAF and it is not stimulable [Henson, 1992].

In addition to its activity on platelets, PAF possesses a broad range of biological activities, such as bronchoconstriction and relaxation of vascular smooth muscle. PAF also has many pro-inflammatory properties [Abbas *et al.*, 1991], [Sturk, 1989].

It is synthesized by pro-inflammatory cells such as neutrophils, monocytes, macrophages, eosinophils, basophils, and mast cells. PAF induces chemotaxis of neutrophils and eosinophils [Shaw *et al.*, 1981], [O'Flaherty *et al.*, 1981], [Wardlaw and Kay, 1986] and activates neutrophils, monocytes and platelets [Benveniste *et al.*, 1972], [Yanaka *et al.*, 1982]. The

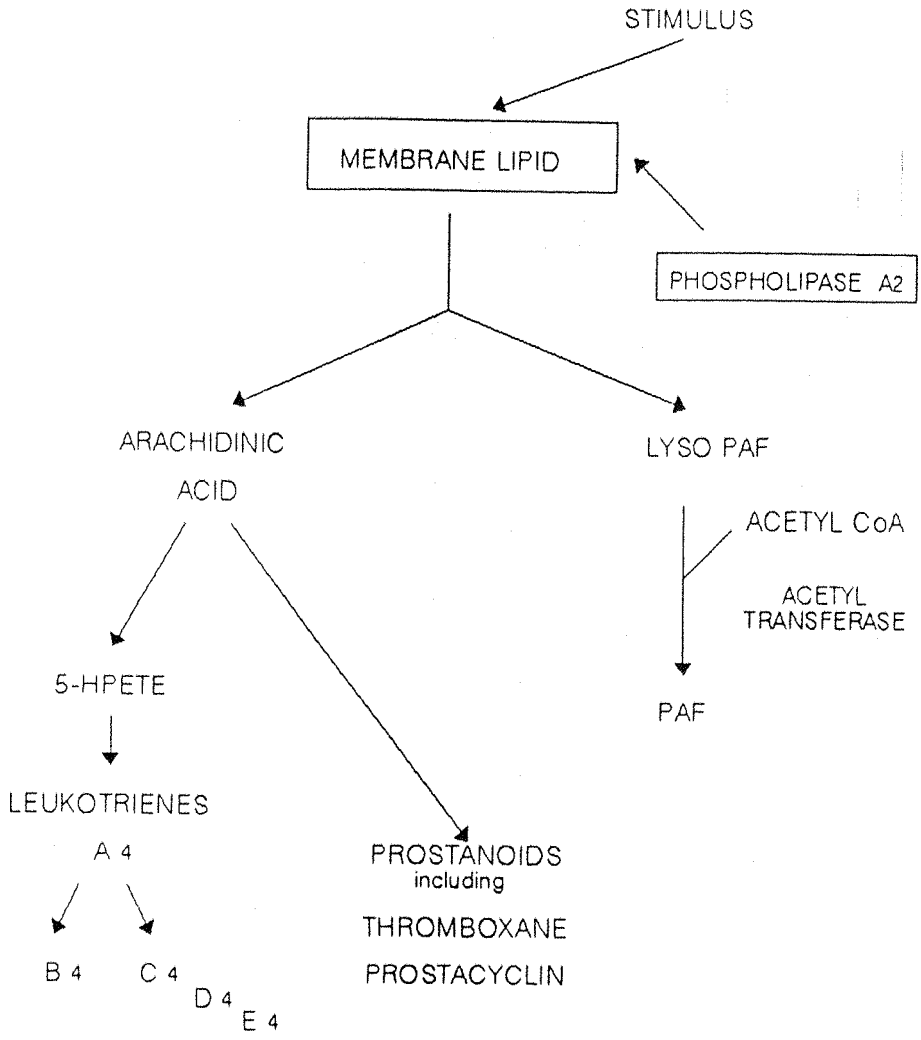

Figure 1.5: Simplified cascade indicating formation from a common precursor membrane lipid of both arachidonic acid and platelet activating factor (PAF) (after Stenmark and Voekel, 1988)

neutrophils also respond by a phenomenon of priming, where the stimulus does not induce a response but alters the cells reactivity to secondary stimuli, so that the eventual response is significantly enhanced. The importance of this phenomenon might be in the concept of two levels of activation of migratory inflammatory cells. Initiation of migration could involve only minimal activation of oxidative and secretory processes. However, at the actual inflammatory site a primed cell would now exhibit an amplified response necessary for the protective processes of inflammation.

Intradermal injection of PAF causes immediate increase in vascular permeability in rats [Hwang *et al.*, 1985] but with a delay in guinea pig and human. In guinea pig addition of prostaglandin E_1 potentiates the intradermal PAF-induced responses [Handley *et al.*,1984]. Hamasaki *et al.* [1984], injecting PAF into a platelet-free perfused guinea pig lung, noted a signifi-

cant increase in airway and pulmonary edema and also synthesis of thromboxane A_2 and prostaglandin I_2 (see figure 1.5). Archer *et al.* [1984, 1985], injecting PAF intradermally in human subjects, demonstrated the expected wheal and flare reaction in 15 minutes, and at 4 to 12 hours there was intravascular accumulation of neutrophils, and extravascular accumulation predominantly of neutrophils and some lymphocytes and monocytes. At 24 hours, the infiltration reduced and was composed predominantly of lymphocytes and histiocytes. Archer *et al* [1985] suggested that PAF has many roles in acute and chronic inflammation.

Use of PAF antagonists like hetrazepines (WEB 2086, WEB 2170 and others) was shown to be effective in several models of altered airways responsiveness following inflammatory reactions in experimental animals. One of the first approaches to the use of antagonists in humans seems likely to be in asthma or in such neutrophil-dominated reactions as the adult respiratory distress syndrome [Henson *et al.*, 1992].

1.5.1 Pulmonary edema

Brown *et al.* [1978] found that infants with CLD had received greater amounts of fluids during the first 5 days of life than the infants who did not develop CLD. Supporting evidence from other groups suggests that increased pulmonary blood flow and/or interstitial fluid can cause a decrease in pulmonary compliance and increase in airway resistance [Bancalari et al., 1977] and consequently increase the risk for CLD.

1.5.2 Airway obstruction

Increased airway resistance has been observed in infants with CLD. Goldman *et al.* [1983] evaluated pulmonary function in a group of infants early during the course of RDS, and they concluded that infants who subsequently developed CLD had increased pulmonary resistance from the first week of life, whereas infants who recovered had lower resistance values. This may open the possibility for early intervention in order to lower airway resistance and possibly reduce CLD incidence.

Factors that may determine the increased airway resistance include hyperplasia and squamous metaplasia of the bronchial epithelium, increased mucus production combined with reduced elimination, mucosal edema caused by inflammation, trauma, infection or increased lung fluid, bronchoconstric-

tion originated by smooth muscle hypertrophy or familial predisposition, and small airway closure due to pulmonary interstitial emphysema [Bancalari and Gerhardt, 1986].

1.6 Surface Tension

Surface tension is an important characteristic of the surfactant system and will be described in this subsection from the theoretical point of view. Following sections will present the influence of surface tension in the pulmonary system and later, in chapter 3, results for surface tension measurements will be shown.

1.6.1 Definition

Surface tension (γ) is a manifestation of surface energy and in the practical world it can be perceived, for example, as the force which keeps a small insect on the water surface or prevents beer from overflowing when its level is above the rim of the glass.

Surface tension is associated with each interface between two phases, such as air-liquid, liquid-solid or two immiscible liquids. It is defined either as the surface force per unit length or as the necessary energy to extend the surface by an unit area. In the International System (SI), its unit is Joule/m^2 or Newton/m, although in laboratory practice and in technical papers it is more common to use the unit dyne/cm.

Contact angle at the intersection of two interfaces is shown in figure 1.6. Figure 1.6.a shows a drop of water over a glass surface in which the water wets the surface and forms an acute angle, figure 1.6.b shows a drop of water over a solid surface of fat in which an obtuse angle is formed due to the hydrophobic characteristics of the surface. Finally figure 1.6.c shows the forces acting at the triple point. The normal and tangential components of the surface force acting on a plate depend on the contact angle and that is the reason for the importance of cleaning and test cleanliness of the Wilhelmy balance platinum float, as it will be seen in section 2.13.

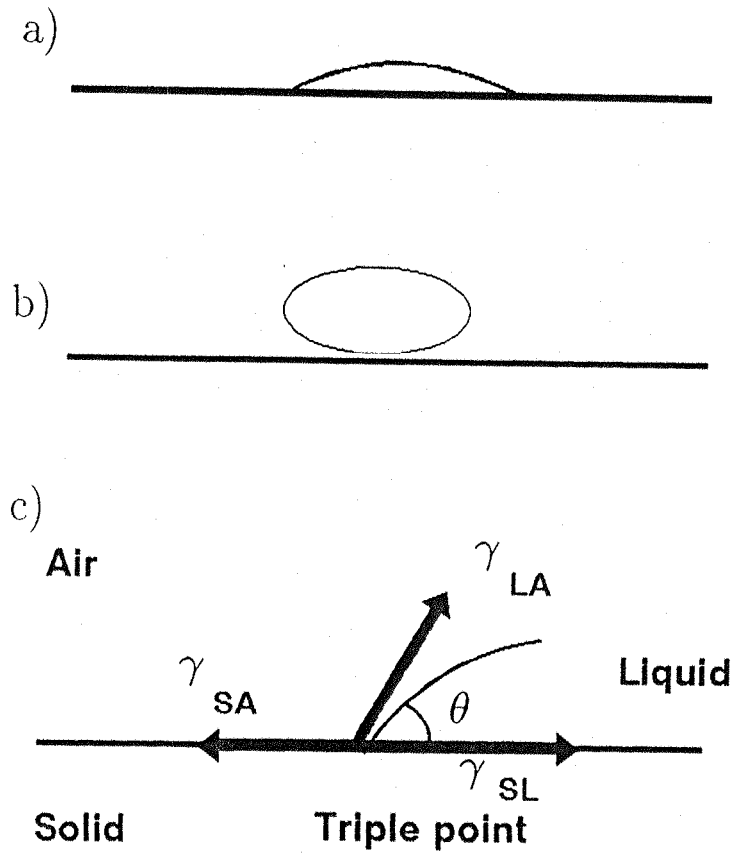

Figure 1.6: a) Contact angle $< 90°$ b) Contact angle $> 90°$ c) Forces acting at the triple point

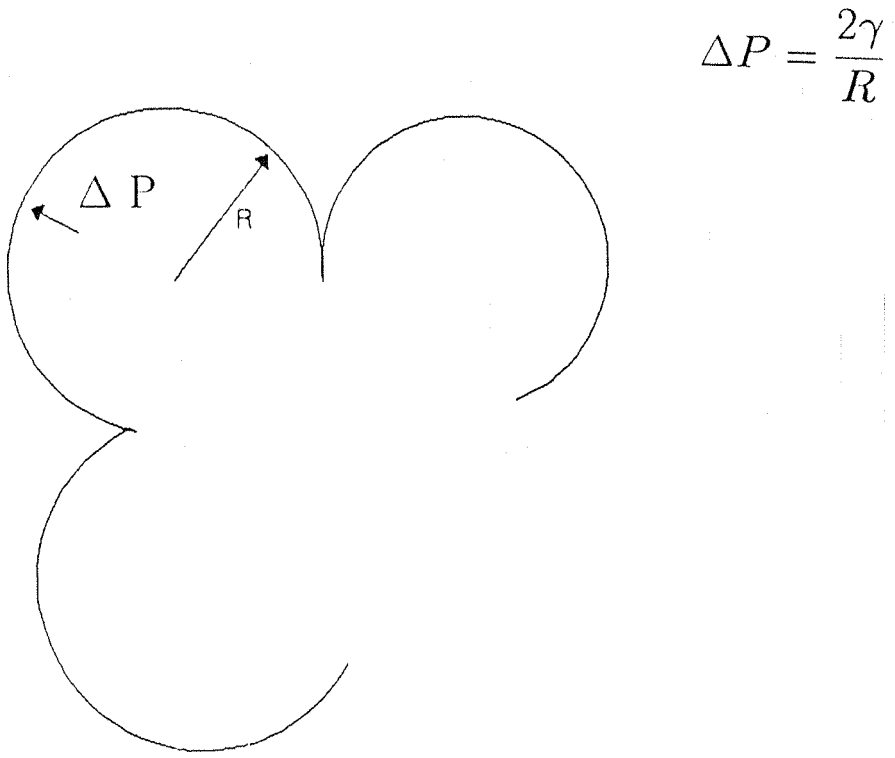

Figure 1.7: Laplace's law

1.6.2 Laplace's Law

The alveolar lining layer, as all human tissues, contains a high percentage of water. Water molecules are polarized and they are strongly attracted to each other by hydrogen bonding and consequently a high surface tension is produced at the water/air interface.

Alveoli can be thought of as spheres or bubbles, where the forces acting obey Laplace's equation

$$\Delta P = \frac{2\gamma}{R} \tag{1.1}$$

where
ΔP = pressure difference
γ = surface tension
R = radius

The pressure difference across a sphere is proportional to the value of surface tension and inversely proportional to its radius (figure 1.7). It means that, if the alveolar lining consisted entirely of water, with no surfactant present, the pressure required to keep the alveoli open would be greater for larger surface tension than for smaller. This situation becomes critical in expiration when the radius is minimum, and much greater pressure would be needed to reopen the alveoli.

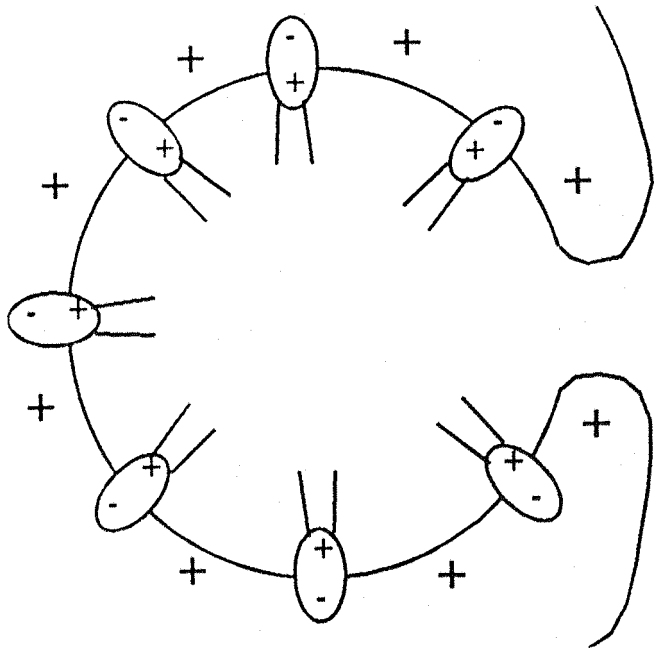

Figure 1.8: Alveoli lining layer with presence of surfactant

1.7 Pulmonary surfactant

Surfactant phospholipids are responsible for the surfactant surface properties. Phospholipids have a polar hydrophilic moiety and an apolar hydrophobic moiety. When surfactant is present at the alveoli lining, the hydrophilic extremity is positioned near the water molecules in the bulk phase and the apolar extremity remains at the surface (figure 1.8). These apolar moieties at the surface produce a lower surface tension by reducing hydrogen bondings of water molecules at the interface.

In the presence of surfactant, surface tension during compression is lower than during expansion. During compression, the surface film is reduced and water molecules move from surface to subphase, increasing surfactant concentration at the surface and consequently decreasing surface tension. During reexpansion surfactant returns to the surface slower than water, lowering surface concentration of surfactants and increasing surface tension. By lowering surface tension, surfactant enables the alveoli to reopen easily.

1.7.1 Characteristics of pulmonary surfactant

Pulmonary surfactant is a mixture of phospholipids, neutral lipids and proteins that coats the interior of the lungs. It spreads as a monolayer in the lungs and reduces both the tendency of alveoli to collapse at end expiration and the transudation of fluid into the air spaces [Clements-1977], [Goerke-1974], [Guyton-1984]. Generally it is achieved by reducing surface tension at the air-liquid interface of the alveoli. In addition to its surface-tension reducing properties, surfactant may also aid in the prevention of pulmonary

edema [Clements-1965] and may act in the lung's system of defense against infection.

A number of physico-chemical properties of natural surfactants are considered to be important to their function; exogenous surfactant either obtained in a natural way or artificially must be compared with these characteristics to predict its applicability and efficiency. Jobe and Ikegami [1987] summarize the surface properties of natural surfactant as follows:

- Should spread within seconds when applied to a surface and establish an equilibrium surface tension.
- Should lower the surface tension on dynamic compression. Multiple compression and expansion cycles should be reproducible, and the surface tension on expansion should return to the equilibrium surface tension, indicating that the surfactant respreads after compression.

After the successful experiment of Fujiwara and his colleagues in 1980, several categories of surfactants have been evaluated for clinical use. The first of them was natural surfactant, either homologous or heterologous, obtained from alveolar washes or from amniotic fluid. Subsequently other experiments were performed using modified natural surfactants, prepared by extraction of lipids with addition and/or removal of compounds. Table 1.1 summarizes several surfactant preparations for clinical replacement with their sources and method of preparation.

Artificial surfactants for clinical use are mixtures of synthetic compounds, some of which are normal components of natural surfactant. A new category, called synthetic natural surfactant by Jobe and Ikegami [1987], can be obtained from phospholipids and human surfactant proteins produced by the new techniques of biotechnology. The genes for the human surfactant proteins have been identified, and recombinant DNA techniques can be used to produce these proteins. Such synthetic natural surfactant is not yet available for clinical use.

1.7.2 Synthesis and metabolism of surfactant

Alveolar surfactant is not a static component of the pulmonary system; it is continuously secreted and catabolised. Wright and Clements [1987] divide surfactant metabolism into three parts: the intracellular metabolism,

Type of surfactant	Specific preparation	Source and method of preparation
Human	Human	Isolated from amniotic fluid aspirated aseptically at caesarian sections of term pregnancies
Bovine and porcine	CLSE (calf lung surfactant extract) and bovine lung surfactant extract	Organic solvent extraction of surfactant from lung lavage of calf and cow lungs.
	Curosurf	Organic solvent extraction and liquid gel chromatography of surfactant from minced porcine lung
	Surfactant-TA (Surfanta)	Organic solvent extraction from minced cow lungs with added phospholipids.
Synthetic	ALEC-Artif. lung expand. compound	7:3(molar ratio) of DPPC:egg PG DPPC=dipalmitoyl phosphatidylcholine PG=phosphatidylglycerol
	Exosurf	Detergent-like substance or emulsifying agents (tyloxapol and hexadecanol) added to DPPC

Table 1.1: Exogenous surfactants for clinical application and methods of preparation

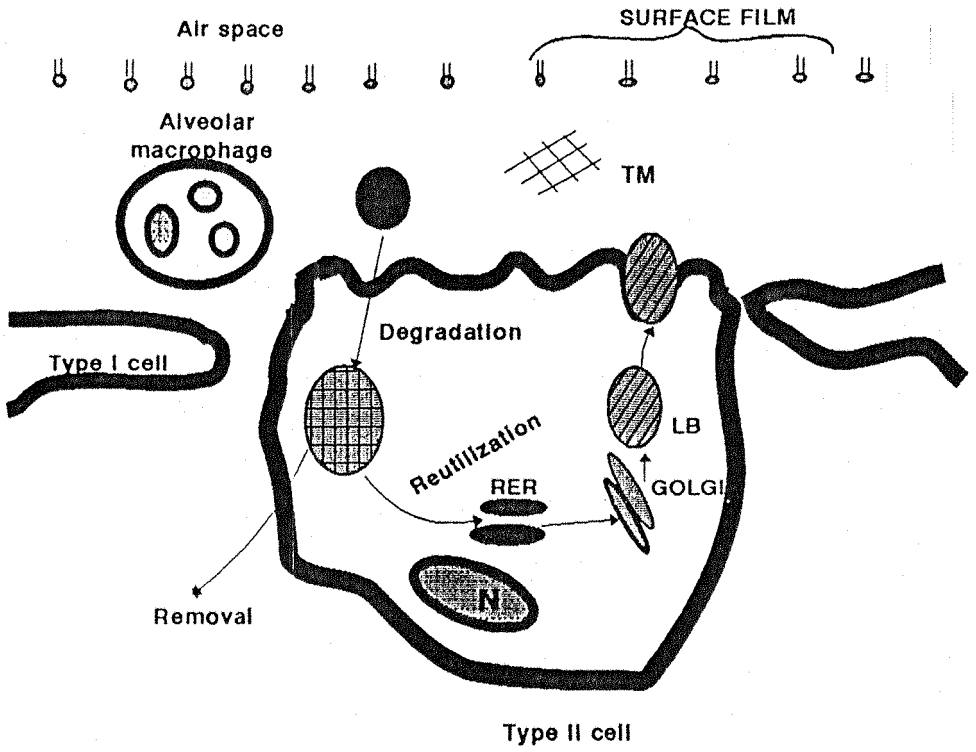

Figure 1.9: Metabolic pathways of lung surfactant (after Wright and Clements [1987])

the intra-alveolar metabolism and clearance. Intracellular metabolism comprises the processes of biosynthesis of lipid and protein components, together with their assembly into mature surfactant.

Figure 1.9 shows a schematic diagram of metabolic pathways of lung surfactant. The lipids of pulmonary surfactant are synthesized in endoplasmic reticulum of type II cells. Then they are transported to the Golgi apparatus. After that, they are tightly packed into a membrane bound organelle, the lamellar body [Gil and Reiss, 1973].

Proteins and apoproteins follow a different route. Proteins are synthesized in the endoplasmic reticulum and they next move to the Golgi, where they are most likely glycosylated [Wright and Clements, 1987]. After that, they move to lamellar bodies by way of multivesicular bodies. Either multivesicular bodies or lamellar bodies may be the location where phospholipids are united with protein.

The surfactant apoproteins are not localized inside the lamellar bodies, but are associated with the periphery of the lamellar body [Walker *et al.*,

1986]. The next step in surfactant's metabolism is the secretion of surfactant from lamellar bodies into the alveolar spaces. Most of the components of surfactant are secreted together.

Concerned with the second item, it is believed that surfactant undergoes metabolic transformations after it is secreted into the alveoli. The principal surface-active ingredient of pulmonary surfactant is phospholipid and in particular the disaturated dipalmitoyl phosphatidylcholine (DPPC). After the secretion of intracellular lamellar bodies into alveolar spaces, the formation of tubular myelin, a unique lattice like structure, from newly secreted lamellar bodies is considered the precursor to the surface film [Paul *et al.*, 1977].

Other physical forms of surfactant-related material have also been observed in the alveoli, including large and small multilamellar vesicles, and disk-like structures thought to be open-ended bilayers [Wright, 1990]. These structural changes are unlikely to be due to changes in phospholipid composition because this was similar for all fractions. It seems likely that some changes in surfactant structure may result from the association or dissociation of one or more surfactant associated proteins with surfactant phospholipid [Williams *et al.*, 1990]. After its secretion into the alveoli, the surfactant undergoes metabolic transformations related to its clearance and recycling.

In the clearance of surfactant, surfactant lipids and proteins can be either degraded, resynthetized and reutilized, or recycled (absorbed and resecreted). Perhaps the most common pathway for clearance of surfactant is the reuptake of surfactant lipids from the alveoli by the Type II cell. The lipids and proteins can be degraded and the degradation products used for synthesis of new surfactant components. Alternatively, surfactant may be incorporated into lamellar bodies and be resecreted [Wright, 1990]. Another route for clearance is the uptake of surfactant by alveolar macrophages [Naimark - 1973]. Another potential pathway for clearance, via the airways, does not seem clear significant amounts of surfactant [Fisher et al, 1979].

Perinatal changes

Several changes occur in the surfactant system during the last few weeks of gestation to ensure that, at birth, fetal pulmonary fluid is absorbed rapidly from the airways and replaced by air. Increasing quantities of whole tissue phosphatidylcholine (PC) and the disaturated component of PC are

produced in the lungs towards term. Lamellar bodies are secreted from type II cells into the pulmonary fluid.

Hallman [1984], analysing human amniotic fluid, observed that the PC and DPPC contents of human amniotic fluid increase markedly after 30 weeks in gestation. The percentage of phosphatidylinositol (PI) decreases and phosphatidylglycerol (PG) increased at approximately 35 weeks of gestation [Hallman *et al.*, 1976]. Surfactant protein A was first detected in human amniotic fluid at approximately 30 weeks gestation and its concentration then increased during the remaining gestation period [King *et al.*, 1975].

Hunt *et al.* [1989] have analysed the developmental changes in individual molecular species of phosphatidylcholine(PC) from fetal lungs of rat, guinea pig and man. They found that both the relative amount and percentage saturation of lung PC rise substantially as gestation progresses. Detailed analysis revealed a progressive increase in two disaturated species: dipalmitoyl PC (PC 16:0/16:0) and myristoylpalmitoyl PC (PC 14:0/16:0) and a fall in palmitoyloleoyl PC (PC 16:0/18:1). In this respect, the guinea pig more closely resembled the human and it has been proposed that guinea pig provides a useful model for human lung prematurity studies [Hunt *et al.*, 1991].

1.7.3 Composition

Lipids

The most active ingredients of surfactant are phospholipids, and associated with other lipids they make up 80 to 90% of surfactant weight. Apart from this, pulmonary surfactant also contains protein (8%) and carbohydrate (2%). The two more important classes of lung phospholipids (PL) are PC, constituting 70% to 80% of the total lipids, and PG, corresponding to 5 to 10%. Among the neutral lipids the most abundant are cholesterol (7.1%) and triglyceride/free fatty acid (7%) followed by diglyceride, cholesterol ester and hydrocarbons [Morgan, Finley and Fialkow, 1965].

About 60% of surfactant PC is DPPC, which is considered to be responsible for the stability of the surface tension-reducing film [Clements and Tierney, 1965]. Figure 1.10 shows the surfactant composition of adult human lung bronchoalveolar lavage. Other components of surfactant phospholipid include phosphatidylinositol (PI), phosphatidylethanolamine (PE)

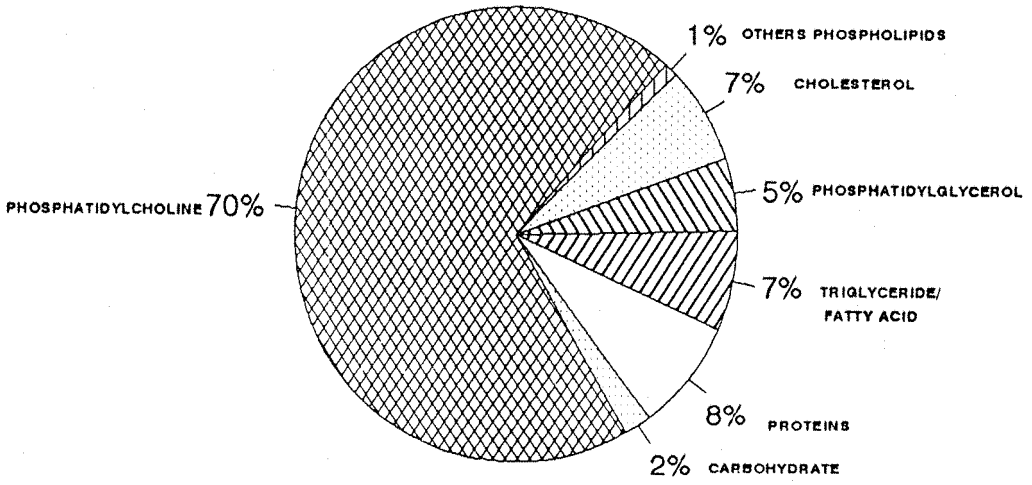

Figure 1.10: Surfactant composition of adult human lung bronchoalveolar lavage

and phosphatidylserine (PS).

Hunt *et al.* [1991] published a comparison of lung PC molecular species from human, guinea pig and rat (Table 1.2). It is interesting to note that the phospholipid composition from various adult mammals are quite similar.

Proteins

Using a surface tension balance, King and Clement [1972] observed that surfactant from which protein was depleted formed stable surface films much more slowly than surfactant which contained protein. Proteins are important components of lung surfactant because rapid surface film formation is necessary for normal respiration. Specific surfactant proteins SP-A, SP-B, SP-C and SP-D have been identified and associated with surfactant lipids and enhance the surfactant properties of the phospholipids [Weaver and Whitsett, 1991].

The predominant surfactant associated protein is the water soluble glycoprotein SP-A, characterized by a collagen like N-terminal domain and variable N-linked glycosylation of the COOH-terminal region. SP-A con-

PC species	PC distribution (Percent sum of major species)					
	Human		Guinea pig		Rat	
	Tissue	Lavage	Tissue	Lavage	Tissue	Lavage
14:0/16:0	9.1±1.8	12.4 ±3.4	12.9 ± 1.6	15.3 ± 3.5	8.4 ± 1.5	13.5 ± 3.0
16:0/16:1	12.0±5.8	16.0 ±5.4	9.1 ± 2.1	9.2 ± 5.9	19.3 ± 5.3	25.9 ± 2.7
16:0/18:2	11.6±2.5	8.2 ±2.2	16.4 ± 1.7	12.3 ± 2.3	11.0 ± 2.0	5.4 ± 1.3
16:0/16:0	45.0±8.0	51.1 ±8.7	44.1 ± 3.6	48.6 ± 6.3	47.0 ± 5.0	49.5 ± 3.4
16:0/18:1	22.3±3.6	12.7 ±2.3	15.4 ± 1.5	13.3 ± 2.0	14.1 ± 2.1	5.6 ± 1.7

Table 1.2: Phosphatidylcholine composition of adult human, guinea pig and rat lung. PC species compositions of whole, unfractioned tissue are shown alongside those of bronchoalveolar lavages of parallel lung samples. Results are presented as percentages of the sum of the principal molecular species of lung PC (means ± S.D.). The following abbreviations are used: PC14:0/16:0 = myristoyl palmitoyl PC, PC16:0/16:1 = palmitoyl palmitoleoyl PC, PC16:0/18:2 = palmitoyl linoleoyl PC, PC16:0/16:0 = DPPC, PC16:0/18:1 = palmitoyl oleoyl PC [Hunt *et al.*, 1991]

sists of a major charge train of 9-13 proteins with molecular weight between 34,000 Da and 36,000 Da and a minor charge train of 3 proteins with molecular weight between 28,000 Da and 30,000 Da [Whitsett *et al.*, 1985].

SP-A enhances phospholipid uptake and inhibits phospholipid secretion by type II cells in vitro. One of its primary biophysical functions in endogenous lung surfactant is in the formation of tubular myelin in the presence of calcium ions. SP-A was demonstrated to improve the resistance to inhibition of lung surfactant extracts and of synthetic mixtures [Venkitaranan, 1990]. SP-A may also be important in the immune defence system of the lung; it has been shown to increase phagocytosis of opsinized erythrocytes and bacteria by macrophages and monocytes [Tenner *et al.*, 1989].

SP-B is a hydrophobic peptide of molecular weight 8,000 Da, it is sparingly soluble in aqueous solution, and can be isolated in the presence of detergent [Kogishi *et al.*, 1988]. SP-B contributes to surfactant properties of phospholipids, to formation of tubular myelin and facilitates turnover of surfactant phospholipids. It also imposes an important molecular organization on phospholipid mixtures [Williams *et al.*, 1991], and markedly enhances the rate of formation of surfactant surface film at an air-liquid interface in vitro [Weaver and Whitsett, 1991].

SP-C is smaller than SP-B, is also hydrophobic and is soluble only in organic solvents. SP-C contributes to surfactant properties of phospholipids and facilitates turnover of surfactant phospholipids. SP-C also enhances the

	Function	SP-A	SP-B	SP-C
1	Contribute to surfactant properties of phospholipids	+	+	+
2	Formation of tubular myelin	+	+	-
3	Facilitate turnover of surfactant phospholipids	+	+	+
4	Inhibition of pulmonary surfactant secretion	+	-	-
5	Facilitate phagocytosis of opsonized particles	+	-	-

Table 1.3: Function of surfactant proteins (after Weaver and Whitsett, 1991)

formation of surfactant surface film, although to a lesser extent than SP-B. Table 3 shows the summary of surfactant protein function as reviewed in [Weaver and Whitsett, 1991].

SP-D consists of a charge train of proteins of molecular weight 43,000 Da, with some characteristics similar to SP-A. SP-D is similar to SP-A in that it contains a bacterial collagenase - sensitive domain, hydroxyproline, asparagine - linked oligosacharide and lectin - like activity but differs in the presence of hydroxylysine and hydroxylysine glycosides [Weaver and Whitsett, 1991]. Up to now very little has been reported about SP-D properties and functions.

Surfactant proteins SP-A, SP-B and SP-C can be associated with lipids individually or jointly and have different properties and functions in the organization of tubular myelin, unilamellar and multilamellar vesicles and others. Williams *et al.*, [1991] have performed interesting work using electron microscopy to determine the changes in structure of simple phospholipid mixtures produced by adding one or combinations of the three proteins.

Apart from these major groups of proteins, other proteins not specific to surfactant have been reported by several investigators. These proteins include albumin, immunoglobulin G [King *et al.*, 1973], secretory immunoglobulin A [Paciga *et al.*, 1980] and actin [Postle *et al.*, 1985]. It is believed that some of these proteins may also help in the defence against infections.

1.8 Model systems

Animal testing is an essential approach to the study of surfactant metabolism, composition and function. Phospholipid compositions for total lung and lavage have been reported for rat, rabbit, guinea pig, monkeys, sheep, cow,

dog, pig, chicken, turkey, turtle and frog. Ohmo *et al.*, [1978] and Sanders [1982a, 1982b] presented a review including a number of species concerning fetal and neonatal surfactant and its biosynthesis.

1.8.1 Rabbit

For some time the rabbit has been used by many investigators to analyse surfactant appearance and its accumulation and disposition in fetal and neonatal lung. One of the advantages of using rabbit is the number and size of the pups.

Rabbit gestation term is around 31 or 32 days. Kikkawa and his colleagues [1968, 1971] found that maturation of fetal lungs started by day 26 of gestation, then the number of lamellar bodies increased rapidly and, in terms of morphology, the lung appeared competent by day 28.

Metcalf and coworkers [1983] observed that the number of preterm rabbits capable of establishing regular air-breathing increased from 10% on day 27 of gestation, through 50% on day 28, to almost all the pups by day 29 of gestation. Rooney *et al.* [1979] found a marked increase in the incorporation of choline into phosphatidylcholine during this transition between 26 and 28 days gestation.

Possmayer [1984] presented in detail the biochemistry of the last days of rabbit gestation, including the alteration in phospholipids, the increase of intracellular surfactant in the form of lamellar bodies and composition of alveolar surfactant obtained from neonatal and adult rabbit lung. A marked alteration in the PG/PI ratio in alveolar phospholipids and in lamellar bodies, which can be associated with the period of pulmonary maturation occurs shortly after birth, even with postmature delivery [Jobe, 1980].

1.8.2 Rat

Rat gestation is around 22 days, and by the day 19 pulmonary canaliculi become evident [Blackburn, 1973],[Williams, 1977]. Lamellar bodies appear on day 19, some Type I cells are present on day 20 and pratically all fetuses delivered on day 21 survive [Weinhold and colleagues, 1980 and 1981].

Tordet and co-workers [1981] reported that the phospholipid content of rat lung doubled during the last 5 days of gestation and the proportion

of PC increased to 50% of total phospholipid before delivery. The percent saturated PC increases from 15% to 20% of total PC during the later stages of fetal development and then rises to 30-35% at birth [Maniscalco *et al.*, 1978], Katyal *et al.* [1977] found that the amount of surfactant phospholipid that could be isolated from fetal rat lung homogenenates increases 20 times, between 19 days and 22 days.

Comparing saline extracts from neonatal and adult lungs, it has been observed that the ability to reduce surface tension of a Wilhelmy balance are practically the same, but surfactant obtained from neonates exhibits greater spreadability [Possmayer, 1984], which means that if it is placed on a clean surface of physiological saline, the surface tension falls rapidly to its equilibrium value.

1.8.3 Monkeys

Primate models of neonatal RDS have been used by some investigators based on the consideration that the development of the terminal air spaces and airways in the fetal monkey is quite similar to the modifications in the respiratory system of prenatal human [Boyden, 1976].

Some of the investigations have used rhesus (Macaca mulatta) or the pigtail (Macaca nemestrina) monkey and more recently baboons. Gestation time is approximately 168 days and after 153 gestation days the fetus can be considered mature [Palmer *et al.*, 1977].

Possmayer [1984] presents a review of the biochemical modifications in pulmonary fetal monkey, including the increase of lung phospholipids and the appearance of lamellar bodies and of intracellular surfactant in the lung.

Palmer *et al.* [1977] and Kotato *et al.* [1977] observed over the latter 20% of gestation a increase in the L/S and the PC/protein ratios of surfactant and an improved ability of this material to reduce the surface tension on a Wilhelmy balance. These changes were accompanied by marked increases in total lung volume and in lung stability during deflation.

Prueitt *et al.*, [1979] reported that 45% of pigtail monkeys delivered during the transitional period at approximately 135 days of gestation developed severe RDS including chest wall retraction, tachypnoea and patchy atelectasis. Furthermore, they exhibit decreased lung distensibility, unstable terminal air spaces and an inability to maintain adequate gaseous exchange.

Related to research with baboons, Escobedo *et al.*, [1982] have developed a model of BPD. De Lemos *et al.*, [1987] and Coalson *et al.*, [1988] analysed oxygen toxicity in the premature baboon with RDS and the diffuse alveolar damage in the evolution of bronchopulmonary dysplasia

1.8.4 Guinea pig

Although baboon model provides good analogy both clinically and histologically with pulmonary disease seen in the premature human neonate, it requires an considerable financial commitment to be maintained.

As an alternative some researchers have developed a model of prematurity in the guinea pig. Merritt [1982] used neonatal guinea pigs for the investigation of the lung inflammatory cell response as a model for critically ill human neonates. He determined elastase activity in lung lavage of neonatal guinea pigs exposed to either room air or hyperoxia, and compared these results with analyses in human neonatal tracheal aspirates. Neonatal guinea pigs were submitted to oxygen exposure Fi $O_2 > 0.9$ for 72 hours. Lung damage resulted in lung cell injury with enhanced chemotaxis for polymorphonuclear leukocytes and macrophages cells that release proteolytic enzymes into pulmonary fluid. His studies indicate that oxygen exposure in human newborns may result in a similar pattern of pulmonary injury as demonstrated in the neonatal guinea pig.

Sosenko and Frank [1987] analysed Anti oxidant Enzymes (AOE) in guinea pig lung development and related this to premature survival in high oxygen exposure. AOE activities increased before birth in parallel with lung surfactant maturation, probably in response to the increased metabolic activity of the lung. Rickett and Kelly [1990] also studied the development of AOEs in guinea pig lung. One of their prominent findings was the important role played by catalase (CAT) during fetal guinea pig lung development. McElroy [1991] also found similar increase of CAT activity during human lung development.

The ontogeny of surfactant phospholipids in the guinea pig is largely prenatal, compared with considerable post-natal maturation in the rats. The comparison of molecular species composition of PC in fetal and neonatal guinea pig, rat and human lung (section 1.7.2) [Hunt *et al.*, 1991] demonstrated a close resemblance in the pattern of maturation of surfactant PC.

Kelly *et al.* [1991a,1991b] and Town [1990] studied the biochemical

maturation of the guinea-pig lung and survival following premature delivery. They analysed preterm guinea pigs delivered by Caesarian section at 65 days gestation (normal gestation 68 days). The survival of pre-term animals was lower than that of term animals after exposure to 95% O_2 (pre term 42% versus term 79% at 96 hours, $P < 0.05$). Pulmonary histology in preterm compared with term animals revealed more evidence of acute lung injury with atelectasis, pulmonary edema, fibrin deposition and inflammatory cell infiltration. They concluded that the preterm guinea pig is more susceptible than the term animal to lung injury after O_2 exposure, and thus represents an appropriate small-animal model in which to investigate the pathogenesis of acute and chronic lung injury in the pre-term infant.

1.9 Analytical systems

1.9.1 Surface tension

Pulmonary surfactant is characterized by rapid adsorption, an equilibrium surface tension and a high resistance to surface compression (see section 1.5). Some instruments measure stationary characteristics of lung surfactant, while others take into account dynamics effects, although in them the effect of viscosity is also included [Hawco *et al.*. 1981].

Shake Method

The simplest method to analyse surface tension in amniotic fluid is the shake method [Clements, J.A. , 1972] which has been used to indicate fetal lung maturity. Amniotic fluid is mixed with ethanol to prevent foaming, and formation of a stable ring of bubbles at the meniscus of the mixture indicates fetal lung maturity. No bubbles suggests that the lung is still imature. This test gives only a qualitative assessment of surface tension, and is too insensitive and error-prone to have been widely adopted into clinical practice.

Wilhelmy Balance

The principle of Wilhelmy (1863) for measuring the surface tension of liquids has been adopted for analysis of lung surfactant function. It has

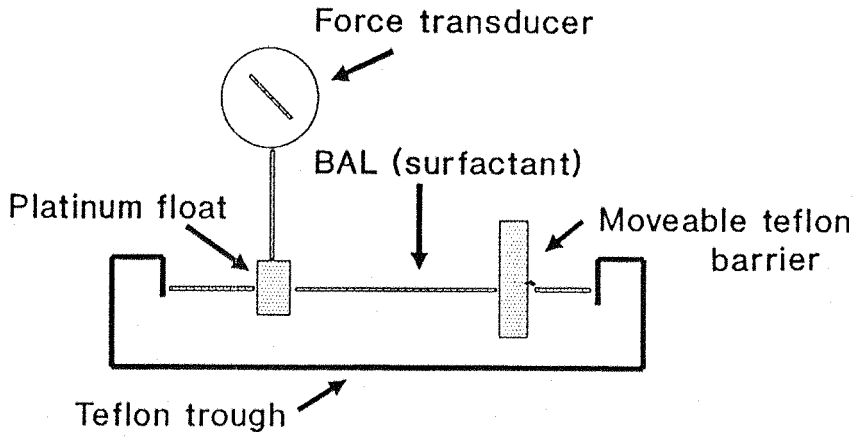

Figure 1.11: Wilhelmy balance (schematic diagram).

two great advantages. First, the Wilhelmy method is compatible with the Langmuir trough with many benefits in controlling the composition and other parameters of the surfactant monolayer. The second advantage is the facility for providing continuous recordings of expansions and compressions in the same surface monolayer.

Clements [1962] described in detail the surface tension measurement in the Wilhelmy balance. The most popular version of the Wilhelmy balance employs a platinum plate dipping into the sample combined with a moveable barrier that compresses the surface monolayer in a continuous recycling regime. Surface tension parameters are usually evaluated by measuring the traction force exerted by surface tension on the platinum plate. Figure 1.11 shows a schematic diagram of the Wilhelmy balance with its main components, and figure 1.12 shows the surface forces acting in the platinum float. Although contact angle problems have been reported in the Wilhelmy method, this can be avoided by scrupulous cleaning and sometimes roughening of the platinum float.

Generally the most useful parameters of surfactant activity to be evaluated with the Wilhelmy balance are spreading rate, equilibrium surface tension, film compressibility and minimum surface tension. The cycling speed usually applied in the classical Wilhelmy balance system (0.33 to 1 cycle/min) is slow in relation to normal breathing patterns but rapid enough to demonstrate the desired stability of pulmonary surfactant films

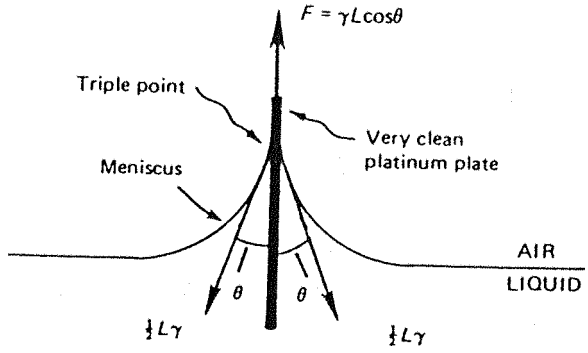

Figure 1.12: Surface forces acting in the platinum plate

[Van Golde *et al.*, 1988].

Pulsating Bubble

The pulsating bubble technique introduced by Adams and Enhorning [1966] consists of using a 'pulsator unit' to cause a small bubble to pulsate. The system is connected hydraulically to a pressure transducer whose reading can be related by a mathematical equation to the surface tension and the bubble whose variation in size is followed by microscope (see figure 1.13).

This method has several advantages: it automatically maintains 100% humidity at the ambient temperature which can easily be set at 37°C, the bubble can be easily pulsated at respiratory frequency (12 to 20 cycle/min), only a small volume of sample is required ($20\mu\ell$), contact angle artefact are avoided and surfactant leaks are eliminated [Hills, 1988].

Although the pulsating bubble constitutes a useful and rapid method for evaluation of lung surfactant activity, there are some inherent methodological problems. The measurements are made on only the very small fraction of the suspended sample that is in the interface. There is no way of knowing what components have been recruited to the air-aqueous interface or in what concentrations [Notter, 1984], and also sometimes the position of the bubble can prevent or delay the establishment of very low surface tension during surface compression [Van Golde, 1988].

A difficulty occurs in measuring near-zero values for surface tension.

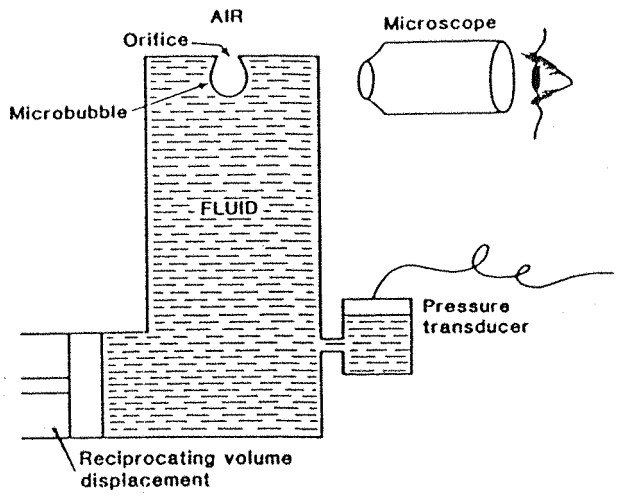

Figure 1.13: A schematic diagram of pulsating bubble method

Without surface tension, the bubble has no shape at all. This may explain why the bubble has been seen to be deformed [Enhorning, 1977]. In this case a much more complex form of the Laplace's equation should be used, and in practical terms very low values for surface tension (less than 2dyne/cm) measurement may be critical [Enhorning, 1977], [Slam, Schoedel and Hanson, 1971].

1.9.2 Phospholipids

Several techniques can be used to estimate the total phospholipid content and composition of lung surfactant. They can include mincing or rinsing the tissue with an aqueous fluid, physically processing the aqueous homogenate or lavage fluid, solvent extraction of lipid content, phospholipid precipitation, separation of individual phospholipids, quantitative estimation of each phospholipid.

One of the first technique to estimate phospholipids in amniotic fluid (AF) was applied in 1971 by Gluck and Kulovich to measure the ratio of lecithin(L) or PC to sphingomyelin(S) in samples of AF, using the finding that towards the end of gestation L rises sharply while S remains relatively constant. Expressing the results as a ratio instead of expressing absolute quantities avoids inaccuracies due to dilutional variations.

They demonstrated changes in lecithin and sphingomyelin concentrations throughout the last weeks of gestation, with a sharp increase in lecithin from 34 weeks to a peak at 36 weeks. Sphingomyelin tended to peak at 30 to 32 weeks and fell after 35 weeks gestation. They have used the ratio of lecithin to sphingomyelin (L/S) as a quantitative measure of amniotic fluid surfactant. Calculating a ratio avoids the problems of variable concentrations of components of amniotic fluid, and have been used to predict RDS. When the L/S ratio exceeds 2.0, the infant is unlikely to have respiratory distress; between 1.5 and 1.9, the distress will be mild; and a ratio of 1.0 to 1.49 is predictive of immaturity of the lung and moderate to severe respiratory distress. Contamination of the sample by blood or meconium gives less accurate results because they also contain lecithin and sphingomyelin in measurable amounts. Complications of pregnancy such as diabetic pregnancy, erythroblastosis foetalis and intrauterine growth retardation are known to produce unreliable results [Wagstaff, 1974].

In the Bartlett method, total phospholipid content is measured directly as the phosphate content of the sample following lipid extraction [Bartlett, 1959], [Fisk and Subbarrow, 1925]; a direct relationship exists between the organic phosphate in the sample and the measurable phosphate after digestion of the sample. The organic phospholipid is digested to release the inorganic phosphate, which is subsequently reacted with ammonium molybdate to give a blue phosphomolybdate complex. This is measured by optical densitometry at 830 nm. Although this method is accurate it involves much work, is time consuming (about 4 hours), and uses toxic substances such as H_2SO_4 and HCl which produce irritating vapours and may cause severe burns. It also uses ammonium molybdate and H_2O_2 which are irritating to skin eyes and respiratory system and may cause mutagenic effects.

Some methods use chromatographic techniques, including thin layer chromatography (TLC), gas-liquid chromatography (GLC) and the high performance liquid chromatography (HPLC). In TLC, a mobile phase is applied to separate the phospholipids using silica gel layers. Touchstone and Alvarez [1988] present a list of mobile phases to give complete separation of the six major phospholipids and a list of some reagents that can be used for detection of phospholipids. Using this technique, all PLs except PE and PS are well separated but effect of temperature and atmospheric humidity are pronounced and variability of results have been reported [Touchstone and Alvarez, 1988].

To measure DPPC fraction, gas-liquid chromatography (GLC) may be used. Palmitic acid in amniotic fluid originates from DPPC. Measurements of palmitate in amniotic fluid have been used to assess lung maturity by GLC

[Warren *et al.*, 1973]. The procedure involves lipid extraction and hydrolysis to release the fatty acids which are subsequently methylated. The fatty acid methyl esters are then separated and measured by GLC. The determination of fatty acid concentration is again susceptible to dilutional variation, and this measurement is more useful when expressed as a ratio palmitic:stearic acid [O'Neil, 1978]

HPLC, developed by Patten *et al.* [1982] can be used for separation and quantitation of phospholipids. Early methods relied upon ultraviolet absorption which depended upon the degree of unsaturation and therefore DPPC (being disaturated) was not detected. In 1987, Postle introduced a technique using post column fluorescence detection which successfully identified and measured individual phospholipid species including DPPC. The PC species are resolved by reversed phase HPLC and detected using the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene. Use of HPLC with post-column fluorescent detection provides rapid non-destructive quantification of PC species and allows quantitation of individual molecular species variations which might provide clues to enrichment mechanisms. Hunt *et al.*, [1991] used the above technique to compute the ratio of DPPC to palmitoyl oleoyl PC (P/O ratio) and demonstrated that P/O ratio provided a sensitive marker of fetal lung maturity. An alternative quantitative detection system is the falling drop mass detector (Applied Chromatography Systems). The sensitivity of this HPLC detector for PC species has yet to be evaluated.

Another technique, first described for plasma, uses enzymatic hydrolysis to liberate the choline moiety in PC [Artiss *et al.*, 1979]. The sample for quantitation is exposed to phospholipase D, an enzyme which hydrolyses PC to release choline. The free choline, in the presence of choline oxidase, forms hydrogen peroxide. Subsequently, the hydrogen peroxide is reacted with phenol and 4-amino antipyrene and the absorbance of the chromogen product is measured at 492 nm. This test, the so called PLD assay is relatively rapid (approximately an hour and a half to complete), involves a small sample volume and does not require lipid extraction. It can measure large sample number simultaneously and provide a practical aid to the clinical setting.

1.10 Aims

The overall aim of this thesis is to combine several experimental techniques to establish an approach for evaluation of lung surfactant, composi-

tion and function.

The specific aims were:

1. To develop an easy and sensitive assay for measurement of PC concentration using small volume BAL.
2. To apply this technique to analyse PC in BAL of guinea pig, as an animal model of RDS; to evaluate changes in PC concentration due to hyperoxia.
3. To determine the role of hyperoxia in the biophysical properties of guinea-pig surfactant BAL, using the Wilhelmy balance.
4. To determine total protein concentration changes in guinea pig model of RDS.
5. To raise a polyclonal antibody in rabbit, against guinea pig surfactant associated protein SP-A.
6. To evaluate the concentration of surfactant associated protein SP-A in term and preterm guinea pig.
7. To investigate the role of PAF antagonist WEB 2086 in the preterm guinea pig model of RDS, measuring PC concentration, total proteins and surface tension in BAL.

Chapter Two
Materials and Methods

This chapter describes the materials and methods that have been applied in this study. Some of the methods utilized are standard ones and will be described in a simplified way. Other methods are less conventional and need additional information.

2.1 Guinea pig

2.1.1 Animals

Virgin female Hartley strain guinea pigs were assessed for their oestrus cycle and introduction of a male before next ovulation determined the pregnancy date of conception (day 0 of gestation). At day 65 of gestation (term 68 days), pups were delivered by Caesarian section, under halothane anaesthesia (2 - 4%) maintained with nitrous oxide (0.4 litre/min) and O_2 (1.5 litres/min), within 4 - 5 minutes after initiation of the procedure.

A lactating surrogate dam and up to six pre-term pups were placed in 25 litre purpose built perspex cages containing food, water and hay. Cages were exposed either 95% O_2/ 5% N_2 or room air at a rate of 3.5 litres/min, and monitored for O_2 concentration and temperature (20 - 24°C). Dams were never exposed to hyperoxia for more than 24 consecutive hours.

Pups lavage were performed after 3 days exposed to 95% O_2 or 21%. Another group of preterm guinea pig were submitted to hyperoxia or air for 3 days and 5.0mg/kg/day of PAF antagonist (WEB 2086) were injected i.p. twice a day; after 3 days lavage was performed as described below.

The *in vivo* work was performed by Mr. Gary Phillips (Department of Nutrition, University of Southampton) as part of a study of inflammation in the pathogenesis of CLD in preterm newborn guinea pigs.

2.1.2 Preterm samples

Preterm guinea pig pups were anesthetized by i.p. injection of pentobarbitol (50 mg/Kg). The trachea was exposed, a tracheotomy was performed and a 14 - gauge cannula inserted and secured. The lungs were lavaged with 10ml of sterile saline at 37°C, of which 80 - 90% was recovered. Bronchoalveolar lavage (BAL) was centrifuged at 200 g for 10 minutes at 4°C.

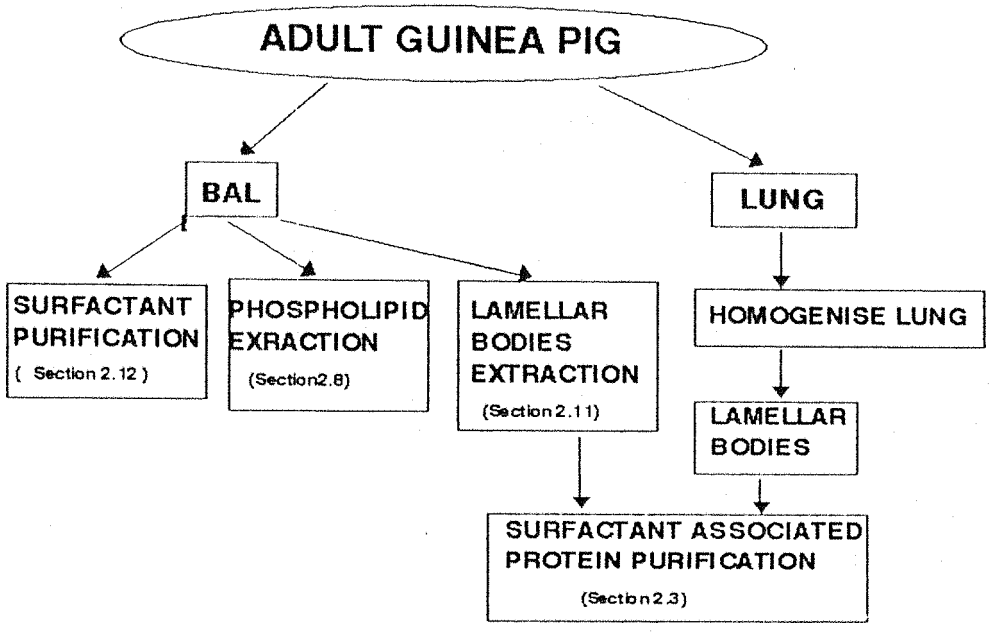

Figure 2.1: Scheme for pulmonary surfactant analysis

The supernatant was stored frozen at $-70°C$, and then freeze dried in a Modulyo-Edwards freeze drier for analysis.

2.1.3 BAL and lungs from adult guinea pig

Adult guinea pigs were anaesthetized by intraperitoneal injection of pentobarbitol (50 mg/Kg). Tracheotomy was performed as described above, and the pulmonary circulation was flushed with saline. Lungs were lavaged with 20 ml of sterile saline at 37°C. After that lungs were taken in STC buffer ($0.32M$ sucrose, 10mM Tris/H Cl, 1mM Ca Cl_2, 1mM PMSF buffer pH 7.4). Lungs were homogenized in the same buffer and either submitted to several centrifugation steps for lamellar bodies extraction as described in section 2.9 or used for purification of surfactant-associated proteins (section 2.3). Figure 2.1 summarizes the processes to which BAL and lungs were submitted.

2.2 Proteins - Bicinchoninic Acid Assay

2.2.1 Basic principle

Proteins reduce alkaline Cu(II) to Cu(I) in a concentration dependent manner [Lowry, 1951]. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I) forming a purple complex with a maximum absorbance at 562 nm [Tikhonov and Mustafin, 1965], [Mazonski *et al.*, 1963] which is directly proportional to protein concentration.

2.2.2 Reagents

The following reagents were supplied by Sigma Chemical Co.:

- Bicinchoninic acid (BCA), containing bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 N NaOH, pH11.25
- Copper(II) sulphate pentahydrate 4% (w/v) aqueous solution
- Protein standard solution was made up using bovine serum albumin (BSA) at a stock concentration of 0.5 mg/ml
- Protein reagent, prepared according to the required amount on the day of use, prepared by adding 4% Cu into BCA (1:50, v/v).

2.2.3 Procedure

The BCA protein assay was performed in microtitre plates; standard BSA (0.5-10 μg) or BAL sample was added to wells in a final volume of 20μl. Protein reagent (200μl/well) was added, the plate incubated for 30 minutes at 37°C and absorbance read in a Multiscan Plus spectrometer at 560nm. A standard curve of absorbance vs standard protein was prepared, and used to determine protein concentration of the BAL samples (fig. 2.2).

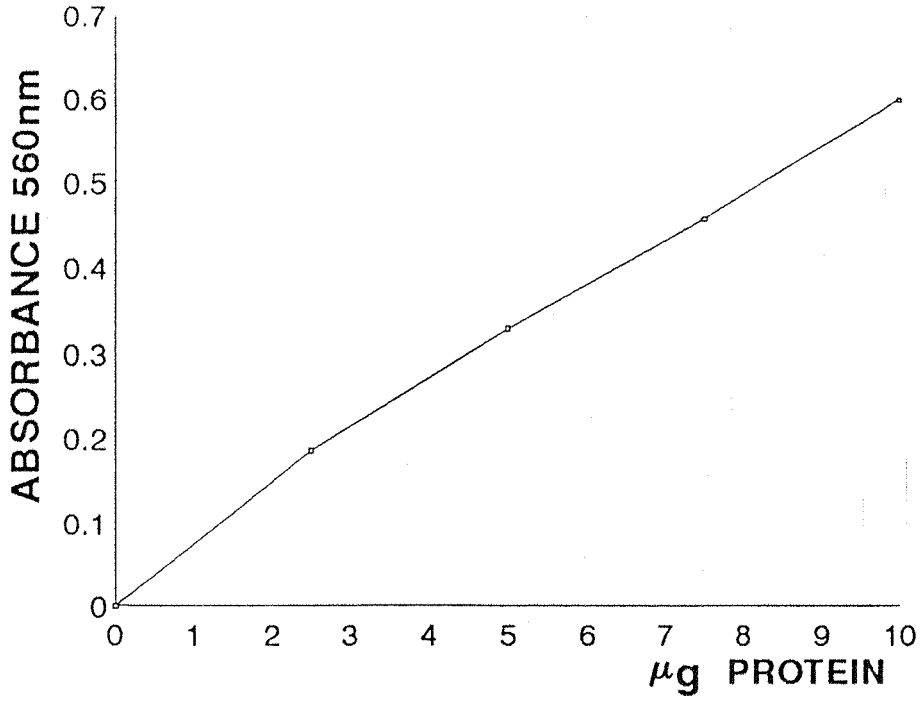

Figure 2.2: Standard curve for Bicinchoninic Acid Protein Assay

2.3 Surfactant Associated Protein Purification (SP-A, SP-B, SP-C)

Surfactant-associated proteins were purified from both guinea pig BAL and homogenized lung by the methods of Hawgood *et al.* (1987)

2.3.1 Reagents

The following reagents were used:

- 1-Butanol (from Sigma laboratory)
- Chloroform / methanol / 0.1 M HCl, 1:1:0.5 (v/v)
- Sephadex LH 20 (from Pharmacia Laboratory)
- 5 mM Tris / HCl pH 7.4
- 5 mM Tris / 150 mM NaCl / 20 mM octyl-β-D-glucopyranoside, pH 7.4 (octylsolution)

2.3.2 Procedure

Isolation of Hydrophobic Protein (SP-B, SP-C)

Pulmonary surfactant was isolated by differential centrifugation of both homogenized lung and BAL (subsection 2.1.3). The surfactant suspension in water was extracted in 1-butanol (1:50, v/v) at room temperature. The mixture was centrifuged twice at 10,000 g for 20 minutes at room temperature.

The supernatant of the butanol mixture was dried by rotary evaporation and the residue was resuspended in chloroform / methanol / 0.1 M HCl, (1:1:0.5, v/v). After that, the sample was applied to a 0.5cm×20cm column of sephadex LH-20 at 4°C. Elution fractions were assayed for protein using an LKB Biochrom Ultrospec II at 280nm, and for PC concentration using the PLD assay. Aliquots of eluted fractions were analysed in SDS-Page using the method of Schägger and Von Jagow (section 2.5) and detection by silver staining.

Isolation of SP-A

The initial precipitate obtained during the butanol extraction, containing water soluble proteins, was dried under nitrogen. Pellets were resuspended in 20ml of 5mM Tris/HCl 150 mM NaCl/20mM octyl- β- D- glucopyranoside pH 7.4 and washed twice. The insoluble protein was resuspended in 30 ml of 5mM Tris/HCl pH 7.4, and dialyzed for 48 h against four changes of the same buffer. The dialyzed material was centrifuged at 100,000 g for 30 minutes to provide a supernatant containing SP-A. Aliquots were assayed for protein concentration measurement and analysed by SDS/Page using the method of Laemmli (section 2.5.a).

2.4 Proteins - SDS-Polyacrylamide Gel Electrophoresis

The method used was based on that of Laemmli[1970].

PROTEIN	MOLECULAR WEIGHT
	(3.5 mg/vial)
Bovine albumin (25 mg/vial)	66,000
Egg Albumin (25 mg/vial)	45,000
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle 5mg/vial)	36,000
Carbonic anhydrase (bovine erythrocytes 5mg/vial)	29,000
Trypsinogen (Bovine pancreas, PMSF treated 25 mg/vial)	24,000
Trypsin inhibitor, (soybeam 5mg/vial)	20,100
α - Lactalbumin (Bovine milk ,5mg/vial)	14,200

Table 2.1: Molecular weight standards (MW-SDS-70L kit Sigma laboratory)

2.4.1 Reagents

The following reagents were used:

- Resolving gel buffer - 3M Tris - HCl (×8) pH 8.8, stored at 4°C up to 1 month
- Stacking gel buffer- 0.5M Tris, HCl pH 6.8 (×4), stored at 4°C up to 1 month
- 1.5% Ammonium persulphate (w/v) - made up fresh
- Running gel buffer - 0.25M Tris, 192 mM glycine pH 8.3 and 0.1% SDS (×10) stored at room temperature up to 1 month
- Sample buffer - 62.5mM Tris - HCl pH 6.8, 4% SDS (w/v), 20% glycerol(w/v), 10% dithiotreitol(DTT)(w/v) (added only before use), 0.01% bromophenol blue (w/v) - dye
- Protein standards (MW-SOS-70L kit Sigma laboratory, Table 2.1)
- 25% Methanol

2.4.2 Procedure

The resolving gel was 12.5% acrylamide and the stacking gel 6%, as shown in table 2.2. A slab gel casting apparatus (Pharmacia Ltd, Milton Keynes, Bucks) was used to cast either 2 or 4 gels, dimensions of 80mm×140mm×2mm, simultaneously.

COMPONENT	RESOLVING GEL	STACKING GEL
	12.5%	6%
Acrylamide	9.38 g	2.4 g
Bisacrylamide	0.250 g	0.250 g
SDS	0.075 g	0.040 g
Resolving buffer	9.38 ml	
Stacking buffer		10ml
Ammonium persulphate	3.75 ml	2.0*ml*
Distilled water	To a final volume of 75 ml	To a final volume of 40 ml
Temed	38μl added just before use	20μl added just before use

Table 2.2: Resolving gel and stacking gel

The gel components were added as described in table 2.2 and the solution poured between the glass plates overlaid with 10μl of 25% methanol and left to polymerize for about 40 minutes. After that the methanol was removed and the stacking gel poured, the well comb placed in the top (6 wells with 80 μl capacity) of the stacking gel, and solution allowed to polymerize. After polymerization, the comb was removed and gels placed in the electrophoresis tank which contained running buffer. The upper reservoir was then filled with running buffer.

2.4.3 Sample preparation

Samples and molecular weight markers analysis were mixed in a 1:1 ratio with sample buffer, boiled for three minutes and then centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and added into the wells of the polyacrylamide gel. Electrophoresis was carried out at a constant current of 30 mA per gel until the dye front was 0.5 cm from the gel bottom. Gells were prepared for nitrocellulose blotting or coomassie stain as required.

2.5 Proteins - SDS-Page, Schägger and Von Jagow's method

This method is a modification of the discontinuous procedure of Schägger and Von Jagow [1987], which has been shown to yield consistent resolution

COMPONENT	MOLECULAR WEIGHT
Myoglobin(Polypeptide backbone 1-153)	16,950
Myoglobin (I+II, 1-131)	14,440
Myoglobin (I+III, 56-153)	10,600
Myoglobin (I, 56-131)	8100
Myoglobin (II, 1-55)	6210
Glucagon	3,480
Myoglobin (III, 132-153)	2510

Table 2.3: Molecular weight markers, MW-SDS-17S, Sigma laboratory

of polypeptides with molecular weight below 10,000.

2.5.1 Reagents

The following reagents were used:

- Acrylamide stock solution- 48% acrylamide (w/v), 1.5%N,N'-methylene- bis-acrylamide, stored at 4°C up to 1 month
- Gel buffer - 5M Trizmabase, 0.5% SDS, stored at 4°C up to 1 month
- Sample buffer - 20% SDS - 4ml + glycerol 2.4ml + 1M Tris.HCl 1ml + brilliant blue G 0.01%, made up to 20ml with H_2O
- 10% Ammonium persulphate, made up fresh
- Anode buffer - 0.2M Tris.HCl pH 8.9
- Cathode buffer - 0.1 M Tris.HCl pH 8.2, tricine, 1%SDS, made up fresh
- Molecular weight markers, MW-SDS-17S (Table 2.3), Sigma

2.5.2 Procedure

This method uses three different polyacryamide gel concentration: 16.5% separating gel, 10% spacer gel and 4% stacking gel, made up as shown in table 2.4.

An LKB slabgel (Pharmacia) was used for 2 gels each time. The separating gel solution was carefully poured, avoiding bubble formation, filling

SOLUTION	STACKING GEL (1-2cm) 4%	SPACER GEL (2-3cm) 10%	SEPARATING GEL (10cm) 16.5%
Acrylamide solution	1.0 ml	6.1 ml	10.0ml
Gel buffer	3.1ml	10.0ml	10.0ml
Glycerol	-	-	3.2ml
Water	8.4ml	13.9ml	6.8ml

Table 2.4: Preparation of electrophoresis gels

the reservoir up to the level of 10cm marked in the gel casting apparatus. After that, quickly and carefully, the spacer gel solution was overlaid about 2cm. This operation was completed in less than 15 minutes.

Finally, 20μl of water was overlaid and the gel allowed to set up for 1 hour. The water was removed, the stacking gel poured and the well comb placed in the top (10 wells, 80 μl/well) and allowed to polymerize. After the gel had set, it was left to equilibrate at 4°C overnight.

2.5.3 Sample preparation and analysis

Samples and molecular weight markers were mixed in a 1:1 ratio with sample buffer, incubated for 30 minutes at 40°C and allowed to cool at room temperature. After that, samples were added to the wells of the polyacrylamide gel.

Gels were placed in the electrophoresis tank. Anode buffer was added to the lower reservoir and cathode buffer to upper reservoir (see Table 2.4). Electrophoresis gels were run at a constant current of 20 mA per gel for 1 hour and the current was increased to 30 mA per gel and, then maintained constant throughout the remainder of the run (about 16 hours).

2.6 Staining for Polyacrylamide Gel

2.6.1 Coomassie staining

Reagents

- Coomassie stain: Coomassie blue R250 (0.025% w/v) in distilled

water /methanol/acetic acid (50:40:10) (v/v/v)

- Destain solution: Methanol/acetic acid/distilled water (30:10:60)
- Pre-drying solution: 4% glycerol (v/v), 5% acetic acid (v/v) in distilled water

Procedure

Electrophoresis gels were placed in a plastic box and covered with Coomassie stain overnight at room temperature. The following morning several washes of the gel were performed, using destain solution to remove stain. The gels were then incubated in pre-drying solution overnight and dried on filter paper under vacuum.

2.6.2 Silver staining

Reagents

- Fixing solution: 12% trichloroacetic acid (TCA) in 50% methanol or 12% acetic acid in 50% methanol
- Dithiothreitol (DTT) solution: stock solution 0.05% DTT in water, stored at 4°C up to 1 week. Working dilution 1:100 in distilled water
- Silver solution: 0.2% silver nitrate in distilled water. Prepared immediately before use
- Developer solution: 3% sodium carbonate with 0.5 ml/l formalin (or 0.05% v/v)
- Stop solution: 1% acetic acid

Procedure

The gels were carefully taken off the plates, without touching the surface, and incubated overnight with fixing solution. The following morning gels were washed three times with distilled water, and then incubated with DTT solution for 30 minutes. DTT solution was replaced, without rinsing, with silver stain solution and incubated for 30 minutes under fluorescent light. Silver stain was decanted into a waste silver stain containing excess NaCl.

Then developer solution was added and reaction were stopped when desired. After that, gels were dried as previously described.

2.7 Immunoblotting

2.7.1 Reagents

The following reagents were used:

- Renaturing solution - 4 M Urea / 10mM Tris / 50mM NaCl, pH 7.0
- Transfer buffer - 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% SDS containing 20% (v/v) methanol
- Blocking buffer - 5% dried semi-skimed milk (w/v), 10mM Tris/HCl, 150 mM NaCl, 0.5% Tween 20 (w/v) at pH 7.4
- Antibody buffer - 0.15% BSA (bovine serum albumin, from Sigma laboratory), 10mM Tris/HCl, 150mM NaCl, 0.5% Tween 20 (w/v) pH 7.4
- Wash buffer - 10mM Tris/HCl, 150mM NaCl, 0.5% Tween 20 (w/v) pH 7.4
- Phosphate buffered saline (PBS) - 8mM $Na_2HPO_4 \bullet 12H_2O$, 1.5 mM KH_2PO_4, 140mM NaCl pH 7.4
- Substrate solution - 1% diaminobenzidine (DAB), 0.04% hydrogen peroxidase made up in PSB
- Anti-rabbit IgG (whole molecule) Biotin conjugate, from Sigma Laboratory
- Streptavidin - HRP from Sigma laboratory 0.5 mg diluted in 0.5 ml PBS, stored at -20°C

2.7.2 Antibody

Antibody were raised in New Zealand rabbits by immunizing with the SP-A purified by SDS Page from guinea pig lamellar bodies (section 2.4). The area of gel corresponding to 36 kDa band was cut out and freeze dried, then rediluted in 1 ml of saline and passed through a syringe to be fluidized. The rabbit was injected twice at intervals of 15 days.

2.7.3 Procedure

Proteins were transfered from polyacrylamide gel to nitrocellulose (Hyperbond super 0.45 micro Amersham), using a immunoblot transfer apparatus (Trans-Blot TM Cell, Bio-Rad). Gels were placed for 2 hours in renaturing buffer and nitrocellulose membrane was soaked in distilled water for 20 minutes.

After that, polyacrylamide gel, nitrocellulose membrane, four filter papers (Whatman, number 4) and two scotch brite pads were soaked in transfer buffer for 30 minutes. Then, for assembling cassettes, 2 filter papers were laid on top of one sheet of scotch brite, followed by positioning of the gel. Nitrocellulose membrane was then laid on the top of the gel, followed by a further 2 filters papers and the second scotch brite pad. Care was taken to avoid bubbles between the layers and to ensure that the nitrocellulose was on the anode side of the gel. The gel was transfered for 48 hours at 4°C at a constant current of 35mV. A stirring bar was placed in the bottom of the transfer apparatus to ensure uniform heat distribution.

The nitrocellulose membrane was then placed into a plastic tray containing block buffer for 2 hours at 37°C. Then the nitrocellulose membrane was rinsed with wash buffer and first antibody in antibody buffer was added and incubated 16 hours (overnight) at room temperature. The membrane was washed three times with wash buffer, each time for 20 minutes, and once with antibody buffer for 30 minutes. Then it is incubated with biotinylated secondary antibody, either anti-rabbit IgG peroxidase conjugate or anti-mouse IgG peroxidase conjugate, for 2 hours at room temperature.

After that, the membrane was rinsed with wash buffer 3 times for 5 minutes each and then incubated for 1 hour with streptoavidin-HRP 0.01 ml in 40 ml of antibody buffer. The membrane was then rinsed 5 times, 5 minutes each, with wash buffer and once with PBS. The membrane was immerse in substrate solution for 5 to 10 minutes and the reaction was stoped by running distilled water over the membrane.

2.8 Dot Blotting

This method consists in applying samples directly on the surface of a nitrocellulose membrane forming dots. Samples of adult guinea pig homogenized lung, lamellar bodies or purified SP-A were analysed in this study.

NAME	TITRE
PE-10 anti-human SPA, monoclonal Kindly provided by Prof. T.Akino Sapporo University - Japan	1:200
Anti-rat SP-A monoclonal Kindly provided by Dr. M. Post Hospital for Sick Children Toronto - Canada	1:200
Anti-guinea pig SPA polyclonal	1:20

Table 2.5: Antibodies used for immunoblotting

The guinea pig samples were assayed for SP-A detection by using western blotting as described in section 2.7.

2.9 Lipid Extraction [Bligh and Dyer, 1959]

2.9.1 Procedure

Bronchoalveolar lavage from adult guinea pig was centrifuged at 200 g for 10 minutes at 4°C to remove cells. After that 1.0 ml of supernatant was taken and 1ml chloroform, 2ml methanol, 1ml distilled water were added and between each addition the sample was mixed. After that, the sample was left in freezer at -20°C for 1 hour and two phases were achieved.

The lower layer contains the lipids and was dried under nitrogen at 40°C. The dried material was then kept in a freezer until use. When required, the powder was diluted either in 1ml of isopropanol, distilled water, chloroform (2:1:0.5) (v/v/v) or in 1ml of chloroform.

2.10 Bartlett Lipid Phosphorus Analysis

This method was described by Bartlett in 1959.

2.10.1 Reagents

- 5M H_2SO_4
- 30% H_2O_2
- 5% Ammonium molybdate in 2M HCl (w/v)
- Fiske - Subbarrow reducer - 0.5g 1-amino-2-naphtol-4-sulphonic acid in 200ml 15% $Na_2S_2O_5$ + 1g Na_2SO_3 filtered and stored in an amber bottle
- 200μM Na_3PO_4 standard

2.10.2 Procedure

A standard curve of phosphorus concentration was prepared in duplicate, using Na_2PO_4 (0 to 200 nmoles) in standard test-tubes. The unknown samples were added into test-tubes in duplicates and volume of both samples and standard were adjusted with distilled water to 2ml. After that 0.5ml of 5M H_2SO_4 were added to sample and standard and heated on a dry block heater at 180°C for 3 hours. Each tube were then incubated with 100 μl of 30% H_2O_2 for a further 1 hour. Distilled water (4.4 ml), 5% ammonium molybdate (200μl) and Fiske - Subbarow reducer (200 μl) were added, mixed and boiled at 100°C for 7 to 10 min. Absorbance were read at 830 nm against phosphate free blank, in a LKB Ultrospec II.

2.11 PLD assay

2.11.1 Principle of the method

Phospholipase D from a *Streptomyces* species liberates choline from phosphatidylcholine. Choline reacts with choline oxidase to yield hydrogen peroxidase and betaine. Hydrogen peroxidase in the presence of peroxidase, 4-aminoantipyrine and phenol induces formation of a red quinone dye, whose absorbance can be measured at 492 nm (see reaction scheme on figure 2.3).

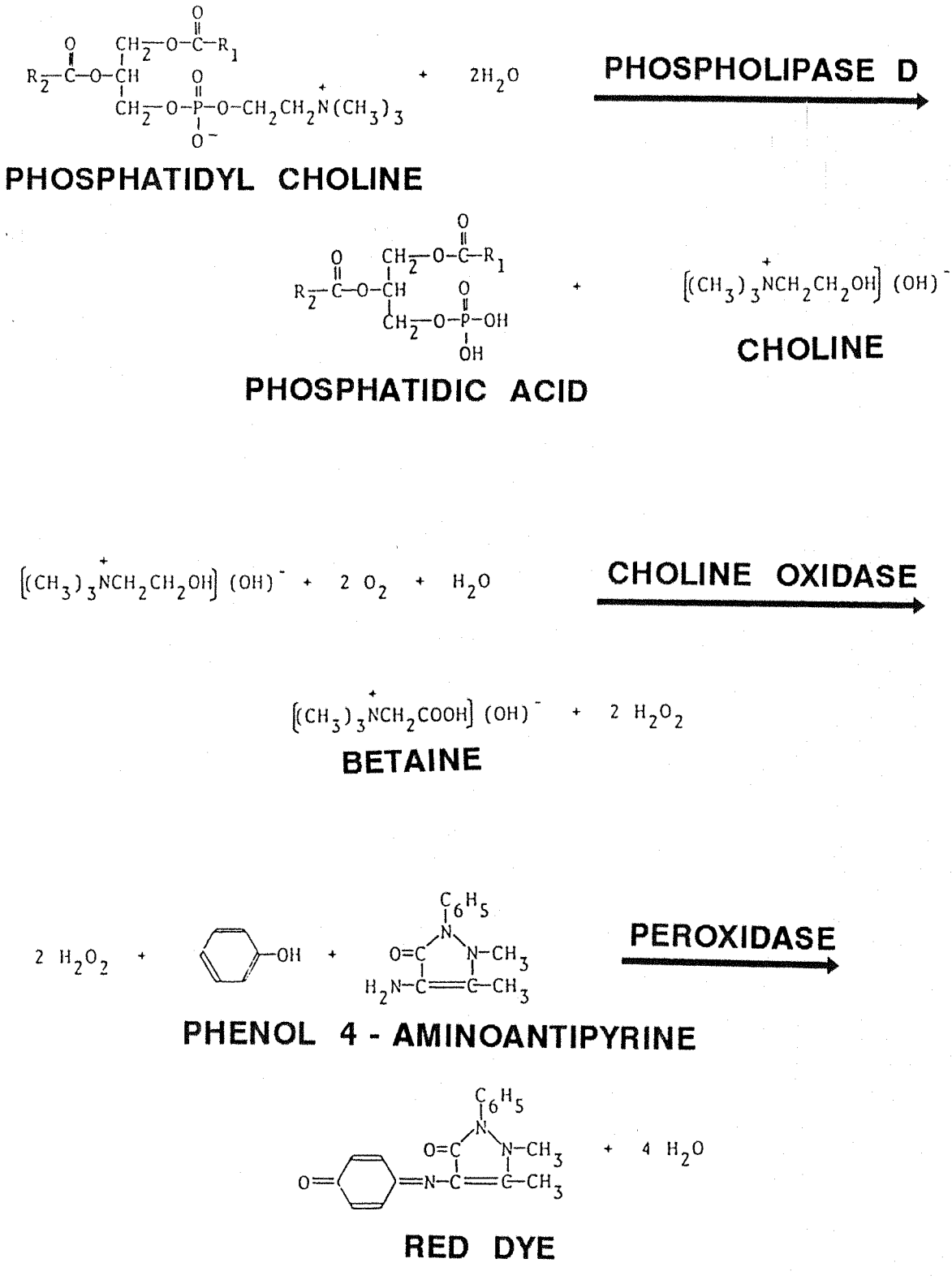

Figure 2.3: Reaction scheme for the enzymatic determination of phosphatidylcholine

2.11.2 Reagents

The following reagents were used:

- Phospholipase D (PLD) from *Streptomyces chromopicus* was obtained from Genzyme as a freeze dried powder at a concentration of 44 U/mg. A solution was made up to 0.1 U/ml in 10mM Tris/HCl buffer pH 8.0 and 0.1% Trition X-100 and aliquoted and stored at -20°C.
- Standard DPPC (dipalmitoyl phosphatidylcholine), molecular weight 734, in a concentration of 1mM in 10 mM Tris/HCl buffer pH 8.0 and 0.25% Trition X-100.
- Choline oxidase (15.7 U/mg) obtained from Genzyme laboratory
- Peroxidase (44 U/mg)obtained from Sigma laboratory
- Chromogen reagent containing 20 units of choline oxidase, 15.8 units of peroxidase, 2mg phenol, 3mg 4-aminoantipyrene diluted in 10ml with 100 mM Tris / HCl buffer pH 8.0.

The sample to be analysed was prepared by adding 10μl of 0.025% Trition X-100 into 100μl of sample.

2.11.3 Procedure

Using a microtitre plate, a standard curve was prepared, in duplicate over a range of 0 to 50 n moles DPPC. Volume was adjusted with 100 mM Tris/HCl + 100mM $CaCl_2$ (4:1 v/v) buffer pH 8.0. Samples were added in duplicates, and volume was adjusted again with 100 mM Tris/HCl + 100mM $CaCl_2$ (4:1 v/v) buffer pH 8.0. To each well, 10μl PLD was added and incubated for 50 minutes at 42°C. After that 100μl chromogen reagent was added and tray incubated 50 minutes at 37°C. Absorbance was then measured using a Titretek Multiscan Plus Spectrofluorimeter at 492 nm, against sample free blank. Results were analysed using the standard curve (absorbance versus nmoles DPPC) and adding unknown sample absorbance against it (figure 2.4).

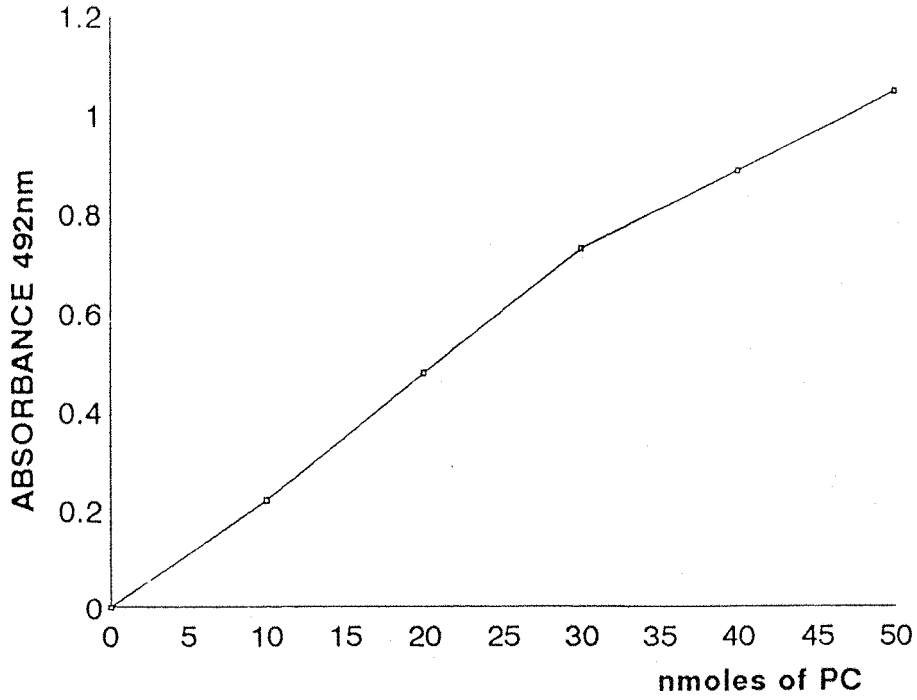

Figure 2.4: Standard curve for PLD assay

2.12 Lamellar body extraction [Gill and Reis, 1973]

2.12.1 Reagents

The following reagents are used for lamellar body extraction:

- STC buffer
- 0.55 M sucrose made up in 0.16 M NaCl pH 7.4
- 0.45 M sucrose made up in 0.16 M NaCl pH 7.4

2.12.2 Procedure

Lungs were taken, as described in section 2.1.3, and homogenized with a teflon glass homogenizer using 4 ml buffer/g tissue. The homogenate was centrifuged at 1000 g for 5 minutes at 4°C, followed by a further centrifugation of the supernatant at 20,000 g for 15 minutes at 4°C.

Pellets from the second centrifugation were resuspended in STC buffer and layered over a sucrose density gradient composed by 4ml of 0.55 M

sucrose overlaid with 2ml of 0.45 M sucrose. The gradients were centrifuged in an MSE superspeed 65 ultracentrifuge in a 6×14ml titanium swing-out rotor for 1 hour at 90,000 g and 4°C. The layers formed between 0.55 M sucrose and 0.45 M sucrose, and the layers formed between 0.45 M sucrose and STC buffer were removed and washed twice with 20 ml of STC. The pellets were stored frozen (-20°C) until use.

2.13 Surfactant Purification

2.13.1 Reagents

- 0.75 M sucrose prepared in 0.9% NaCl
- 0.9% NaCl (w/v)

2.13.2 Procedure

Adult guinea pig BAL was obtained as described in section 2.1 and centrifuged at 30,000 g for 30 minutes. The precipitate was resuspended in 5ml of 0.9% NaCl and layered over 6 ml of 0.7 M sucrose and centrifuged in a MSE superspeed 65 ultracentrifuge using a 6×14ml titanium swing-out rotor at 90,000 g for 1 hour. The interfacial layer was removed by aspiration and suspended in 0.9% NaCl and centrifuged twice at 90,000 g. All procedure were performed at 4°C. The pellet was resuspended in 2ml of 0.9% NaCl and kept at -20°C until use.

2.14 Surface tension measurement

2.14.1 Procedure

The Wilhelmy surface tension balance requires special care before use. It is very important make sure it is placed on a horizontal vibration-free table. The apparatus should not be exposed to extreme light or other heat sources. Other cares are related to cleaning, operation, calibration, sample and measurement itself.

2.14.2 Cleaning

- Float
  Initially the platinum float was taken from the hook, rinsed with methanol and immersed in chloroform for one or two minutes. After that, the lower part of the platinum float was heated in a flame and the float was remounted making sure it was parallel with the trough.

- Trough and moveable barrier
  The trough and barrier were immersed in hot water containing 0.1% of Decon 90 (v/v) for 3 to 5 minutes. After that, rinsing with hot water for about 1 minute, they were washed sequentially with methanol and chloroform, dried and inserted into the balance.

2.14.3 Operation

The Wilhelmy balance was switched on 1 hour before use to ensure correct operation. The calibration procedure was then performed.

2.14.4 Calibration

Calibration was performed using 0.9% NaCl at 37°C. Zero adjustment was made with the float above the surface of the liquid. Maximal surface tension was adjusted to 72.9 dyne/cm using the gain control, after the float was immersed 1mm into the fluid. After that the balance was left to run for three cycles to check the plate had no surface active material left in it. The screen should show the same value (G=72.9 dyne/cm) after each cycle and a difference of ± 0.8 between γ(maximun) and γ(minimum) was acceptable.

2.14.5 Sample

Bronchoalveolar lavage (BAL) samples prepared as previously described (sections 2.1.2 and 2.1.3) were warmed in a water bath at 37°C before adding either a constant concentration of PC (80 - 85 nmoles) or a constant volume

of 5ml to the saline surface. Before starting measurement the sample was left to spread over the surface for 5 minutes.

2.14.6 Measurement

The balance was set to perform a cycle each 3 minutes (speed 0.33 cycles/min) and the 1st, the 6th and the 12th cycle were recorded. γ(maximum) and γ(minimum) and area of hysteresis were recorded and printed.

2.15 High Performance Liquid Chromatography (HPLC)

The HPLC system was used for analysis of DPPC concentration after hydrolysis by PLD. DPPC concentration were detected using a post-column fluorescence agent [Postle, 1987].

2.15.1 Reagents

- Mobile phase - acetonitrite:methanol:buffer (380:120:25 v/v/v)
- Buffer - 15mM $Na_2PO_4 \bullet 2H_2O$ and $NH_4H_2PO_4$ at pH 5.5
- Detection reagent - 3mM 1,6-diphenyl - 1,3,5 hexatriene (DPH) in tetrahydrofuran was diluted to 0.045 mM in water

2.15.2 Procedure

The HPLC system column used in this study was an aminopropyl silica stationary phase packed in a 25cm×4.6mm stainless steel column (Jones chromatography, Hengoed, Wales) at room temperature. The mobile phase was delivered at 1ml/min by a Waters 6000 A pump (Millipore, Harrow, U.K.)

The HPLC column was mixed on-line with an aqueous stream (3ml/ml) of DPH reagent. This was made by adding 150μl of a 3 mM solution of tetrahydrofuran to 1l distilled water, followed by sonication in a water bath for 20 min. The post column mixture was passed through a teflon string mixer (1m×0.15mm) at 50°C and through an HPLC fluorimeter equipped with a 20μl flow cell. PC was quantified by fluorescence, excitation 340nm, emission 460nm, and integration of peak area.

Chapter Three
Surface Tension

3.1 Wilhelmy balance measurements

Surface tension of pulmonary surfactant was described in sections 1.6 and 1.7. The Wilhelmy balance was described in section 1.9 and in section 2.13 the technique of using the Wilhelmy tensiometer was outlined, including cleaning, calibration and sample preparation.

The surface tension parameters are evaluated by measuring the traction force exerted by surface tension on a platinum plate dipping into the subphase over which the surfactant was spread as a monolayer. During cycling of alternate surface film compression/expansion, the relationship between surface tension and surface area shows a hysteresis, which gives qualitative information about the surfactant characteristics. Generally, increased areas of hysteresis correlate with good pulmonary surface tension properties, and the area of the hysteresis loop can be computed as a parameter of surfactant activity.

3.2 Calibration of the Wilhelmy balance

Scrupulous attention to cleanliness of the trough and the float and roughening of the platinum plate are very important in the use of Wilhelmy balance. Standard cleaning, as described in section 2.13 was made before each measurement.

In this study it was found that high temperature increased the calibration values with time and low temperature affected the phospholipid spreading during the measurements. Surface tension is extremely dependent on the temperature and the measurements were not reproducible when temperature variation occurred. In some cases strong variations of the surface tension results were found for the same sample. This agrees with, for example, Notter *et al.* [1980] who used a mixture of DPPC:Cholesterol (9:1) and obtained less than 1 dyne/cm for the surface tension at 23°C but as high as 25 dyne/cm at 37°C.

In this study, the temperature was maintained at 37°C with maximum variation of 0.4°C during the whole experiment, to preserve physiological characteristics. To avoid heat losses, during this work an additional cover was designed to enclose the trough and the float. The air trapped between the cover and the equipment acted as a thermal insulation and the cover proved to be an efficient modification to preserve the temperature.

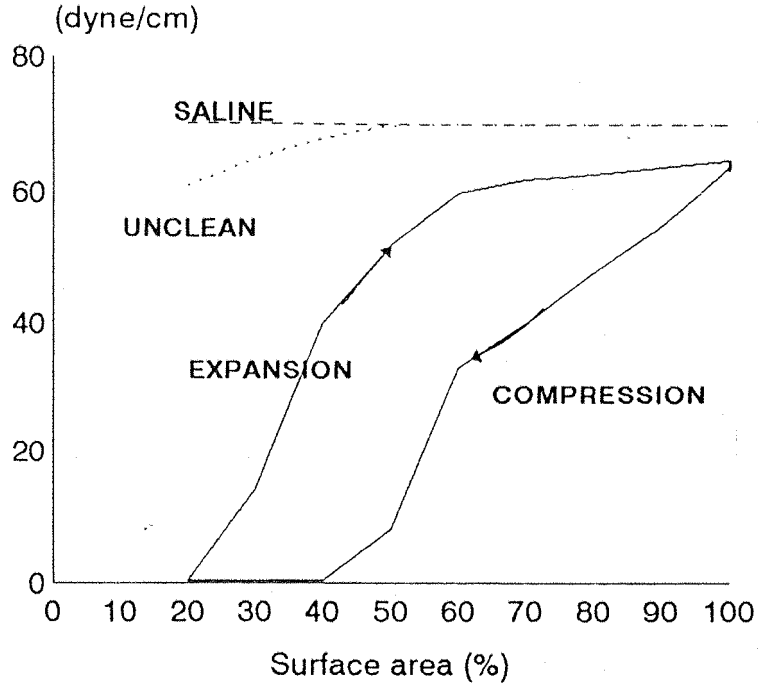

Figure 3.1: Surface tension measurements with the Wilhelmy balance, showing results for saline in ideal conditions, saline with imperfect cleaning and the hysteresis loop for DPPC

Another important point found in this research using the Wilhelmy balance was the high sensitivity to vibration. Small perturbation such as another person performing experiments on the same bench, a sudden door shutting, or the draught coming from an open window were enough to produce oscillations in the balance and deteriorate the measurements. The cover described above proved also to be useful against wind produced vibrations; apart from this, care had to be taken to avoid any other kind of vibration.

For calibration, saline at 37°C was added to the trough, and barrier was run back and forth about three times for a 5:1 compression of the surface area of the pool. For the saline the minimal and the maximal surface tension should be equal and the value of 70.2 dyne/cm should be obtained in the measurement. In practical terms a deviation less than 0.8 dyne/cm between the maximal and the minimal surface tension can be tolerated. For any greater disparity (figure 3.1), the whole cleaning procedure was repeated.

For validation, synthetic DPPC at 37°C was applied over the saline subphase in the balance. The minimum surface tension (γ_{min}), obtained at maximum compression, on the 6th. cycle was near zero and the maximum surface tension was 64.6 dyne/cm. Although the transition temperature of DPPC is 41°C, this was of minimal importance when applied in chloroform

DPPC:PG (7:3 w/w)	Number of cycles	γ_{min} dyne/cm	γ_{max} dyne/cm	area hysteresis
0.01 mg	4	20.3	68.7	0.86
0.02 mg	2	19.6	67.1	0.35
0.1 mg	3	0.3	57.8	1.8
0.25 mg	4	3.2	68.0	1.33

Table 3.1: Surface tension measurements with DPPC:PG (7:3 w/w) applied over 15 ml the balance subphase

solution to the surface of the Wilhelmy balance trough. Under these conditions, spreading was rapid, and a large area of hysteresis and low γ_{min} were achieved (figure 3.1). However, this monolayer was relatively unstable and collapsed after cycling between 100% and 20% area compression for 1 hour.

3.3 Artificial surfactant - DPPC:PG mixtures

A mixture of phospholipids was prepared as an artificial pulmonary surfactant. Surface tension parameters of various concentrations of DPPC:egg PG (7:3 w:w) were determined at 37°C. Results are summarized in table 3.1. Although the values for maximum surface tension were in the expected range, it can be noted that for small concentration of the mixture (0.01-0.02 mg), the values of the minimum surface tension were higher than expected ($\gamma_{min} > 10$ dyne/cm). The values obtained for γ_{min} and γ_{max}, when 0.1 mg DPPC:PG was added to the balance, were satisfactory. These values were considered to correspond to the necessary amount of DPPC to form a monolayer over the subphase, and were used for standardize the sample BAL PC concentration. The number of mols were calculated and divided by the surface area of the saline in the trough gave 4.7 nmols/cm^2 of DPPC as the amount needed to complete cover the subphase (approximately 85nmols PC per sample).

Very large amount of DPPC:PG caused problems in using the Wilhelmy balance. Figure 3.2 shows the results of an experiment performed with 0.5 mg DPPC:PG applied to the balance. The shape of the curve was distorted and inconsistent with the usual trace in the Wilhelmy balance.

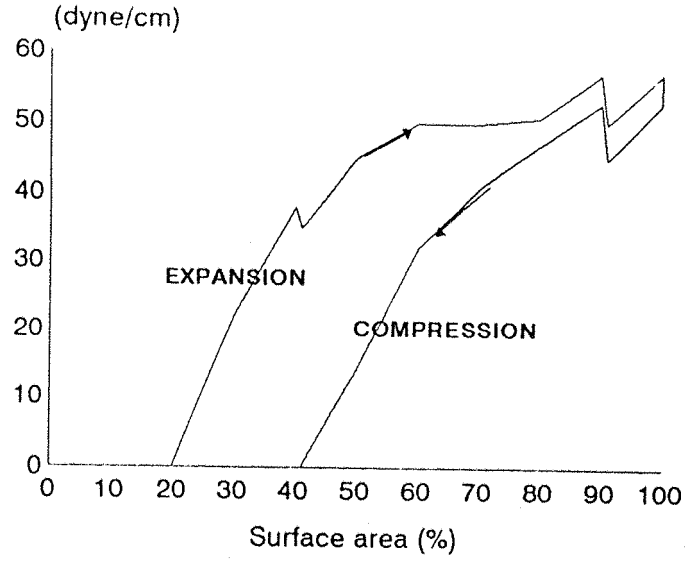

Figure 3.2: Surface tension measurement using high concentration of DPPC:PG (23.5 nmols/cm^2

3.4 Standard lavage

After calibrating and validating the balance, bronchoalveolar lavage (BAL) from adult guinea pig was used routinely as a quality control standard. BAL samples were obtained as described in section 2.1, centrifuged at 200 g for 10 minutes to remove contaminant cells, and stored in aliquots at -20°C until use.

The ability of surfactant to adsorb and spread as a monolayer is one important factor in surface tension analysis by the Wilhelmy tensiometer. A typical adsorption trace is shown in figure 3.3. Surface tension at maximal surface area against time for DPPC:PG (0.1 mg) and adult guinea pig BAL (85 nmol) is shown. Equilibrium surface tension was achieved rapidly by 5 minutes and was unchanged after 10 minutes for DPPC:PG, and almost immediately for BAL. Routinely, sample applied to the balance trough was left to adsorb for 5 minutes before compression analysis, to make sure that complete spreading had been obtained.

During recycling the following cycles do not retrace the first due to processes of selective squeezing out of unsaturated PC, polyunsaturated PC and saturated PC from the monolayer. By the fourth to sixth cycle a stable configuration was reached and continued for several cycles. If samples were then left cycling for long periods, γ_{max} reduced, while γ_{min} and area of hysteresis did not change (figure 3.4).

Bronchoalveolar lavage, purified surfactant, phospholipids extracted from BAL and BAL concentrated by freeze drying were applied to the surface of the trough subphase (0.9% NaCl) to choose the best preparation for use

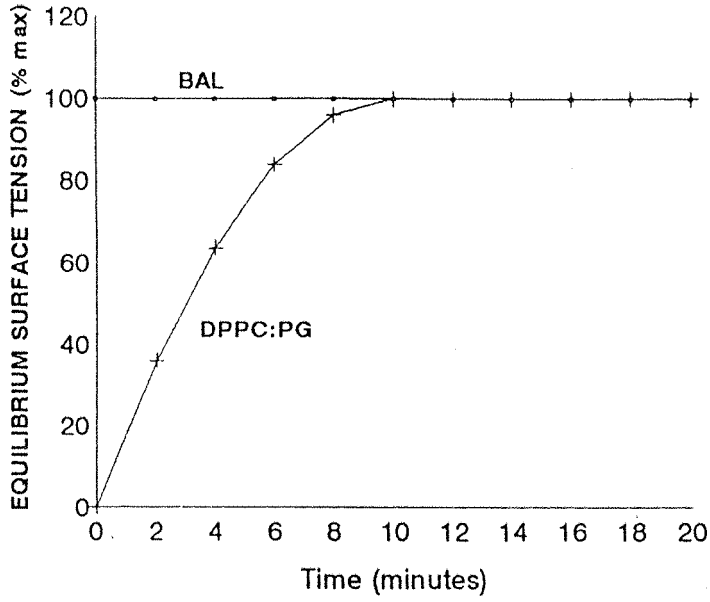

Figure 3.3: Equilibrium surface tension (% max). Samples were put over the balance subphase. Adult guinea pig BAL presents an almost instantaneous spreading and DPPC:PG (7:3) takes around 10 minutes to spread completely

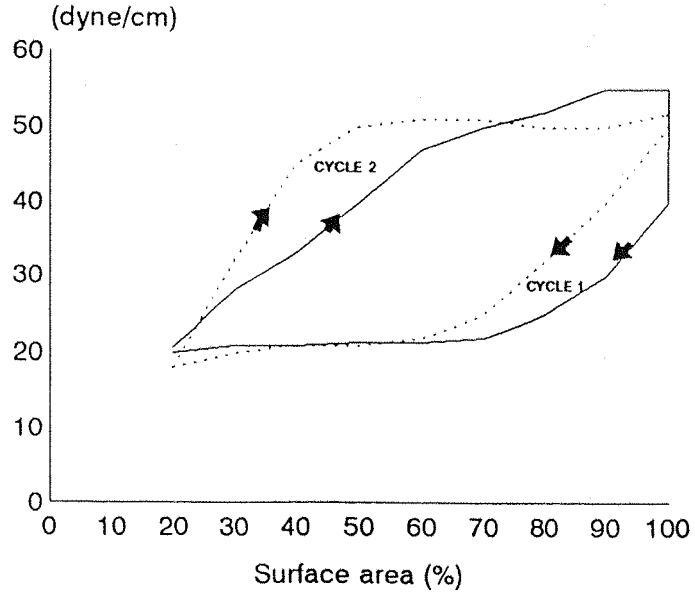

Figure 3.4: Surface tension measurement with guinea pig BAL - different cycles. Subsequent $\gamma \times A$ cycles do not retrace the first due to alignment of the molecules. If samples were left cycling for long periods, γ_{max} tended to reduce

Sample	1st cycle			4th cycle			12th cycle		
	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area
BAL	22.9 ±2.3	61.8 ±2.8	1.24 ±0.36	21.7 ±1.98	64.5 ±6.36	1.23 ±0.03	20.6 ±1.21	61.8 ±5.52	1.43 ±0.35
Purif surfact	22.5 ±0	47.9 ±0.64	1.14 ±0.25	17.2 ±7.5	50.1 ±2.4	1.43 ±0.06	17.9 ±5.1	50.2 ±15.5	1.38 ±0.1
PL extract	20.6 ±0.7	30.1 ±3.7	1.60 ±0.88	20.4 ±1.1	57.4 ±5.3	0.83 ±0.23	21.2 ±0.85	58.1 ±6.2	1.13 ±0.62
Concent. BAL	23.9 ±0.2	47.1 ±1.9	1.00 ±0.13	22.7 ±0.2	65.4 ±3.6	0.91 ±0.08	20.6 ±4.3	68.9 ±1.8	0.66 ±0.03

Table 3.2: Effect of surface tension parameters from adult guinea pig comparing equivalent concentrations of BAL, purified surfactant, phospholipid extract and concentrated BAL. Samples were applied to the saline subphase and left to adsorb for 5 min before cycling

in the Wilhelmy balance. Results were similar for all the different options. Use of purified surfactant produced lower values for surface tension, but the difference was not statistically significant. Therefore, in this study, BAL and rediluted concentrated BAL were used. Concentrated BAL was then assessed for the PC concentration to apply in the surface tension balance as explained in section 3.3. Approximately 85 nmoles total PC gave satisfactory results, and was used in some of the measurements. The samples were freeze dried; part of them were used for surface tension analysis and part for PC measurements and protein concentrations.

In addition the effect on surface tension of inclusion of calcium chloride in the subphase was evaluated (table 3.3). A set of measurements were done using saline (0.9% NaCl) in the balance trough and another set using saline with calcium chloride (0.9% NaCl + 3mM $CaCl_2$. This second compound was added to better simulate the alveoli lining over which the surfactant is located. Comparison of results in both cases did not demonstrate any significant changes neither in surface tension values nor in the value of the hysteresis area.

Sample	1st cycle			4th cycle			12th cycle		
	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area
BAL	22.6 ±0.7	60.4 ±6.9	1.49 ±0.25	21.6 ±0.63	67.9 ±7.7	1.36 ±0.06	17.9 ±4.7	64.2 ±11.7	1.52 ±0.21
Purif surfact	22.0 ±1.9	50.1 ±11.7	0.90 ±0.26	16.1 ±7.3	46.8 ±12.4	1.10 ±0.02	18.4 ±0.42	51.5 ±0.1	1.33 ±0.08
PL extract	23.1	27.5	1.00	21.2	53.7	0.67	20.6	55.9	1.57

Table 3.3: Effect of inclusion of calcium chloride in the subphase on surface tension parameters from adult guinea pig. Comparing equivalent concentrations of BAL, purified surfactant and phospholipid extract. Samples were applied to the 0.9% NaCl + 3mM $CaCl_2$ of the subphase and left to adsorb for 5 min before cycling

3.5 Experimental measurements

3.5.1 Term guinea pig

Several samples of BAL and of rediluted concentrated BAL from term guinea pig was used to analyse the surface tension characteristics. Table 3.4 summarizes the results obtained with 5 ml of BAL which corresponds to approximately 475 nmols in 21% O_2 and 800 nmols in 95% O_2 applied to the saline subphase of the Wilhelmy balance. Table 3.5 shows the results of a similar set of experiments using concentration of 85 nmols PC after redilution from concentrated BAL. As described in the previous section, this amount should provide a complete monolayer over the saline subphase.

In the first case, the values obtained for maximal and minimal surface tension of BAL from pups submitted to 21% O_2 concentration were higher than for those exposed to 95% O_2. However the difference was not statistically significant and the hysteresis areas were similar.

For the second group, summarized in table 3.5, there were no significant differences between the results for 1 hour old pups and three days old pups submitted to 21% or 95% O_2.

Sample	1st cycle			4th cycle			12th cycle		
	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area
21% O_2	21.5	36.3	1.52	18.1	50.6	1.33	16.0	45.2	1.48
n=5	±1.9	±11.6	±0.38	±3.3	±5.2	±0.26	±3.1	±8.1	±0.24
95% O_2	15.8	28.2	1.03	12.8	42.6	1.35	10.2	44.8	1.46
n=5	±5.2	±7.4	±0.35	±3.6	±9.2	±0.17	±7.4	±7.6	±0.17

Table 3.4: The effect of oxygen exposure in term guinea pig. BAL from term guinea pig exposed to either 21% O_2 or 95% O_2. 5 ml of sample were applied to the saline subphase

Sample	1st cycle			6th cycle			12th cycle		
	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area
1h old	25.1	50.9	1.08	23.9	66.7	1.01	20.7	70.1	1.06
n=6	±0.4	±4.2	±0.25	±3.4	±6.4	±0.20	±6.3	±2.7	±0.16
21% O_2	22.6	43.2	1.12	22.8	66.9	1.20	22.7	68.3	1.02
n=4	±0.8	±7.8	±0.19	±0.4	±2.13	±0.39	±0.8	±2.2	±0.25
95% O_2	22.3	47.5	1.16	20.5	67.0	1.25	18.8	69.1	1.07
n=4	±1.6	±8.0	±0.21	±4.2	±2.1	±0.41	±8.4	±0.8	±0.49

Table 3.5: The effect of exposure to 21% O_2 or 95% O_2 on BAL surface tension of term guinea pig. Samples containing approximately 85 nmols PC were applied to the saline subphase.

Sample	1st cycle			4th cycle			12th cycle		
	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area
21% O_2	21.9	47.3	1.13	18.2	50.2	1.25	17.2	47.8	1.31
n=8	±2.0	±13.6	±0.28	±4.3	±10.9	±0.26	±4.9	±7.2	±0.31
95% O_2	21.1	47.5	1.07	18.8	46.9	1.18	18.0	41.9	1.19
n=9	±1.8	±7.9	±0.26	±2.6	±8.2	±0.26	±1.9	±7.9	±0.24

Table 3.6: Preterm guinea pig BAL was obtained from 3 day old pups exposed to either 21% O_2 or 95% O_2. 5 ml of sample were applied to the saline subphase.

3.5.2 Preterm guinea pig

Samples of BAL from preterm guinea pig (65 days of gestational age) were assessed for surface tension. Table 3.6 summarizes the results for 5 ml of sample which corresponds to approximately 2100 nmols for 21% O_2 and 2820 nmols for 95% O_2 applied to a saline subphase. There were no significant differences between the results for 3 day old pups submitted to either 21% or 95% O_2.

Another set of experiments was performed using rediluted concentrated BAL containing approximately 85 nmols PC applied over the saline subphase (table 3.7). There were no significant differences between 1 hour old pups and 3 day old pups submitted either to 21% or 95% O_2. This set of measurements with constant number of mols agreed with the previous one with constant volume.

Comparing the surface tension values of BAL from term guinea pig with the values of preterm guinea pig, no significant changes were observed neither for pups exposed to room air nor for pups submitted to hyperoxia.

3.5.3 PAF antagonist study

A set of surface tension measurement was performed to analyse the effect of PAF antagonist administration. 5 mg/kg/day of WEB 2086 was injected i.p. twice a day, for three days, and the control group received saline as placebo. Table 3.8 presents the surface tension results; again no significant variation was observed.

Sample	1st cycle			6th cycle			12th cycle		
	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area
1h old	23.4	48.9	1.06	20.2	67.8	0.95	20.5	68.6	0.86
n=4	±1.8	±3.2	±0.06	±5.6	±2.2	±0.20	±6.7	±1.3	±0.15
21% O_2	22.8	53.2	1.53	22.4	67.4	1.41	21.9	69.3	1.25
n=5	±0.5	±1.0	±0.17	±0.6	±1.6	±0.23	±0.7	±1.3	±0.23
95% O_2	21.3	53.2	1.70	21.5	67.5	1.28	20.1	68.2	1.12
n=4	±0.5	±2.7	±0.09	±1.3	±1.2	±0.26	±2.2	±1.7	±0.26

Table 3.7: Preterm guinea pig BAL obtained from 1 hour old pups or 3 day old pups exposed to either 21% O_2 or 95% O_2. Sample containing approximately 85 nmols PC were applied over the saline subphase.

Sample	1st cycle			6th cycle			12th cycle		
	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area	γ_{min}	γ_{max}	Area
21% O_2	22.8	53.2	1.53	22.4	67.4	1.41	21.9	69.3	1.25
saline	±0.5	±1.0	±0.17	±0.6	±1.6	±0.23	±0.7	±1.3	±0.23
95% O_2	21.3	53.2	1.70	21.5	67.5	1.28	20.1	68.2	1.12
saline	±0.5	±2.7	±0.09	±1.3	±1.2	±0.26	±2.2	±1.7	±0.26
21% O_2	22.3	52.5	1.65	22.1	68.7	1.52	21.5	69.2	1.32
WEB2086	±0.7	±2.5	±0.20	±0.5	±3.2	±0.25	±0.8	±2.6	±0.29
95% O_2	22.0	52.2	1.67	21.3	68.1	1.44	20.6	68.3	1.25
WEB2086	±0.5	±2.2	±0.11	±2.4	±1.5	±0.15	±3.9	±0.6	±0.14

Table 3.8: PAF antagonist study. Preterm guinea pig BAL, from 3 day old pups submitted either to 21% O_2 or 95% O_2, either using PAF antagonist WEB 2086 or saline. Sample containing approximately 85 nmols PC were applied over the saline subphase of the balance trough

3.6 Discussion

The functional properties of guinea pig BAL were measured using the Wilhelmy balance. It was found that cleanliness of the trough and the platinum float are very important for the calibration procedure. In this study, it was found that constant temperature was essential for a proper measurement. The experiments were performed at 37°C to simulate physiological characteristics and an additional cover was designed to enclose the trough and the float avoiding heat losses. The Wilhelmy balance demonstrated to be very sensitive to vibration and special care had to be taken to avoid any kind of oscillation and perturbation.

In table 3.2 the subphase was saline and in table 3.3 the subphase was saline + 3mM $CaCl_2$. No significant difference was observed in the measurements. Perhaps for the concentration used in this analysis the calcium effect was too small to be detected.

Figures 3.4 and 3.5 shows that surface tension on compression was lower than on expansion, what was due to the presence of surfactant. The critical properties of native surfactant depend on the 3-dimensional organization of the surfactant components [Jobe and Ikegami, 1987]. If the surface film is compressed as in expiration, the molecules become crowded together, giving a higher concentration of surfactant, leading to a further reduction in surface tension. Another geometrical feature is that saturated phospholipids such as DPPC have straight fatty acid chains and so permit a greater packing density of phospholipid molecules in the surface film. By occupying less area, more phospholipid molecules can interact with water, causing a more effective reduction on surface tension. Films consisting of a high proportion of saturated phospholipids have the ability to remain very stable on repeated compression.

Scarpelli *et al.*, [1965] gave an additional reason for the variation of surface tension during compression and expansion. Compression causes water molecules to move from the surface to the subphase. During the early phase of reexpansion, water or hydrated ions or both must move into the surface, effectively lowering the surface concentration of surfactants and increasing surface tension. Surfactants return to the surface more slowly throughout reexpansion and the original surface concentration is gradually restored.

Using the Wilhelmy balance for several consecutive cycles, it was noted that the subsequent $\gamma \times A$ cycles did not retrace the first cycle. In the ex-

periments performed in this study a reproducible loop was reached between the 4th. and the 6th. cycle. Table 3.2 to 3.8 presents the results for the 1st. cycle, either for the 4th. or the 6th. cycle, and the 12th. cycle. This last one was included to give a confirmation that the loop had reached a stable configuration. This changes in the first cycles have also been reported by Clements *et al.* [1961] and Kuenzig *et al.* [1965].

In two sets of measurements, with term and preterm guinea pig, constant PC concentration has been adopted to analyse which other factors could influence the surface tension. There was no significant differences between 1 hour old pups and 3 day old pups submitted either to 21% or 95% O_2 (tables 3.5 and 3.7). A hypothesis to explain this fact could be that surface tension only depends on PC concentration or perhaps the surfactant concentration was so low that no protein influence could be detected.

The values obtained for minimal surface tension at 37°C were in the vicinity of 20 dyne/cm. Although at room temperature smaller values would be obtained, this would not represent the physical condition in the lungs. Two school of experience have been reported: one which claims 'near zero' values for minimum surface tension [King and Clements, 1972], [Notter et al., 1984] and the other who found values for minimal surface tension around 20 dyne/cm [Boudurant and Miller, 1965], Morgan [1971], [Hills, 1988]

The second of the two above options is more likely to be true. Jobe and Ikegami [1987] mentioned this problem of measurement sensitivity to the surface and aqueous bulk phase concentrations of the surfactant, *i.e.* at high concentration, 2 surfactants can have equivalent surface properties; however, at limit dilution, the 2 surfactants can have distinctly different properties [Notter and Finkelstein, 1984], [Notter *et al.*, 1985]. And they concluded that dilution may be critical as only a small fraction of a surfactant suspension may confer the surface properties to the suspension.

In the PAF antagonist analysis (table 3.8) the surface tension results did not change with the administration of WEB 2086. Perhaps the PAF antagonist doses were not enough to produce any variation or the 95% of O_2 exposure have produced only small inflammation which not modified the surface tension characteristics of the rediluted concentrated BAL.

An alternative method for further research would be to measure surface tension using an oscillating bubble. The oscillating bubble requires much less sample volume to measure minimal and maximal surface tensions, so samples with higher surfactant concentrations can be prepared.

Chapter Four
Phosphatidylcholine

An enzymatic method, based on hydrolysis by PLD, was set up in microscale for measuring total PC in BAL. Measurement of PC and other PL is essential for the assessment of surfactant composition. Previous work from this department has shown that the molecular species composition of BAL PC from preterm and term guinea pigs changes with postnatal age. The major effect of hyperoxic exposure, however, was to increase BAL PC concentration without altering the species composition [Kelly *et al.*, 1991], [Merritt, 1982]. Consequently, the development of a routine and convenient assay for BAL PC concentration was the aim of this part of my thesis.

4.1 Principles of the method

In 1977, Takayama *et al.* described an enzymatic method for determination of choline containing phospholipids in serum. PC was hydrolysed by PLD to liberate choline, which was subsequently oxidized by choline oxidase to betaine and hydrogen peroxide. In the presence of peroxidase, 4-aminoantipyrine and phenol, hydrogen peroxide yielded a red quinone dye whose absorbance was read at 500 nm. This method presented high sensitivity and specificity.

The original method used egg PC as standard dissolved in an aqueous solution containing 5g triton X-100 per litre. The colour reagent contained 45 units PLD, 100 units choline oxidase, 12 mg 4-aminoantipyrine, 20 mg phenol, 8 mg calcium chloride dihydrate dissolved in 100 $m\ell$ of 50 mM Tris/HCl buffer (pH 7.8 and containing 2g Triton X-100 per litre). Sample (20 μl) of sample was added to 3.0 ml colour reagent, incubated at 37°C for 20 min. The calibration curve showed linearity up to 1000 mg PC/100 ml. In this current project, Takayama's method was set up in a microscale for measuring PC in BAL. To establish the final method, several processes were performed as described below.

4.2 Method Validation

4.2.1 Adaptation of choline assay to microtitre plate

The chromogen reagent concentration was adjusted by using choline chloride (0 to 50 nmoles) as standard. Different concentration of the com-

	A	B	C	D	E	F
Choline oxidase	20 U	40 U	20 U	20 U	40 U	40 U
Peroxidase	15.8 U	15.8 U	31.6 U	15.8 U	31.6 U	31.6 U
4-Aminoantipyrine	3 mg	3 mg	3 mg	6 mg	3 mg	6 mg

Table 4.1: Optimization of chromogen reagent concentrations. For all the samples 100 mM Tris/HCl + 100 mM $CaCl_2$ pH 8.0 (4:1) were added to a final volume of 10 ml

pounds (choline oxidase, 4-aminoantipyrene and peroxidase) were added into microtitre plate (table 4.1) and incubated for different times from 30 to 60 minutes. The absorbance was read at 492 nm every 10 minutes and was plotted against choline cloride.

The colour development was completed at 50 min at 37°C, but the calibration curve (figure 4.1) did not reproduce the linearity of Takayama's curve. For reagent concentration indicated in table 4.1, the colour development was acceptable, and did not present significant differences for variations in chromogen reagent enzyme or substrate concentrations (figure 4.2). For lower concentrations than indicated on table 4.1, the colour development was very poor. Therefore the values of column A were chosen because they were the most cost effective ones and presented satisfactory colour development, which was not enhanced at higher concentration of chromogen reagent.

4.2.2 PLD hydrolysis rate determination

Initial experiments for analysis of PLD hydrolysis rate used egg PC (0 to 50 nmols) as standard, and the rate of choline liberation was compared with the colour formation of standard choline chloride. Three enzyme concentrations (2.5×10^{-4} U, 5.0×10^{-4} U and 1.0×10^{-3} U) were added to either 20 or 50 nmols egg PC in a microtitre plate at 37°C. The plate was incubated from 20 to 60 min, chromogen reagent was then added and the plate incubated for 50 min at 37°C.

PC hydrolysis rate changed in a direct proportion of enzyme concentration and time incubation. For example, 50 nmols of PC incubated at 37°C for 20 min with 2.5×10^{-4} U PLD hydrolysed 30.3%, with 5.0×10^{-4} U PLD hydrolysed 41.0% and with 1.0×10^{-3} U PLD hydrolysed 63.9%. For 40 min of incubation time, the pattern was similar (see figure 4.3) but the hydrolysis rate increased , for instance, to 73.5% with 1.0×10^{-3} U PLD. The

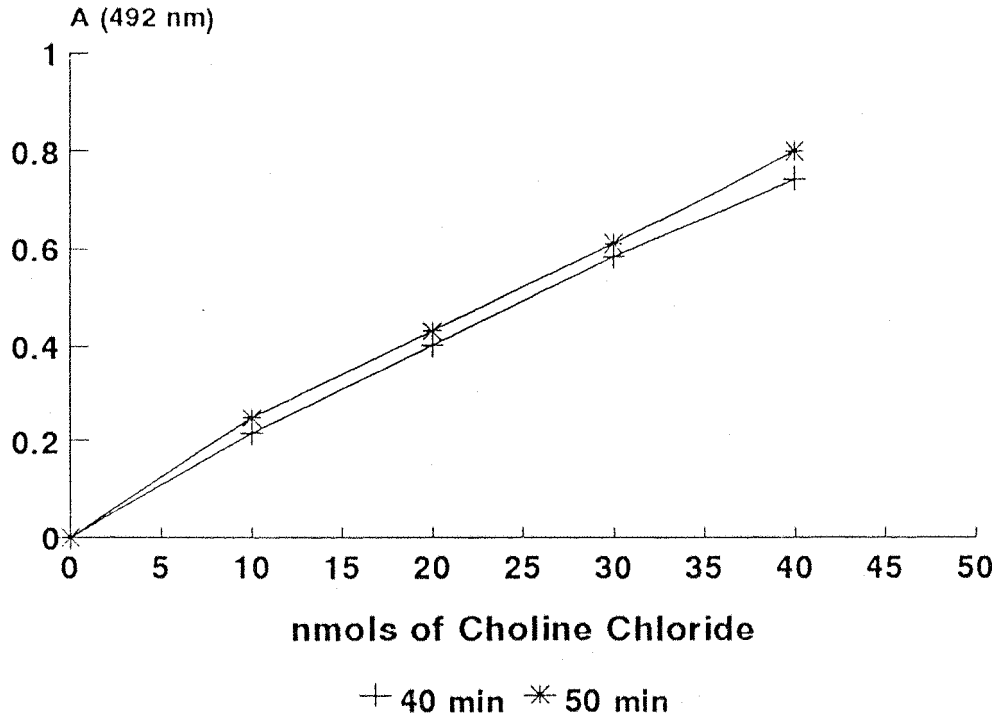

Figure 4.1: Effect of time incubation of chromogen reagent. Absorbance versus number of mols of choline chloride for different incubation time. The following compounds were used: Choline oxidase - 20 U, Peroxidase - 15.8 U, 4-Aminoantipyrine - 3 mg

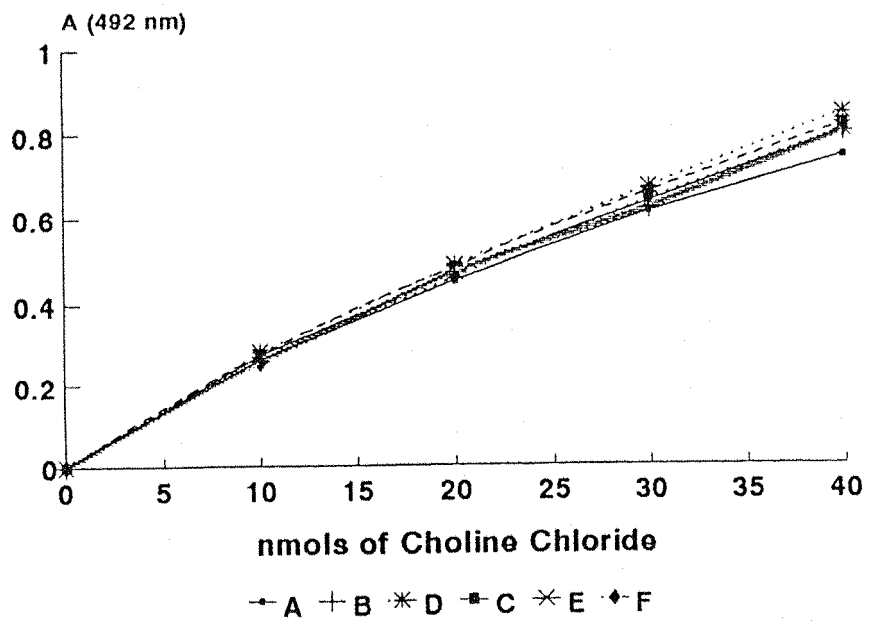

Figure 4.2: Effect of chromogen reagent concentration. Absorbance versus number of mols of choline chloride for several compound concentrations as described in table 4.1.

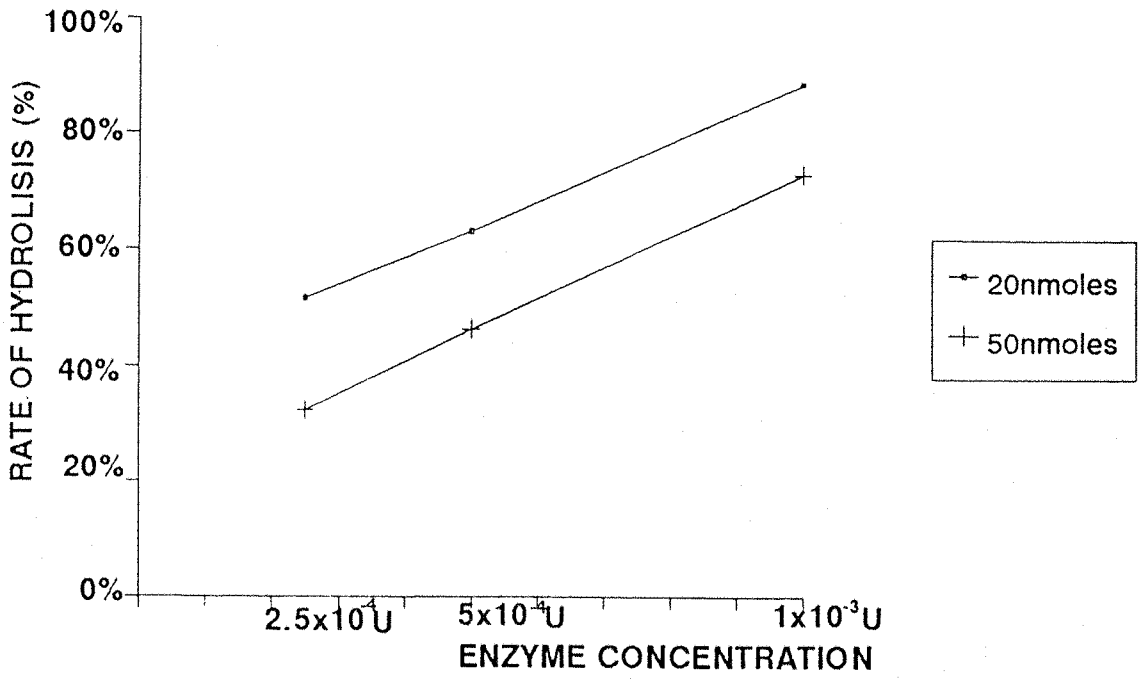

Figure 4.3: PC hydrolysis rate, using different concentrations of PLD incubated for 40 minutes. Results are expressed as percentage of choline chloride absorbance values at the same concentration

hydrolysis rate was satisfactory with 1.0×10^{-3} U PLD, and all experiments were then performed using this concentration.

The major component of BAL surfactant PC is DPPC, and it was considered important to determine whether conditions used to assay egg PC were suitable to quantify DPPC. Egg PC and DPPC over the range 0 - 50 nmols/well were incubated with 1.0×10^{-3} U PLD at 37°C for 40 min and a comparison is shown in figure 4.4.

Results were analised in percentage of hydrolysis and demonstrated that using 1.0×10^{-3} U PLD and incubating for 40 min at 37°C the overall hydrolysis rate of DPPC was lower than that of standard egg PC (fig. 4.5). Therefore it was important to determine the appropriate condition for assay DPPC rather than egg PC.

4.2.3 Adaptation of method for assay DPPC

The rate of hydrolysis of DPPC was analysed by HPLC to provide independent confirmation of the completeness of reaction. As described above (section 4.2.2), the hydrolysis rate increased with temperature and with enzyme concentration. The temperature was increased to 42°C to accelerate the rate of hydrolysis of DPPC, since the transition temperature of DPPC solid to liquid is 41°C. An amount of 50 nmols DPPC was used, and incu-

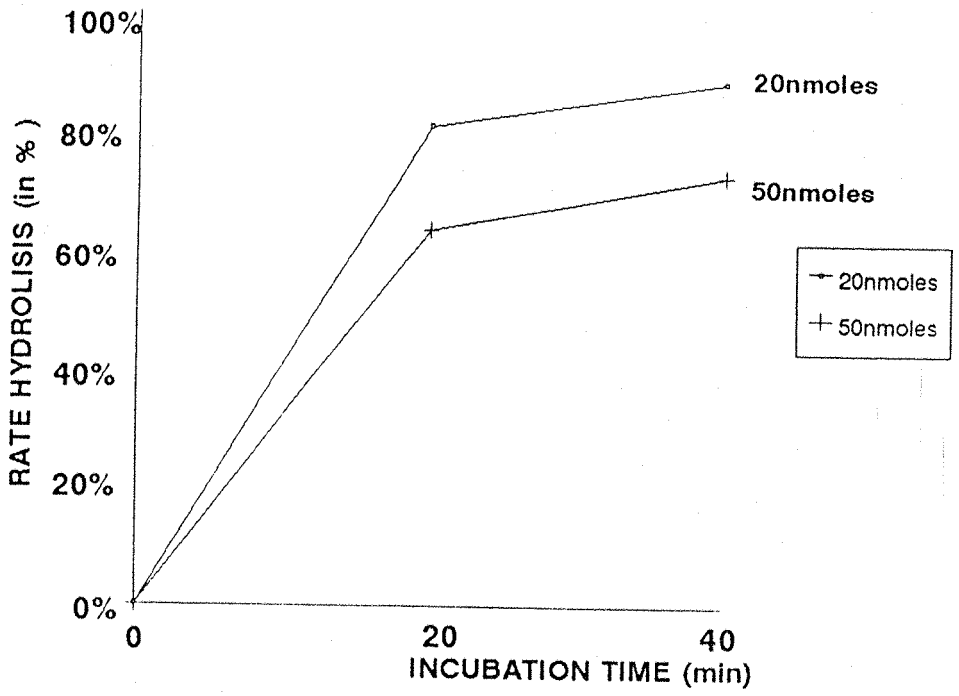

Figure 4.4: PC hydrolysis rate of egg PC and DPPC with 1.0×10^{-3} U PLD incubated for 40 minutes, again compared to choline chloride function

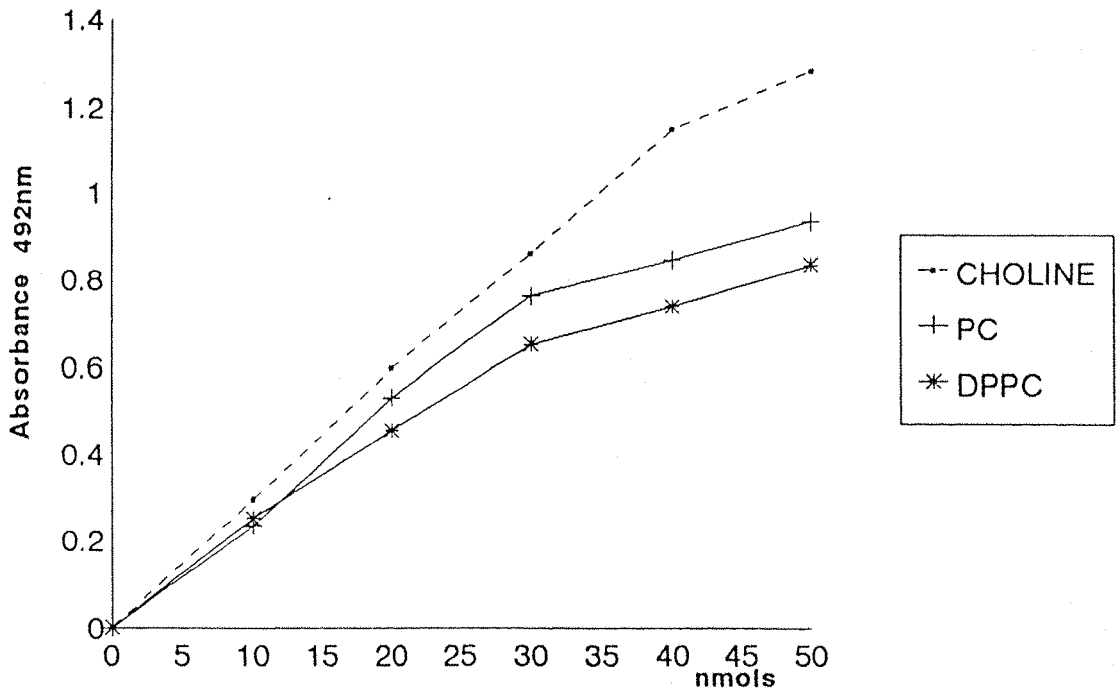

Figure 4.5: Absorbance of choline cloride, PC and DPPC for 40 min incubation time at 37°C with 1.0×10^{-3} U PLD. For the same incubation time PC reaches a higher hydrolysis rate than DPPC

Incubation time	Hydrolisis rate 1.0×10^{-3} U PLD 42°C
20 min	52.0%
30 min	60.0%
40 min	76.0%
50 min	86.0%
60 min	100.0%

Table 4.2: Percentage of DPPC hydrolisis at 42°C.

bated for periods from 0 to 60 minutes. DPPC was hydrolysed by 50% at 20 min, as shown in figure 4.6, and at 60 min the hydrolysis was completed.

4.2.4 Final method

The final method used DPPC as standard with the reaction being performed at 42°C. An incubation time of 50 minutes was chosen for both PLD hydrolysis and chromogen reagent development. PLD was added at 10^{-3} U/well, and 10ml of the chromogen reagent containing 3mg 4-aminoantipyrene, 20 U choline oxidase and 15.8 U peroxidase. Under these conditions, DPPC and choline chloride gave equivalent colour formation in the assay (figure 4.7)

4.2.5 Reproducibility of the assay

The reproducibility of the assay was determined by randomly assessing one sample which was divided into 10 fractions and used as a quality control standard. The PC content of each fraction was then determined in 10 separated assays using the technique described above. The coefficient of variation for this standard was 6.0%, at a concentration of 3.5 $\pm$ 0.72 μmols/BAL (mean $\pm$ S.D.).

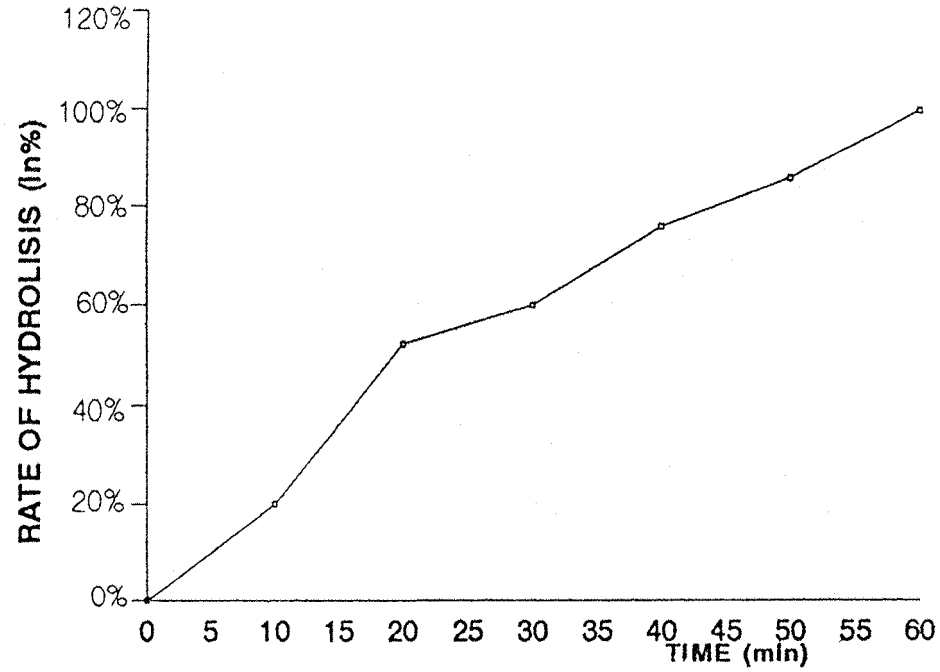

Figure 4.6: Percentage of DPPC hydrolysis with 1.0×10^{-3} U PLD at 42°C.

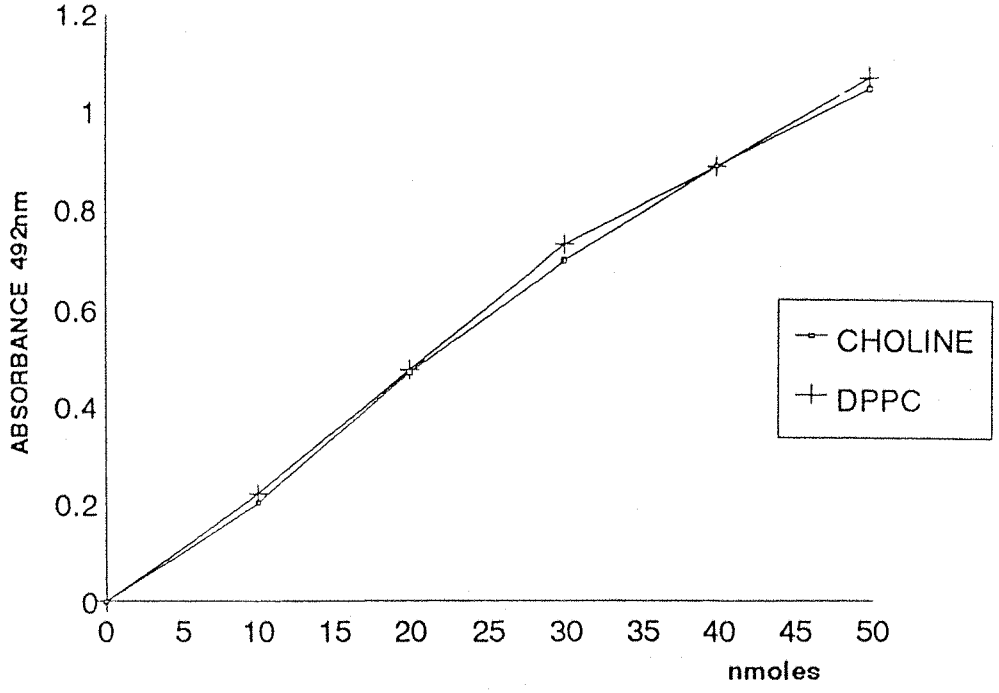

Figure 4.7: Final method of PLD assay. Diagram shows good agreement of absorbance of DPPC and choline cloride

O_2 exposure	Number of samples	PC measurement (μmol/lavage) mean $\pm$ standard deviation
1 hour old	6	0.95 $\pm$ 0.20
72 hours at 21% O_2	4	0.95 $\pm$ 0.15
72 hours at 95% O_2	4	1.60 $\pm$ 0.60

Table 4.3: PC concentration in BAL from term guinea pig. Mean and standard deviation are expressed in μmol/lavage

4.3 BAL measurement results

4.3.1 Term guinea pig exposed to hyperoxia

BAL PC concentration was analysed for three groups of term guinea pig pups. Measurements were made at the first hour after birth, and after exposure to either 21% O_2 or 95% O_2 for 72 hours. There was no change in BAL PC content of pups between 1 hour of age and 21% O_2 exposure for 3 days (table 4.3). BAL PC content appeared to increase after hyperoxic exposure but, due to a large variation and small numbers, this change was not significant.

4.3.2 Preterm guinea pig exposed to hyperoxia

BAL PC concentration was analysed for preterm guinea pig pups. Measurements were made at the first hour after birth and after exposure to either 21% (n=5) or 95% (n=4) O_2 for 72 hours. Oxygen exposed pups were then left at room air and subsequent measurements were made after 2 days and 4 days.

Figure 4.8 summarizes the variations of PC concentration. This increased over the first 72 hours for those pups submitted to 95% hyperoxia ($P < 0.025$). While PC also increased for the group exposed to room air, this change was statistically not significant. When hyperoxic- exposed pups were transfered to room air, BAL PC concentration decreased. By 7 days of age the concentration approximately equalled the initial value, figure 4.8.

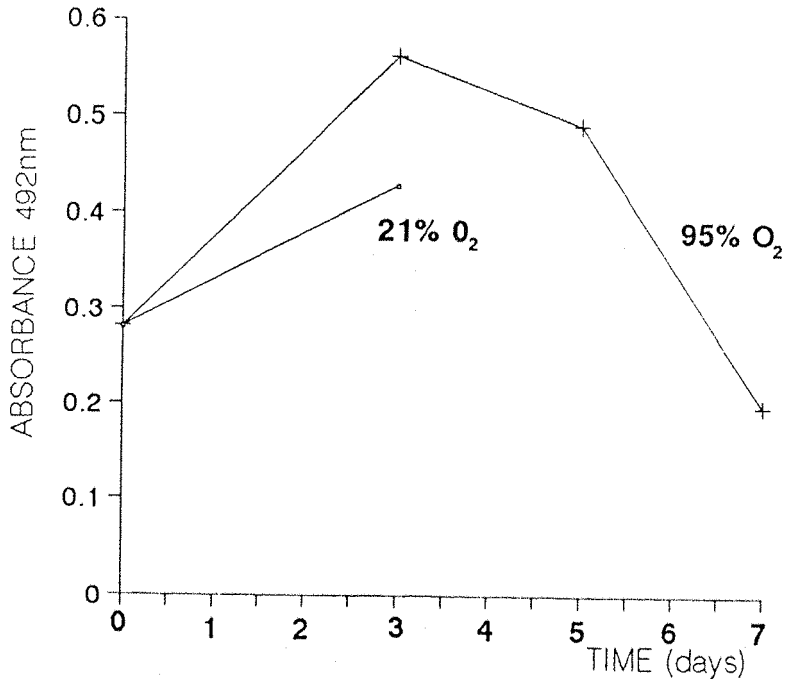

Figure 4.8: PC concentration in BAL from preterm guinea pig exposed to either 21% or 95% O_2 for 72 hours and then left at room air

4.3.3 Effect of PAF antagonist (WEB 2086)

A parallel study by a colleague (Gary Phillips) in the department has investigated the role of PAF on the inflammatory component of hyperoxic lung damage in the preterm guinea pig. BAL samples from this study were analysed for PC content. The same protocol of exposure to 21% or 95% O_2 as described above were used, together with administration of 5.0 mg/kg/day of PAF antagonist WEB 2086, injected twice a day i.p. during the first three days. Results are summarized in table 4.4, indicating PC mean concentration and standard deviation.

For the pups exposed to 21% O_2, administration of WEB 2086 produced an increase of PC concentration ($P < 0.01$) compared with saline control group. For the pups exposed to 95% O_2, PAF antagonist administration produced a slight decrease in BAL PC concentration, which was not statistically significant.

4.4 Discussion

A PLD assay based on Takayama's original method was established, for PC determination in BAL. The major modifications were to scale reagent volumes down to be usable in a microtitre plate, the use of DPPC as a

O_2 exposure	Treatment	Number of samples	PC concentration (μmol/lavage) mean $\pm$ standard deviation
72 h at 21% O_2	saline	5	4.30 $\pm$ 0.80
72 h at 21% O_2	WEB 2086	5	6.19 $\pm$ 0.92
72 h at 95% O_2	saline	4	5.64 $\pm$ 1.24
72 h at 95% O_2	WEB 2086	5	4.58 $\pm$ 0.95

Table 4.4: PC concentration in BAL from preterm guinea pig submitted either to 21% or 95% O_2 together with administration of PAF antagonist (5.0 mg/Kg/day of WEB 2086) injected twice a day during three days

standard, and the employment of increased temperature for the reaction (42°C). In addition, the method used lower choline oxidase and peroxidase concentrations, and was therefore more cost-effective. The method was sensitive, accurate and reproducible. Analysis of PC concentration were possible down to 10 nmols/assay. This would require approximately 5% of a typical guinea pig pup lavage. Advantages of this method over alternative such as phospholipid phosphorous determination (Bartlett) include no handling of harmful compounds and a relatively small time to perform the experiment (90 min).

Furthermore, it may be useful for routine clinical determination of BAL PC and also for studies of the physiological or clinical significance of choline-containing PL in comparison with total PL.

Results for PC concentration in BAL from term guinea pig is shown on table 4.3. Standard deviation for the measurements with 1 hour old pups is larger than for 72 hour old pups at room air. One explanation for this finding is the rapid changes immediately after birth. BAL from newborn pups, before the first breath, contains little surfactant; the alveolar surfactant pool changes quickly in the first hour of life as the mechanism for surfactant secretion and recycling become established.

For the 72 h old guinea pig exposed to 21% O_2, the average PC concentration was similar to the average PC at the first hour, probably because there was no inflammation. This agrees with Kelly *et al.*, [1991] who found no evidence of lung injury in term animals exposed to 21% O_2.

For the pups submitted to 95% O_2 during the first 72 h, a trend of increase of PC concentration was found, which was similar to the findings of Ward and Roberts [1984]. Probably, this is correlated to the inflammatory changes ocurring in the lungs. Pulmonary histological findings of Kelly *et al.*, [1991] revealed also that lung injury was not pronounced for term pups

submitted to hyperoxia.

Previous studies in the preterm guinea pig has documented that 95% oxygen exposure during the first three days was associated with progressive pulmonary inflammation that was maximal at 96 hours [Kelly and Phillips, 1989]. As the mortality at 96 hours was very high, the exposure duration was reduced, in this study, to sublethal time (72 hours).

PC measurements in preterm guinea pig demonstrated a trend of increase in concentration for pups submitted to 21% O_2 and a increase in PC concentration ($P < 0.025$) for those exposed to 95% hyperoxia. This increase in PC concentration is possibly due to increased release from damaged cells. This also agrees with histological findings of Kelly *et al.* [1991] which showed acute lung injury, atelectasis, pulmonary oedema, fibrin deposition and inflammatory cell infiltration in preterm animals exposed to hyperoxia.

Although, the number of samples of term and preterm pups were relatively small (between 4 and 6), the rise in PC concentration was more pronounced in preterm than in their term counterpart.Probably in preterm the antioxidant system is still immature and fails to prevent inflammation. These results confirms that the preterm guinea pig is more susceptible than the term animal to lung injury due to O_2 exposure.

After ceasing hyperoxia exposure, PC concentration started to decrease slowly during the following 2 days, and then markedly decreased over the next 2 following days. PC concentration at day 7 approximately equalled the initial value at birth. This probably reflects the timescale of lung recovery from inflammation.

For the pups submitted to 21% O_2, administration of WEB 2086 produced an increase of PC concentration ($P < 0.01$) compared with saline control group (table 4.4). The reason for this increase is not clear, especially as no comparable increase was observed to WEB 2086 in the hyperoxic-treated guinea pig. The concentration of surfactant PC in the alveolus results from a complex interaction between synthesis, secretion and uptake. These parameters would need to be studied in greater detail to determine the mechanism of action of the PAF antagonist. The slight decrease in BAL PC for the hyperoxic treated guinea pig pups in response to WEB 2086 was associated with reduction in inflammation determined by differential white cell count and assay of lavage protein [Mr. G. Phillips, Personal communication and section 5.2.3]

An effect of this PAF antagonist in the respiratory system has already been reported. The bronchoconstriction caused by 10 or 30 ng PAF was suppressed when the isolated guinea pig lung was perfused with WEB 2086 (0.1 μM) [Pretolani *et al.*, 1987]. Continuous intravenous infusion of PAF at 30 ng/Kg/min in guinea pigs produced a continuous decrease of respiratory flow, and inhalation of WEB 2086 for 3 min at 0.25 to 0.5 mg/ml 5 min before infusion of PAF slowed down the decrease of respiratory flow [Casals-Stenzel *et al.*, 1987].

With respect to PAF-associated contribution to inflammation of the airways and edema formation *in vivo*, WEB 2086 has been shown to inhibit PAF-induced microvascular leakage in the guinea pig in a dose-dependent manner, as measured by the extravasation of IV injected Evans blue dye in the airways [Evans *et al.*, 1988].

Chapter Five
Protein

5.1 Introduction

Pulmonary surfactant contains approximately 8% of proteins which on their own exhibit only minimum surface activity. However surfactant associated proteins are very active in promoting the surface activity of phospholipids and they are also important for the formation of tubular myelin, the turnover of surfactant phospholipids, the inhibition of pulmonary surfactant secretion and the phagocytosis of opsonized particles [Weaver and Whitsett, 1991].

Measurement of total protein and identification of individual proteins contained in pulmonary lavage is necessary to characterize surfactant composition and to determine the protein contribution towards lung surfactant properties.

In this study, total protein was determined using the bicinchoninic acid protein assay. Protein identification was performed using gel electrophoresis (SDS-Page) and comparing the electrophoretic mobility with known protein markers. Immunoassay western blotting was also performed to characterize protein SP-A, using a polyclonal antibody against guinea pig SP-A.

5.2 Protein total measurement in BAL

Total protein in BAL sample was analysed using the BCA method (section 2.2). Although total protein in BAL is not a specific parameter for analysing inflammation, other studies had correlated an increase in total protein with increase of neutrophil in BAL of guinea pig exposed to hyperoxia [Kelly *et al.*. 1991], [Merritt, 1982].

5.2.1 Total protein concentration in term guinea pig

Total protein concentration in BAL was analysed in three groups of term guinea pig pups. BAL sample measurement were made at the first hour of birth, and after 3 days exposed to air (21% O_2) or hyperoxia (95% O_2).

Results are summarized in table 5.1 and demonstrated an increase in total protein concentration of pups between 1 hour old and 21% O_2 for 3 days (P=NS). There was a marked raise in BAL total protein concentration

	Number of samples	Total protein (mg/lavage) mean ± standard deviation
1 hour old	6	0.16 ± 0.05
21% O_2	4	0.52 ± 0.18
95% O_2	4	3.04 ± 1.79

Table 5.1: Total protein concentration of BAL from term guinea pig submitted to either 21% O_2 or 95% O_2 during 72 hours. Mean and standard deviation are expressed in mg/lavage

	Number of samples	Total protein (mg/lavage) mean ± standard deviation
1 hour old	4	0.76 ± 0.29
3 days, 21% O_2	5	2.30 ± 0.54
3 days, 95% O_2	4	5.81 ± 3.39
3 days, 95% O_2 +2 days RTP	5	1.47 ± 0.74
3 days, 95% O_2 +4 days RTP	3	0.91 ± 0.27

Table 5.2: Total protein concentration of BAL from preterm guinea pig submitted to either 21% O_2 or 95% O_2 during 72 hours. Mean and standard deviation are expressed in mg/lavage

in pups exposed to hyperoxia for 3 days comparing either with 1 hour old ($p < 0.005$) or 3 days old 21 ($P < 0.025$).

5.2.2 Total protein in preterm guinea pig

BAL total protein concentration was determined in preterm guinea pig pups at 1 hour old, exposed 3 days either to 21% O_2 or to hyperoxia (95% O_2). After that, the pups were left in room to recover for 2 days or 4 days before measurement.

After three days of exposure to hyperoxia (table 5.2), BAL protein concentration increased seven-fold compared with concentration at birth ($P < 0.01$) and was double the concentration of pups submitted to room air ($P < 0.05$).

After ceasing the hyperoxia exposure, the protein level decreased markedly. After 2 days, it reduced to one third of previous concentration ($P < 0.025$) and after 4 days it reduced to one sixth of concentration at 72 hours ($P < 0.025$).

	Treatment	Number of samples	Total protein (mg/lavage) mean ± standard deviation
3 days, 21% O_2	saline	5	2.30 ± 0.54
3 days, 21% O_2	WEB 2086	5	2.65 ± 1.31
3 days, 95% O_2	saline	4	5.81 ± 3.39
3 days, 95% O_2	WEB 2086	5	3.11 ± 1.12

Table 5.3: Total protein concentration of BAL from preterm guinea pig exposed to either 21% O_2 or 95% O_2 and submitted to i.p. injection either of PAF antagonist WEB 2086 or saline. Mean and standard deviation are expressed in mg/lavage

5.2.3 Effect of PAF antagonist (WEB 2086)

BAL samples of preterm guinea pig from this study were analysed to determine total protein concentration. Preterm guinea pig were submitted either to 21% O_2 or 95% O_2, a group of pups received 5.0 mg/Kg/day i.p. of PAF antagonist WEB 2086 twice a day and a control group received saline.

Table 5.3 summarizes the results. Administration of WEB 2086 produced no changes in protein concentration of BAL from pups submitted to 21% O_2. For the pups exposed to hyperoxia, administration of PAF antagonist reduced the concentration level of proteins to two thirds of concentration in the control group but with low level of statistical significance (P=NS).

5.3 Purification of surfactant associated proteins (SP-A, SP-B and SP-C)

The initial research project included setting up an ELISA for analysing changes in SP-A concentration in BAL due to hyperoxia. Surfactant associated proteins were purified by using lamellar bodies extracted from BAL and homogenized lung from adult guinea pig. The method of Hawgood *et al.*, [1987] was then performed as described in section 2.3.

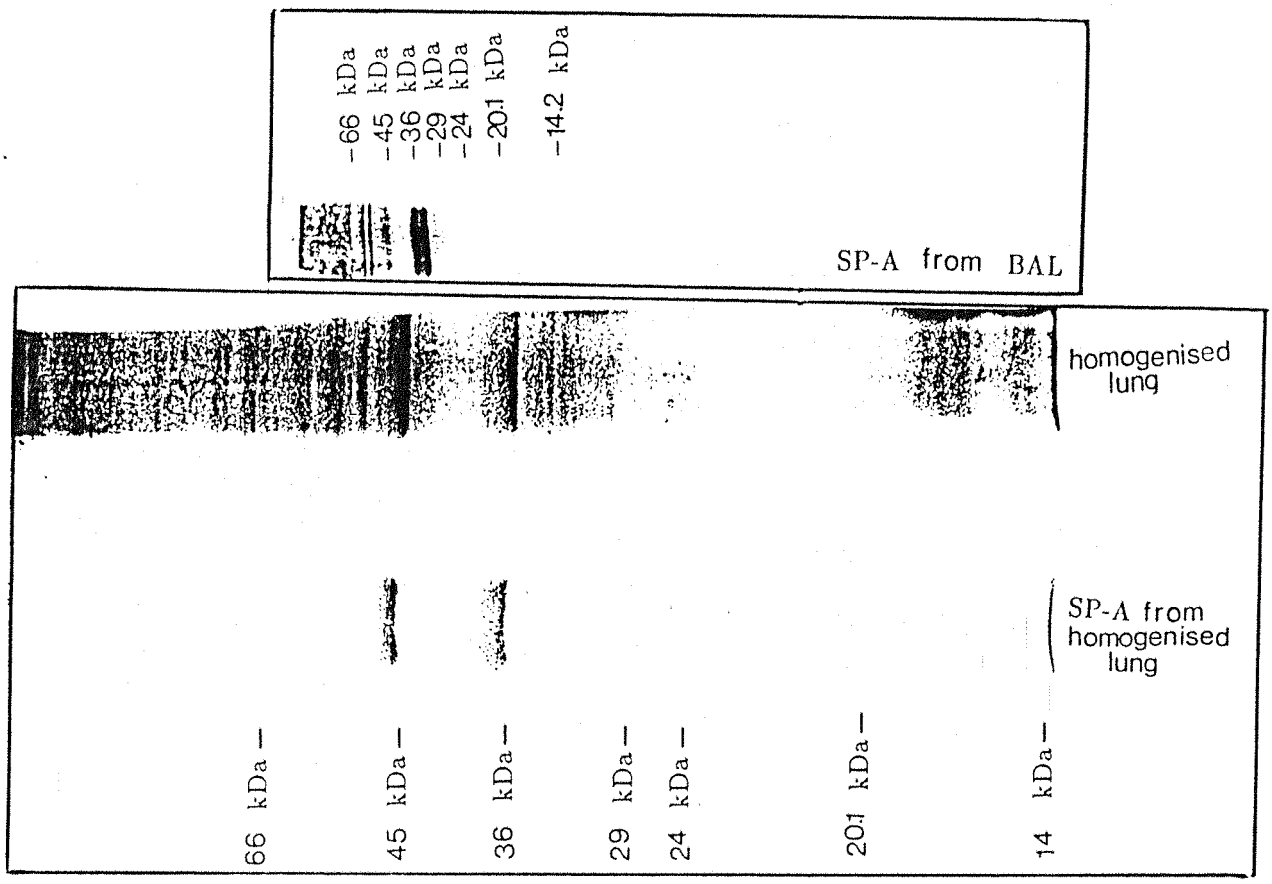

Figure 5.1: Bands of SDS-Page gel electrophoresis for SP-A purification

5.3.1 SP-A

The insoluble fraction obtained after the butanol extraction was processed as described in section 2.3, aliquoted and stored at −20°C until use. Aliquots of the fractions obtained from homogenized lung and other from BAL containing 0.1 mg of total protein, was analysed by SDS-Page gel electrophoresis and compared with homogenized lung and lamellar bodies fraction. The 12.5% polyacrylamide gel, fraction obtained from homogenized lung, showed two bands: one at approximately 36 KDa, which is probably the SP-A monomer and another at 45 KDa which may be SP-D (figure 5.1). The gel containing the fraction obtained from BAL showed four bands: two between 36 and 29 KDa and two around 60 KDa.

Total protein content were analysed by BCA method, section 2.2, and the result demonstrated a recovery, 1.2 mg of protein out of 6 homogenized lungs. Also an aliquot was kept for immunoanalysis (section 5.4).

5.3.2 SP-B and SP-C

After the butanol extraction, the soluble fraction was processed as described in section 2.3. Elution fractions obtained in the acidified Sephadex LH-20 column were assayed for PC concentration using the PLD enzymatic

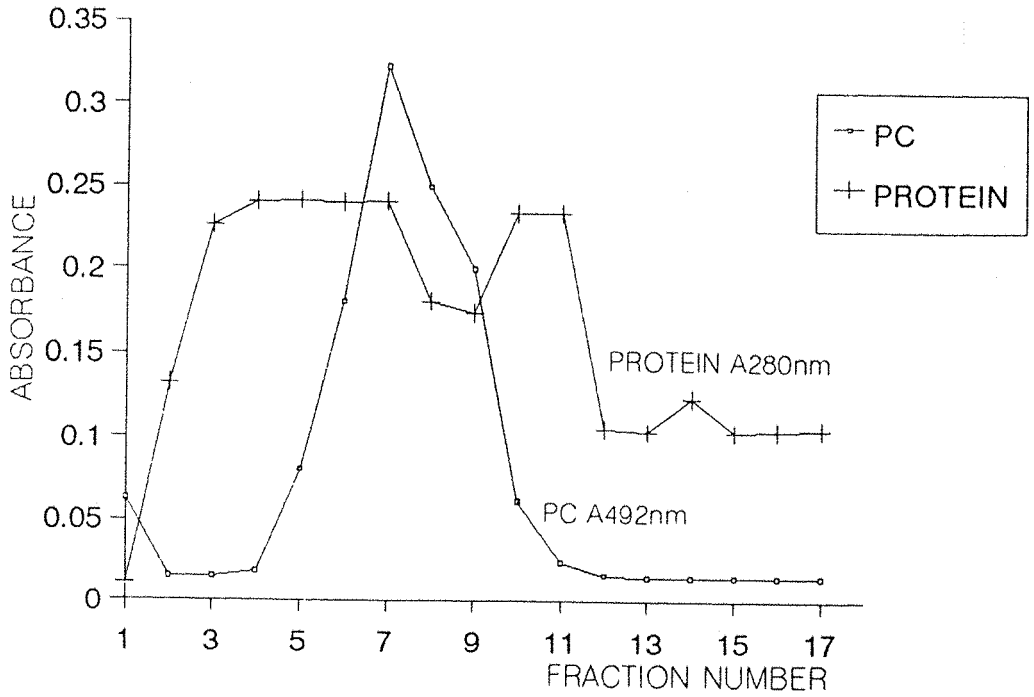

Figure 5.2: Resolution of SP-B and SP-C from adult guinea pig. Absorbance *vs* elution fraction of BAL

method, section 2.10, and total protein concentration using absorbance of 280 nm. Results were plotted as absorbance *vs* eluted fraction number (figure 5.2). Subsequently, the elution fraction were analysed in SDS-Page using the method of Schägger and Von Jagow, as described in section 2.6, and presented in figure 5.3.

5.4 SP-A evaluation

Initial experiments for SP-A identification were performed using anti-rat SP-A (kindly supplied by Dr. Martin Post, from the Hospital for Sick Children, Toronto, Canada). Results demonstrated that anti-rat SP-A cross reacted with guinea pig homogenized lung.

However, the amount available of this antibody was limited and a new antibody against guinea pig SP-A was raised in rabbit, as described in section 2.7, by injecting the 36 KDa band of lamellar bodies extracted from adult guinea pig antigen lung. After two injections with 36 KDa band, blood was collected from the two injected rabbits and serum aliquoted. Aliquots were stored at $-20°C$.

Dot blotting was performed, and results demonstrated that both rabbit

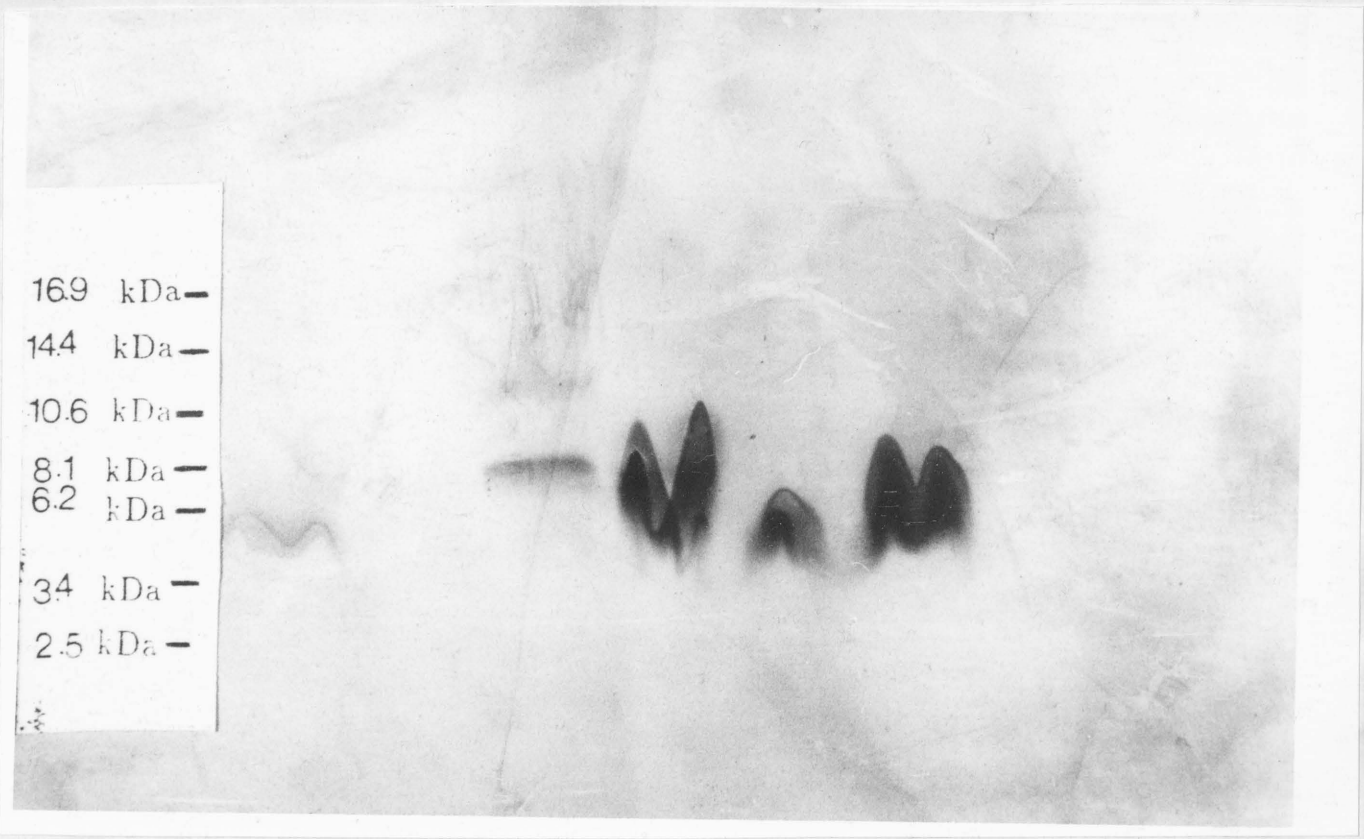

Figure 5.3: Polyacrylamide gel containing eluted fractions from the Sephadex LH20 column.

antibodies (A and B) reacted in dilution of 1:200 with either denaturated or lamellar bodies applied directly in the nitrocellulose membrane. However, the antibody A had shown no specificity when a dot blotting was performed using fetal homogenized lung (55 and 68 days of gestational age), adult guinea pig and purified SP-A. Antibody B was more specific (figure 5.4).

A variety of conditions were evaluated in attempts to identify SP-A in guinea pig lung homogenate or lamellar body preparation by Western blotting using antibody B. Immuno blotting was performed both with denatured protein and with renatured protein by incubating gel with renaturing solution (section 2.7). Denatured protein did not react with antibody B, even when antibody was concentrated (1:20) and a second antibody was used. However reaction was obtained with renatured protein, again using antibody B at 1:20 dilution and the biotinylated 2nd. antibody (section 2.7). Results are shown in figure 5.5.a and demonstrate that antibody B cross reacted with some lung proteins as well as 36 KDa protein band.

PE-10 [Kuroki, 1985], a monoclonal antibody against human SP-A, kindly provided by Prof. T. Akino from Sapporo University, was used to evaluate the possibility of establishing an ELISA assay. PE-10 cross reacted with guinea pig SP-A in a western blotting concentration of 1:200 (figure 5.4). PE 10 reacted with the same 36kDa protein band also detected by anti guinea pig SP-A (antibody B).

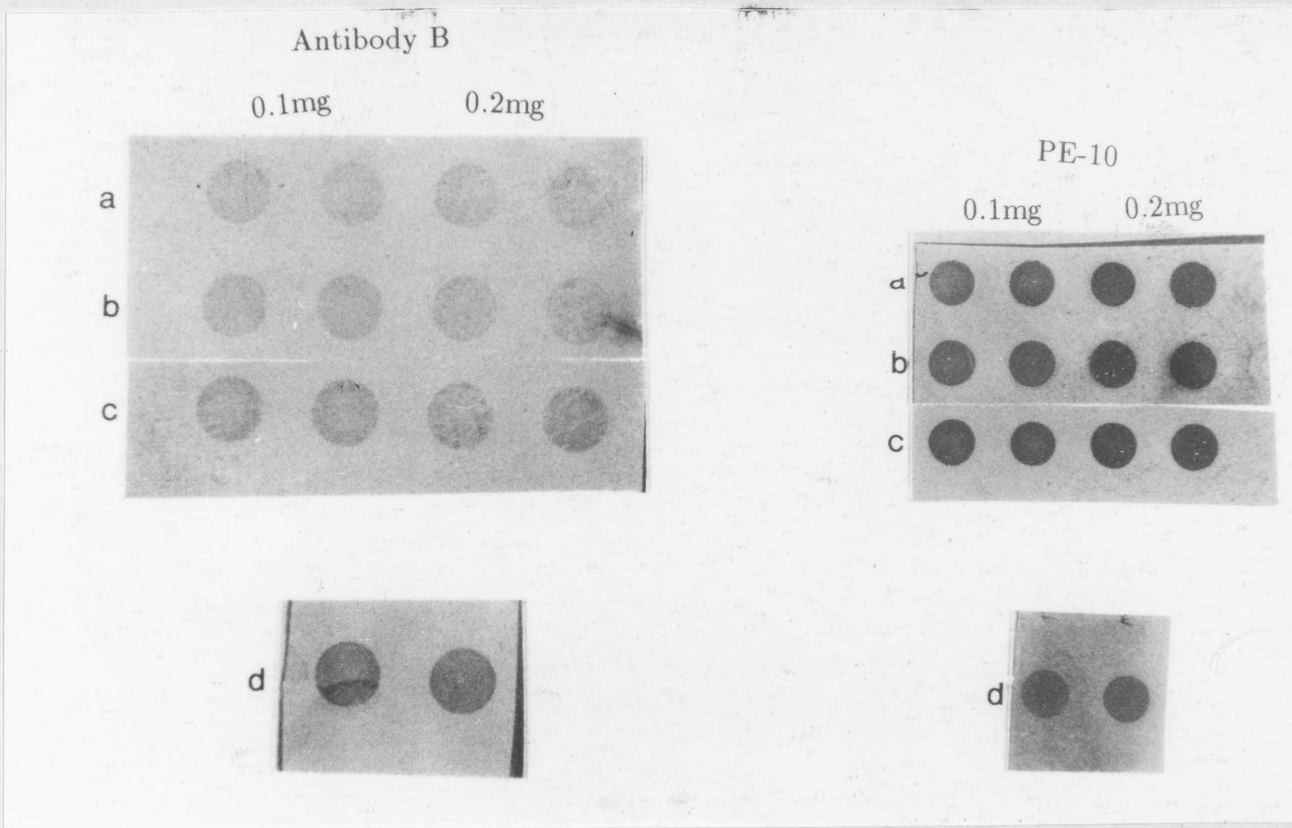

Figure 5.4: Nitrocellulose membrane of a dot blotting containing the same total protein concentration of homogenized lung (0.1 mg and 0.2 mg) of a)fetus, day 55 b)fetus, day 68, and c) adult guinea pig d) purified SP-A

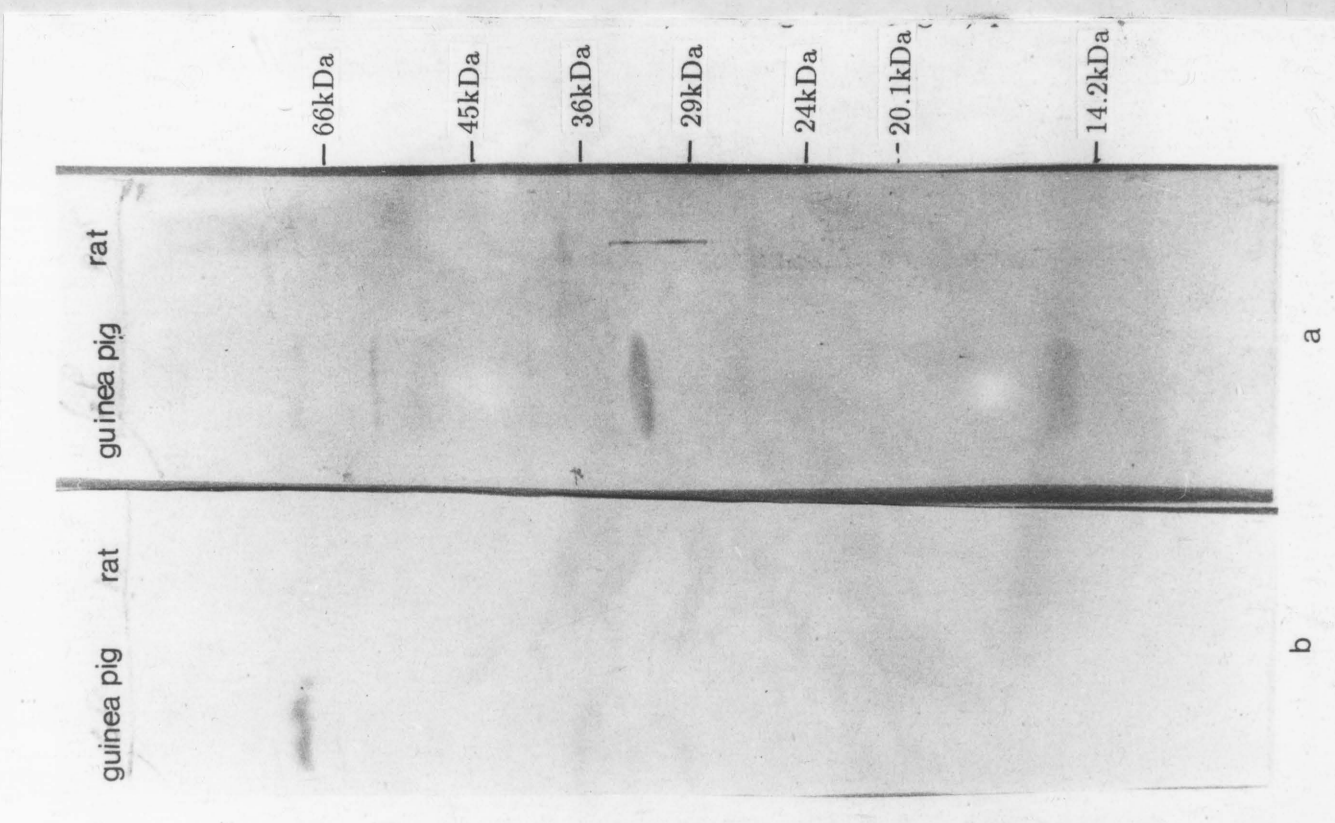

Figure 5.5: Western blotting. a) adult rat and guinea pig homogenized lung crossed with antibody B. b) adult rat and guinea pig homogenized lung crossed with PE10

5.5 Discussion

5.5.1 Total protein

Total protein in BAL from term guinea pig exposed to 21% O_2 increased three fold compared with protein concentration at birth and for the group submitted to 95% O_2 the increase was almost twenty fold (table 5.1). Merritt [1982] analysing term guinea pig submitted to 90% O_2 found an increase of total protein and albumin within 48 h of oxygen exposure, perhaps due to the effect of high oxygen exposure on vascular permeability.

Analogously, values of total protein concentration after 95% O_2 exposure in preterm animals (table 5.2) were significantly elevated compared with concentration at birth ($P < 0.01$). The protein concentration of the BAL fluid from preterm animals exposed to 21% O_2 was significantly higher (almost double) that from term animals. This pattern is similar to the one observed by Kelly *et al.*, [1991]. They found that 50% of this protein was identified as albumin, probably derived from transudation of serum protein. They also found that lung of preterm pups had a more pronounced inflammation.

In the 95% oxygen treated animals, after the marked elevation of total protein concentration at day 3, there was a decrease of protein concentration when pups were left at RTP to recover until day 7, what can be due to the reduction of inflammatory levels. Town [1990] observed a similar behaviour, although the decrease rate was slower.

In the PAF study, administration of PAF antagonist (WEB 2086) reduced the level of protein concentration for the pups exposed to hyperoxia. It is probable that because PAF may play a role in the inflammation and application ofits antagonist may help the recovery process.

Meredith *et al.*, [1989] found an increase in PAF activity in BAL from ventilated preterm baboons. Many models of altered airways responsiveness following inflammatory reactions in experimental animals can be reduced by PAF antagonist. In pulmonary diseases, PAF is likely to be a participant in inflammatory processes and also there was suggestion that its presence and action may be important in such neutrophil-dominated reactions as adult respiratory distress syndrome [Henson *et al.*, 1992].

5.5.2 Surfactant associated protein purification (SP-A, SP-B and SP-C)

The method was described by Hawgood *et al.*, [1987] using lavage from dog lungs for purification of SP-A, SP-B and SP-C. It had been demonstrated to be useful for the analysis of BAL and homogenized lung lamellar bodies content of SP-A, SP-B and SP-C from guinea pig.

Electrophoresis gel of the water soluble fraction obtained from homogenized lung shows two bands, as described in section 5.3. One which was at 36 KDa is probably SP-A and the other at 45 KDa could be a contaminant protein, for example actin [Postle *et al.*, 1985], or another surfactant associated protein SP-D, which in rat is a 43 KDa protein [Person *et al.*, 1989], [Person, 1988]. The polyacrylamide gel from the BAL fraction showed four bands, as described in section 5.3, and all of them were at molecular weight expected for SP-A monomer (29KDa to 36KDa) and dimer 60 KDa [Kuroki *et al.*, 1985]

The hydrophobic proteins (SP-B and SP-C) have a molecular weight of approximately 18 KDa unreduced and 8-5 KDa reduced [Hawgood *et al*, 1987]. SP-B and SP-C were separated from guinea pig lamellar bodies in acidified Sephadex LH-20. Electrophoresis of eluted fractions demonstrated two bands, one at approximately 8.5 KDa and other in 6.4 KDa (see figure 5.3). The relationship of this bands to SP-B and SP-C was not explored further.

5.6 Antibody specificity evaluation

The dot blotting performed using fetuses (55 days of gestational age), term pups and adult guinea pig was a reliable method for showing antibody B. Less amount of SP-A was found at day 55 than at term birth. From birth up to adult guinea pig the concentration was then maintained constant.

Correlation between production of SP-A and gestational age was found in other mammalian species. SP-A in rat lungs only appears during late fetal development (day 18 or 19), increases markedly during the last few days of gestation and reaches maximal levels in the adult [Katyal and Singh, 1979, 1984, 1985], [Fisher *et al.*, 1988]. For fetal lamb lungs, SP-A was also found only in late gestation [Gikas *et al.*, 1977]. Fetal human lung SP-A was found only after 29 weeks of gestation by King *et al.*, [1975] and was detected very

low (7% of adult) at 25 weeks of gestation [Ballard *et al.*, 1986]. For guinea pig, this correlation between SP-A production and gestational age has not been established yet, but presumedly a similar pattern might apply.

Although antibody B was shown to be specific, it demonstrated poor sensitivity in western blotting and high concentration of the antibody (1:20) was necessary for identification of the 36KDa protein. Also, as mentioned before, the sensitivity had to be increased by using a biotinalated antirabbit IgG and streptavidin. This high titre for the antibody may explain why there were a number of non specific bands in figure 5.5, in addition to the expected bands for SP-A.

Anti guinea pig SP-A, PE-10 and anti-rat SP-A all cross reacted with bands of same molecular weight in the guinea pig lung. Differences in antigenicity between the guinea pig, rat and human for SP-A were demonstrated. While both anti guinea pig SP-A and PE-10 reacted against human and guinea pig SP-A, neither detected the rat protein. However, the anti-rat SP-A successfully detected the antigen present in lung from all these animal species. A further step in this research would be the set up of an ELISA assay, using either purified anti-guinea pig SP-A or PE-10.

Chapter Six
Summary

In this thesis several experimental techniques were integrated to establish an approach for evaluation of lung surfactant, composition and function. Initially this study included the set up of measurement techniques for evaluation of surface tension, PC concentration, total protein and protein identification. After that, the study was concerned with application of the above measurement techniques to analyse BAL from guinea pig as an animal model for RDS.

BAL from term and preterm guinea pig pups were analysed either in a constant concentration or in a constant volume. Samples were obtained at the first hour after birth, or after 72 hours of exposure either to room air or hyperoxia. After hyperoxia exposure, a group of preterm guinea pig pups was left at room air to recover for four days.

6.1 PLD assay

A PLD assay, based on Takayama's original method, was modified in this research project, to a microscale for using in a microtitre plate, applying DPPC as standard. This method was demonstrated to be sensitive and reliable for measuring PC in BAL samples. A cost-effective method was developed, using less amount of choline oxidase and peroxidase, and allowing larger incubation time at a higher temperature. The PLD assay is relatively rapid (approximately an hour and a half to complete) and should provide a practical aid to the clinical setting.

Concerning PC concentration, it was found that term guinea pigs exposed to 21% O_2 had no variation compared with PC values at birth. This is in agreement with previous observations that have described minimal lung inflammation in such control animals. BAL PC content appeared to increase in term guinea pig after hyperoxia exposure, but due to a large variation and small numbers, this change was not significant.

For preterm guinea pig, PC concentration increased over the first 72 hours for those pups submitted to 95% hyperoxia ($P < 0.025$). PC also increased for the group exposed to room air, although this change was statistically not significant. These results confirm that the preterm guinea pig is more susceptible to lung injury due to O_2 exposure and agrees with Kelly *et al.* [1991] histological findings related to lung inflammation in preterm animals exposed to hyperoxia. When hyperoxia-exposed pups were trans-

fered to room air, BAL PC concentration decreased to the initial value by 7 days of age ($P < 0.01$).

6.2 Surface tension

The functional properties of guinea pig BAL were analysed using the Wilhelmy balance. During this project, the cleaning procedures, that would otherwise appear trivial, were shown to be essential.

The effect of surface tension analysis at a temperature at 37°C, was intriguing. Measuring at room temperature (approximately 20°C), has given reduced values for γ_{min}, but this is far from the body temperature. Preserving constant temperature at 37°C was more appropriated, to simulate physiological condition. An additional cover was designed in this study to prevent heat losses from the balance. The thermal insulation cover proved to be an efficient method to preserve a constant temperature during the whole experiment. Concerning with minimal surface tension, two schools of experience have been reported. One represented by Notter [1984] which claims 'near zero' surface tension and another which measures values in the vicinity of 20 dyne/cm [Hill, 1988]. The results obtained in this study are in agreement with this second group.

Another problem which interfered in the balance measurements was vibration, such as draught from an open window, a sudden door shutting, etc. The cover mentioned before was an effective protector against wind vibration. Apart from this, care had to be taken to avoid any other kind of vibration.

Another experiment was performed to analyse the influence of the balance subphase. A set of measurements were done using saline in the balance trough and another set using saline (0.9% NaCl) + 3mM $CaCl_2$. This second compound was added to better simulate the alveolar lining over which the surfactant is located. Comparison of results in both cases did not demonstrate any significant changes neither in surface tension values nor in the value of the hysteresis area.

Lung surfactant from guinea pig was analysed for surface tension characteristics. There are several forms in which pulmonary surfactant can be obtained, handled and stored. In this study, several ways of BAL presentation were tried 1) BAL itself, 2) phospholipids extracted from BAL, 3) surfactant purified from BAL and 4) concentrated BAL. Comparison of the

values measured with the different ways of using BAL, demonstrated no significant differences.

A trend of lower minimal surface tension was observed in pups exposed to hyperoxia, compared with pups exposed to room air for the cases in which constant volumes were analysed, but the difference was statistically not significant.

6.3 Protein assessment

Total protein concentration measurement in BAL was analysed as an indicator of vascular leakage and inflammation due to oxygen exposure. Term guinea pigs had a small increase in BAL protein when exposed to air for three days (P=NS) whereas preterm pups had a significant increase ($P < 0.001$). When exposed to hyperoxia, term guinea pig had a marked raise in BAL total protein concentration over the first three days ($P < 0.005$), and preterm pups also had an increase ($P < 0.01$). Wewers [1988], analysing inflammation parameters, found an influx of serum proteins in acute lung injury.

Protein results associated with PC measurements suggest that preterm pups suffered lung damage even when left at room air during the first 72 hours. Term and preterm guinea pigs exposed to hyperoxia respond with lung injury, but the damage was more pronounced in preterm guinea pig pups. This is thought to develop primarily as a result of oxygen free radical damage to the pulmonary endothelium which results in a protein rich exudate in the lung.

Initial studies used anti-rat SP-A (kindly provided by Dr. M. Post from Hospital for the Sick Children, Toronto, Canada) for SP-A identification in BAL sample from guinea pig. Although it reacts with the 36 kDa band of homogenized lung, the amount of antibody was not enough to perform the assay and a new antibody was raised in this laboratory. Using Western blotting, this anti-guinea pig SP-A antibody only reacted when the concentration was high (1:20) and a biotinylated anti-rabbit IgG/streptavidin reaction was used. This high titre of antibody may explain why there were a number of non specific bands in addition to the expected bands for SP-A.

PE-10, a monoclonal antibody against human SP-A, kindly supplied by Prof. T. Akino from Sapporo University in Japan, was used for comparison with results obtained with the antibody raised in this research. It was found

that PE-10 cross reacted against guinea pig SP-A when in a concentration of 1:200. Next step would include setting up an ELISA assay using either PE-10 or purified anti-guinea pig SP-A for SP-A concentration analysis.

Dot blotting was performed during this study. Results obtained using homogenized lung from fetus (day 55 of gestation), term (day 68) and adult guinea pig are in agreement with the pattern of correlation between SP-A production and gestational age of other mammalian species, in which SP-A is produced during late fetal development [Katyal and Sing, 1979, 1984, 1985], [Fisher *et al.*, 1988], [King *et al.*, 1975], [Ballard *et al.*, 1986], [Gikas *et al.*, 1977]. However, much research has to be carried out to establish the complete correlation for guinea pig fetus.

6.4 PAF study

Another study was related to the PAF role in neonatal RDS or CLD. PAF is synthetized in inflammatory cells such as neutrophils and macrophages. It had been shown that formation of hyaline membranes is correlated to migration of neutrophils and macrophages to the lungs and release of chemotaxis factors. Archers *et al.* [1985] suggested that PAF has many roles in acute and chronic inflammation and Meredith *et al.* [1989] demonstrated an increase in PAF activity in BAL from ventilated preterm baboon.

The use of a PAF antagonist drug (WEB 2086) was analysed to identify the role of PAF on preterm lung inflammation due to hyperoxia exposure. Application of PAF antagonist produced a decrease in PC concentration and in total protein in pups exposed to hyperoxia. This can be associated with reduction of the inflammatory process.

Chapter Seven
References

Bibliography

[1] Abbas, A.K., Lichtman, A.H., Porber, J.S. - In "Cellular and molecular immunology". W.B. Saunders Company, Philadelphia, 1991.

[2] Adams,F.H. and Enhorning,G. - Acta Physiol. Scand., V 68, pp 23:27, 1966.

[3] Alenghat, E., Esterly, J.R. *Pediatrics*, 74, 221-223, 1984.

[4] Allen, R.G., and Balin, A.k. *Free Rad. Biol. Med.*, 6, 631-661, 1989.

[5] Archer, C.B., Page, C.P., Morley, J., MacDonald,D.M. - Br J Dermatology, 110, pp 112-285, 1985.

[6] Archer, C.B., Page, C.P., Paul, W., Morley, J., MacDonald, D.M. - Dermatology, 110, pp 45-50, 1984.

[7] Artiss,J.D.;*et al. Microchem. J.*, 25, 153-168, 1979.

[8] Avery,M.E. and Mead,J. - *Am. J. Dis. Child.*, 97, 517:523, 1959.

[9] Avery,M.E., Fletcher,B.D. and Williams,R.G., - The lung and its disorders in the newborn infant, W.B.Saunders Company, Philadelphia, 1981.

[10] Ballard, P.L., Hawgood, S., Liley, H., Wellenstein, G., Gonzales, L.N., Benson, B., Codell, B., Tyler White, R. *Proc. Natl. Acad. Sci.*(USA) 83, 9527-9531, 1988.

[11] Bancalari, E. - Pathogenesis of bronchopulmonary dysplasia: an overview, 'Bronchopulmonary Displasia', Ed by E. Bancalari and J.T.Stocker, Hemisphere Publishing Corporation, Washington, 1988.

[12] Bancalari,E. and Gerhardt,T. *Pediatric Clinics of North America*, 33, 1-23, 1986.

[13] Bancalari, E., Jesse, M.J., Gelband, H. *et al.* - *J. Pediatr.*, 93, 192, 1977.

[14] Bartlett, G.R. *J. Biol. Chem.*, 234, 466-468, 1959.

[15] Beck, K.C., Lai-Fook, S.J. *J. Appl. Physiol.* , 55, 1277-1283, 1983.

[16] Benveniste, J., Henson, P.M., and Cochrane, C.G. *J. Exp. Med.*, 136, 1356-1377, 1972.

[17] Blackburn, W.R. - Hormonal influences in fetal lung development, Respiratory Distress Syndrome, Edited by C.A. Villee, D.B. Villee and J. Zuckerman, 271-293, Academic Press, New York, 1973.

[18] Bligh, E., Dyer, W. *Canad. J. Biochem. Physiol.* , 37, 911-923, 1959.

[19] Block,E.R., Fisher, A.B. *J. Appl Physiol.*, 42, 33-38, 1977.

[20] Borderon, J.C., Gold, F., Laugier, J. *Biol. Neonate* 39, 1-7, 1981.

[21] Boyden, E.A. *Anat. Rec.*, 186, 15-38, 1976.

[22] Boyton, B., Mannino, F., Davis, R., *et al J. Pediatr.*, 105, 297-302, 1984.

[23] Brown, E.R., Stark, A., Sosenko, I. *et al. J. Pediatr.*, 92, 982, 1978.

[24] Buckingham, S. *Am. J. Dis. Child.*, 102, 521, 1961.

[25] Butler, W.J., Bohn, D.J., Bryan, A.C., *et al Anesth. Analg.*, 29, 577-584,1980.

[26] Caesar, P.A., Wilson, S.J., Normand, I.C.S., and Postle, A.D. *Biochem. J.* 253, 451-457, 1988.

[27] Campbel, A.G.M. *Clin. in Endocrinol. and Metabol.*, 5, n. 1, 55-87, 1976.

[28] Chance, B., Sies, H., Boveris, A. *Physiol. Rev.*, 3, 527-605, 1979.

[29] Chirico, G., Marconi, M., DeAmici, M., Gasparoni, A., Mingrat, G., Chiara, A., Rondini, G., Wgazio, A.G. *Biol. Neonate*, 47, 125-129, 1985.

[30] Clements,J.A. *Arch. Environ. Health*, 2, 280-283, 1961.

[31] Clements, J.A. *Physiologist*, 5, 11-28, 1962.

[32] Clements, J.A. *N. Eng. J. Med.*, 286, 1077-1081, 1972.

[33] Clements,J.A. *Am. Rev. Respir. Dis.*, 115, 67-71, 1977.

[34] Clements,J.A. and Tierney,D.F. - Alveolar instability associated with altered surface tension, Fenn WO, Rahn H., Eds. Handbook of physiology, Section 3: Respiration, Vol II, Washington DC, Am. Physiol. Society, 1565-1583, 1965.

[35] Coalson, J.J., Kuehl, T.J., Prihoda, T.J. and DeLemos, R.A. *Pediat. Res.*, 24, 357-366, 1988.

[36] Cockburn,F. and Drillieu,C.M. - In: Neonatal Medicine, Blackwell Scientific Publication, Oxford, 1974.

[37] Crone, R.K., and Favorito, J. *J. Pediatr.*, 97, 991-993, 1980.

[38] Cross, C.E., Hasegawa, G., Reddy, K.A., Omaye, S.T. *Res. Commun. Chem. Pathol. Pharmacol.* , 16, 695-706, 1977.

[39] Davis, W.B., Rennard, S.I., Bitterman, P.b., *et al. N. Engl. J. Med.*, 309, 878-883, 1983.

[40] DeLemos, R.A., Coalson, J.J., Gertsman, D.R., Kuehl, T.J. and Null, D.M. - *Am. Rev. Respir. Dis.*, 136, 677-682, 1987.

[41] Deneke, S.M., Gershoff, S.N., Fanburg, B.L. *J. Appl. Physiol.* , 54, 147-151, 1983.

[42] Deneke, S.M., Lynch, B.A., Fanburg, B.L. *J. Appl. Physiol.* , 58, 571-574, 1985.

[43] Edwards, D.K., Dyer, W.M. and Northway, W.H. Jr. *Pediatrics*, 59, 839, 1977.

[44] Enhöning, G. *J. Appl. Physiol.*, 43, 198-203, 1977.

[45] Enhörning, G. and Kirschbaum, T.H. - *Am. J. Obst. Gynec.*, 90, 537-545, 1964.

[46] Enhörning,G., Shennan, A., Possmayer, F., Dunn, M., Chen, C.P., Milligan *J., Pediatrics*, 76, 145-153, 1985.

[47] Escobedo, M.B., Hillard, J.L., Smith, F. *et al. Exp. Mol. Pathol*,37, 323-334, 1982.

[48] Evans, T.W., Dent, G., Rogers, D.F., Aursudkij, B., Chung, K.F. and Barners, P.J., *Br. J. Pharmacol.*, 94, 164, 1988.

[49] Farrel, P.M. Lung Development: Biological and Clinical Perspectives. Vol.I, Academic Press, New York, 13-25, 1982.

[50] Feurstein, G. and Hallenbeck, J.M. *Annual Review of Pharmacology and Toxicology*, 27, 301-313, 1987.

[51] Finer, N.N., and Tomney, P.M. *Pediatrics*, 67, 641-646, 1981.

[52] Fisher, H.K., Hyman, M.H. and Ashcraft, S.J. *Fed. Proc.*, 38, 1373a, 1979.

[53] Fisher, J.H., Emrie, P.A., Shannon, J., Sano, K., Hattler, B. and Mason, R.J. *Biochim. Biophys. Acta*, 950, 338-345, 1988.

[54] Fisk, C.H., Subbarrow, Y. *J.Biol. Chem.*, 234, 375-400, 1925.

[55] Frank, L., and Sosenko, I.R.S. *J. Pediatr.*, 110, 106-110, 1987.

[56] Frantz, I.D., Werhammer, J.,and Stark, A. *Pediatrics*, 71, 483-488, 1983.

[57] Fujiwara. T., Maeta, H., Chida, S., Morita, T., Watabe, Y. and Abe, T. *Lancet* 1, 55-59, 1980.

[58] Gikas, E.G., King, R.J., Mescher, E.J., Platzker, A.C.G., Kitterman, J.A., Ballard, P.L., Benson, B.J., Tooley, W.H. and Clements, J.A. *Am.Rev. Resp. Dis.* , 115, 587-593, 1977.

[59] Gil, J., and Reiss, O.K. *J. Cell. Biol.*, 58, 152, 171, 1973.

[60] Gitlin,J.D. Soll, R.F., Parad, R.B.,*et al. Pediatrics*, 79, 31-37, 1986.

[61] Gluck,L., Kulovich,M.V., Borer,R.C., Brenner,P.H., Anderson,G.G. and Spellacy,W.N. - *Am. J. Obstet. Gynecol.*, 109, 440-445, 1971.

[62] Goerke,J. *Biochim. Biophys. Acta*, 344, 241-261, 1974.

[63] Goldman, D.A., Leclair, J., Macone, A. *J Pediatr* , 93, 288-293, 1978.

[64] Goldman, S.L., Gerhardt, T., Sonni, R., *et al J. Pediatr.*, 102, 613-, 1983.

[65] Greenough, A., Morley, C.J., Wood, S., *et al Lancet*, 1-3, 1984.

[66] Gregory, G.A., Kitterman, J.A., and Phibbs, R.H. *N. Engl. J. Med.*, 284, 1333-1340, 1971.

[67] Guyton,A.C., Moffat,D.S., Adair,T.H. - Role of alveolar surface tension in transepithelial movement of fluid, Pulmonary surfactant, Edited by B. Robertson, L.M.G. Van Golde and J.J. Batenburg, 171-185, Elsevier Amsterdam, 1984.

[68] Haasgman,H.P., Van Golde, L.M.G. *Lung*, 163, 275-303, 1985.

[69] Hallman, M. - In "Pulmonary Surfactant" R.Robertson, L.M.G. Van Golde, and J.J.Batenburg (Eds), Elsevier Science Publishers, Amsterdam, 419-448, 1984.

[70] Hallman, M., Feldman, B.H., Kirkpatrick, E. and Gluck, L. *Pediatr. Res.*, 11, 714-720, 1977.

[71] Hallman, M., Kulovich, M., Kirkpatrick, E., Sugarman, R.G. *Am. J. Obstet. Gynecol.*, 125, 613-617, 1976.

[72] Hallman, M. and Gluck, L. - *Pediatr. Res.*, 14, 1250-1259, 1980.

[73] Hallman, M.,Merrit, T.A., Jarvenpaa, A.L., Boynton,B., Mannino, F., Gluck,L., Moore,T. , Edwards, D. *J. Pediatr.* 106, 963-969, 1985.

[74] Hamasaki, Y., Mojarad, M., Saga, T., Tai, H.H., Said, S.I. *Am. Res. Resp. Dis* , 129, 742-746, 1984.

[75] Handley, D.A., Van Valen, R.G., Melden, M.K., and Saunders, R.N. - *Thromb. Haemostasis*, 52, 34-36, 1984.

[76] Hawco,M.W., Davis,P.J. and Keough,K.M.W. - *J. Appl. Physiol.*, 51, 509-515, 1981.

[77] Hawang, S.B., Lan, M.H., and Shen, T.Y. *Biochem. Biophys. Res. Commun.*, 138, 972-979, 1985.

[78] Hawgood,S., Benson,B.J. and Hamilton Jr., R.L. - *Biochemistry*, 24, 184-190, 1985.

[79] Hawgood, S., Benson, B.J., Schilling, J., Damm, D., Clements, J.A., and White, R.T. *Biochemistry*, 84, 66-70, 1987.

[80] Heaf,D.P., Belik, J., Spitzer, A.R., *et al J. Pediatr.* 101, 103-107, 1982.

[81] Henson,P.H., Barnes,P.J. and Banks-Schlegel,S.P. - *Am. Rev. Respir. Dis.*, 145, 726-731, 1992.

[82] Herman, S., Reynolds, E.O.R. *Arch. Dis. Child.*, 48, 612-617, 1973.

[83] Hever, H.O. - In "Handbook of PAF and PAF antagonists", Ed by Pierre Braquet, CRC Press, Boca Raton, Florida, 171-202,

[84] Hills, B.A. - The Biology of Surfactant, Cambridge University Press, Cambridge, 1988.

[85] Hislop, A.A., Wigglesworth, J.S., Desai, R. *Early Hum Dev*, 13, 1-11, 1986.

[86] Hunt, A.N., Kelly, F.J., and Postle, A.D. *Early Hum. Dev.*, 25, 157-171, 1991.

[87] Hunt, A.N., Normand, I.C.S., Postle, A.D. *Biochim. Biophysica Acta*, 1043, 19-26, 1990.

[88] Ikegami, M., Jacobs, H., Jobe, A. *J. Pediatr.*, 102, 443-447, 1983.

[89] Jobe, A. *Pediatr. Res.*, 14, 319-325, 1980.

[90] Jobe, A. and Ikegami, M. *Am. Rev. Respir. Dis.*, 136, 1256-1275, 1987.

[91] Jobe, A., Ikegami,M., Jacobs, H., Jones, S., Conaway, D. - J Appl Physiol, 55, 169-176, 1983.

[92] Jobe, A.H., Ikegami, M., Seidner, S.R., Pettenazzo, A. and Ruffini, L. - *Am Rev Respir Dis*, 139, 352-359, 1989.

[93] Katyal, S.L., Estes, L.W. and Lombardi, B. - *Lab. Invest.*, 36, 585-592, 1977.

[94] Kaytal, S.L., Singh, G. *Pediatr. Res.*, 17, 439-443, 1985.

[95] Kaytal, S.L., Singh, G. *Exp. Lung Res.*, 6, 175-189, 1984.

[96] Kaytal, S.L., Singh,G. *Lab. Invest.*, 40, 562-567, 1979.

[97] Kelly, F.J., Town, G.I., Phillips, G.J., Holgate, S.T., Roche, W.R. and Postle, A.D. - *Clinical Science*, 81, 439-446, 1991.

[98] Kelly, F.J., Gickett, G.M.W., Hunt, A., Town, G.I., Holgate, S.T. and Postle, A.D. - *Int. J. Biochem*, 23, 467-471, 1991.

[99] Kikkawa, Y., Motoyama, E.K. and Gluck, L. - *Amer. J. Path.*, 52, 177-192, 1968.

[100] Kikkawa, Y., Kaibara, M., Motoyama, E.K., Orsalesi, M.M. and Cook, C.D. - *Amer. J. Path.*, 64, 423-442, 1971.

[101] King, R.J. and Clements,J.A. - *Am. J. Physiol.*, 223, 715-726, 1972.

[102] King, R.J., Klass, D.J., Gikas, E.G. and Clements, J.A. - *Am. J. Physiol.*, 224, 788-795, 1973.

[103] King, R.J., Ruch, J., Gikas, E.G., Paltsker, A.C.G., and Creasy, R.K. - *J. Appl. Physiol.*, 39, 735-741, 1975.

[104] Kogishi, K., Kurozimi, M., Fujita, Y., Murayama, T., Kuze, F. and Suzuki, Y. *Am. Rev. Respir. Dis.*, 137, 1426-1431, 1988.

[105] Kotas, R.V., Farrell, P.M., Ulane, R.E. and Chez, R.A. - *J. Appl. Physiol.*, 43, 93-98, 1977.

[106] Kwong,M.S., Egan,E.A., Notter, R.H., Shapiro, D.L. *Pediatrics*, 76,585-592, 1985.

[107] Laemmli, U.K. *Nature*, 227, 680-685, 1970. bibitem Lindroth, M. - *Indian pediatrics*, 25, 654-665, 1988.

[108] Lowy,O.H. *et al. J. Biol. Chem.*, 193, 265, 1951.

[109] MacCarthy, K. , Bhogal, M., Nardi, M., Hart,D. *Pediatr Res*, 18, 483-488, 1984.

[110] MacElroy, M.C. *PhD Thesis*, University of Southampton, 1991.

[111] Maniscaldo, W.M., Wilson, C.M., Gross, I., Gorbran, L., Rooney, S.A., and Warshaw, J.B. *Biochim. Biophys. Acta* , 530, 333-346, 1978.

[112] Marchak,B.E., Thompson, W.D., Duffty, P., *et al. J. Pediatr.*, 99, 287-292, 1981.

[113] Mazonski, T., *et al. Zezyty,Nauk. Politec. Slask. Chem.*, 13, 63, 1963.

[114] Merrit,T.A. *Pediatr. Res.*, 16, 798-805, 1982.

[115] Merrit, T.A., Cochrane, C.G., Holcomb, K., *et al. J. Clin. Invest.*, 72, 656-666, 1983.

[116] Merritt, T.A., Hallman,M., Bloom, B.T.,*et al. N. Engl. J. Med.*, 315, 785-790, 1986.

[117] Merrit, T.A., Stuart, I.D., Puccia, J., *et al. J. Pediatr.*, 98, 949-956, 1981.

[118] Metcalfe, I.L., Pototschnick, R., Bourgoyne, R. and Enhörning,G. J. Appl. Physiol., 53, 838-843, 1983.

[119] Monin, P., Cashore, W.J., Hakanson, D.O. *et al. Pediatr. Res.*, 10, 464, 1976.

[120] Morgan, T.E., Finley, T.N. and Fialkow, H. *Biochim. Biophys. Acta*, 106, 403-413, 1965.

[121] Naimark,A. *Fed. Proc.*, 32, 1967-1971, 1973.

[122] Nilsson, R., Grossman, G., Robertson, B. *Pediatr. Res.*, 12, 249-255, 1978.

[123] Northway, W.H. Jr., Rosan, R.C., Porter, D.Y. *N. Engl. J. Med.*, 276, 357, 1967.

[124] Notter, R.H. - Surface chemistry of pulmonary surfactant: the role of individual components. In: Pulmonary Surfactant, Ed by B. Robertson, L.M.G. Van Golde and J.J. Battenburg, 17-66, Elsevier, Amsterdam, 1984.

[125] Notter, R.H. Egan, E.A., Kwong, M.S., Holm, B.A., Shapiro, D.L. *Pediatr. Res.*, 19, 569-577, 1985.

[126] Notter, R.H., Finkelstein, J.N. *J. Appl. Physiol.*, 57, 1613-1624, 1984.

[127] Notter, R.H., Tabak, S.A., Holcombs, S., and Mavis, D. *J. Interface Sci.*, 74, 370-377, 1980.

[128] Notter, R.H., Taubold, R., and Mavis, R.D. *Exp. Lung Res.* 3, 109-127, 1980.

[129] O'Brodovich, H.M. and Mellins, R.B. *Am. Rev. Respir. Dis.*, 132, 694-709, 1985.

[130] O'Flaherty, J.T., Wykle, R.L., Miller, C.H., Lewis, J.C., Waite, M., Bass, D.A., McGall, C.E., and De Chatelet, L.R. *Am. J. Pathol.*, 103, 70-79, 1981.

[131] Ohno, K., Akino, T. and Fujiwara, T. Reviews in Perinatal Medicine, 2, 227-318, Edited by E.M. Scarpelli and E.V. Cosmi, Raven Press, New York, 1978.

[132] Paciga,J.E., Shelley,S.A. and Balis,J.U. *Biochim. Biophys. Acta*, 631, 487-494, 1980.

[133] Palmer, S., Morgan, T.E., Prueitt, J.L., Murphy, J.H. and Hudson, W.A. *Pediat. Res.*, 11, 1015-1021, 1977.

[134] Patten, G.M. ; *et al. J. Lipid Res.*, 23, 190-196, 1982.

[135] Pattle, R.E. and Thomas, L.C. *Nature(London)*, 189, 1961.

[136] Paul, G.W., Hassett, J.A., and Reiss, O.K. *Proc. Natl. Acad. Sci. USA* 74, 3617-3620, 1977.

[137] Person, A., Chang, D., Rust, K., Moxley, M., Longmore, W. and Crouch, E. *Biochemistry*, 28, 6361-6367, 1989.

[138] Pollitzer, M.J., Reynolds, E.O.R., Shaw, D.G., *et al Lancet*, 346-348,1981.

[139] Possmayer,F. - Biochemistry of pulmonary surfactant during fetal development and in the perinatal period, Pulmonary Surfactant, Edited by B. Robertson, L.M.G. Van Golde and J.J. Batenburg, Elsevier, Amsterdam, 1984.

[140] Postle,A.D. *J. Chromatogr.* 415, 241-251, 1987.

[141] Postle,A.D., Ceasar, P.A., Normand, I.C.S. *Hormonal effects on syntesis of phosphatidylcholine molecular species by organ cultures of human fetal lung.* In "Basic research on lung surfactant" Eds. Von Wichert, P.,and Muller, B., *Prog. Respir. Res.* Basal, Karger, 25, 313-317, 1990.

[142] Postle,A.D., Hunt,A.N. and Normand,I.C.S. *Biochim. Biophys. Acta*, 793, 297-299, 1984.

[143] Prueitt, J.L., Palmer, S., Standaert, T.A., Luchtel, D.L., Murphy, J.H. and Hodson, W.A. Lung Development in the fetal primate *Macaca nemestrina.* III. HMP, Pediat. Res., 13, 654-659, 1979.

[144] Reynolds, E.O.R., Taghizadeh, A. *Arch. Dis. Child.*, 49, 505, 1974.

[145] Repine, J.E., Pfenninger, O.W., Talmadge, D.W., Berger, E.M., Pettijohn, D.E. *Proc. Natl. Sci. USA*, 78, 1001-1003, 1981.

[146] Rickett, G.W.M. and Kelly, F.J. *Development*, 108, 331-336, 1990.

[147] Rooney, S.A. *Am. Rev. Respir. Dis.*, 131, 439-460, 1985.

[148] Rooney, S.A., Gobran, L.I., Marino, P.A., Maniscalco, W.M. and Gross,I. *Biochim. Biophys. Acta (Amst.)*, 572, 64-76, 1979.

[149] Rosenfeld, W., Conception, L., Evans, H., Jhaveri, R., Sahdev, S. Zabaleta, I. *Am. Rev Resp Dis*, 134, 1229-1239, 1986.

[150] Rotimi, V.O., Duerden, B.I. *J. Med. Microbiol.*, 14, 51-62, 1981.

[151] Roux, J.F., Nakamiwu, J. and Frosolow, M. *Am. J. Ostet. Gynecol.*, 119, 838, 1974.

[152] Sanders, R.L. - The chemical composition of the lung, In: Lung Development: Biological and Chemical Perspectives, Vol 1, Biochemistry and Physiology, Edited by P.M. Farrell, pp 179:192, Academic Press, New York, 1982.

[153] Sanders, R.L. - The composition of pulmonary surfactant. In: Lung Development: Biological and Clinical Perspectives, Vol 1. Biochemistry and Physiology, Edited by P.M. Farrell, pp 193:210, Academic Press, New York,, 1982.

[154] Scarpelli,E.M., Gabbay,K.H. and Kochen,J.A. *Science*, 148, 1607-1609, 1965.

[155] Schägger, H., Von Jargow, G. *Anal. Biochem.* , 166, 368-379, 1987.

[156] Shapiro, D.L., Notter,R.H., Morin, F.C., Deluga, K.S., Golub, L.M., Sinkin, R.A., Weiss, K.I., Cox, C. *Pediarics*, 76, 593-599, 1985.

[157] Show, J.O., Pinckard, R.N., Ferrigni, K.S., McManus, L.M., and Hanahan, D.J. *J. Immunol.*, 127, 1250-1255, 1981.

[158] Sinkin, R.A. and Shapiro, D.L. Surfactant Replacement Therapy, Bronchopulmonary Dysplasia, Ed. by E. Bancalari and J.T. Stocker, Hemisphere Publishing Corporation, Washington,1988.

[159] Slama, H., Schoedel, W., and Hansen, E. *Pluegers arch.*, 322, 355-363, 1971.

[160] Smith, J.W., Bloomfield, A.L. *J. Pediatr.*, 36, 51-60, 1950.

[161] Smith, P.K. *et al. Anal. Biochem.*, 150, 76, 1985.

[162] Sosenko, I.R.S. and Frank, L. *Am. J. Physiol.*, 252, R693-698, 1987.

[163] Stark, A.R., Bascom, R., and Frantz, I.D. *Pediatr.*, 94, 439-443, 1979.

[164] Strange, R.C., Cotton, W., Fryer, A.A., Drew, R., Bradwell, A.R., Marshall, T., Collins, M.F., Bell, J., and Hume, R. *Biochim. Biophy. Acta* 964, 260-265, 1988.

[165] Synder, F. - Platellet Activating Factor and Related Lipid Mediators. Plenum Press, New York,1987.

[166] Sprunt, K., Leidy, G., Redman, W. *Pediatr. Res.* , 12, 998-1002, 1978.

[167] Taghizadeh, A. and Reynolds, E.O.R. *Am. J. Pathol.*, 82, 242 , 1976.

[168] Takayama,M., Itoh,S., Nagasaki,T. and Tanimizu,I. *Clinica Chimica Acta*, 79, 93-98, 1977.

[169] Tappel, A.L. *Fed. Proc.*, 232, 1870-1874, 1973.

[170] Tappel,A.L. *Ann. NY Acad. Sci.*, 203, 12-28, 1982.

[171] Tenner, A.J., Robinson, S.L., Borchelt, J. and Wright, J.R. *J. Biol. Chem.*, 264, 13923-13928, 1989.

[172] Tikhonov, U.N., and Mustafin, I.S. *Zh. Anal. Khim.*, 20, 390, 1965.

[173] Tordet, C., Bertin, R., Gardey, C., Richard, M.O., Dameron, F. and Marin, L. *Pediat. Res.*, 15, 787-793, 1981.

[174] Touchstone,J.C. and Alvarez,J.G. *Journal of Chromatography*, 429, 359-371, 1988.

[175] Town, G.I. *Doctor in Medicine Thesis*, University of Southampton, 1990.

[176] Turcotte,J.G., Sacco,A.M., Steim,J.M., Tabak,S.A. and Notter,R.H. *Biochim. Biophys. Acta*, 488, 235-248, 1977.

[177] Van Golde, L.M.G., Batenburg, J.J. and Robertson,B. *Physiological Reviews*, Vol 68, No. 2, April 1988, USA.

[178] Venkitaraman, A.R., Hall, S.B., Whitsett, J.A., and Notter, R.H. *Chem. and Physics of Lipids*, 56, 185-194, 1990.

[179] Walker, S.R., Williams, M.C., Benson, B. *J. Histochem. Cytochem.*, 34, 1137-1148, 1986.

[180] Wardlow,A.J., and Kay, A.B. *J. Allergy Clin. Immunol.*, 77, 236(abstr.) 1986.

[181] Weaver, T.E. and Whitsett, J.A. *Biochem. J.*, 273, 249-264, 1991.

[182] Weibel, E.R.,In The pathway for oxygen. Structure and function in mammalian respiratory system. Cambridge: Harvard University Press, 1984.

[183] Weinhold, P.A., Quade, M.M, Brozowski, T.B. and Feldman, D.A. *Biochim. Biophys. Acta (Amst.)*, 617, 76-84, 1980.

[184] Weinhold, P.A., Feldman,D., Quade, M.M., Miller, J.C. and Brooks, R.L. *Biochim. Biophys. Acta*, 665, 134-144, 1981.

[185] Weitberg, A.B., Weitzman, S.A., Clark, E.P., Stossel, T.P. *J. Clin. Invest.*, 75, 1835-1841, 1985.

[186] White, C. W., In: Bronchopulmonary dysplasia, Edited by E. Bancalari and J.T. Stocker, Hemisphere Publishing Corporation, Washington, 1988.

[187] White, C.W., Repine, J.E. *Exp. Lung Res.*, 8, 81-96, 1985.

[188] Whitsett, J.A., Hull, W., Ross, G. and Weaver, T. *Pediatr. Res.*, 19, 501-508, 1985.

[189] Williams, M., Hawgood, S. and Hamilton, R.L. *Am. J. Respir. Cell Mol. Biol.*, 5, 41-50, 1991.

[190] Williams, M. *Fed. Proc.*, 36, 2653-2659, 1977.

[191] Wright, J.R. *Am. J. Physiol.*, 259, L1-L12, 1990.

[192] Wright,J.R. and Clements,J.A. *Am. Rev. Resp. Dis.*, 135, 426-444, 1987.

[193] Yasaka, T., Boxer, L.A., and Baehner, R.L. *J. Immunol.*, 128, 1939-1944, 1982.

[194] Yeh, T.F., Shibli, A., Leu, S.T., *et al J. Pediatr.*, 105, 603-609,1984.