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UNIVERSITY OF SOUTHAMPTON

THE INFLUENCE OF HYPEROXIA AND DEXAMETHASONE ON PULMONARY PROTEIN SYNTHESIS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

JULIA FUSSELL

SEPTEMBER 1990

This book is dedicated to my mother and father who, at all times, have supported me with magnificent love and enthusiasm

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MAIN ABBREVIATIONS USED IN THE TEXT

CAT	Catalase
CLD	Chronic Lung Disease
DPPC	Dipaltmitoyl Phosphatidylcholine
eIF-2	Eukaryotic Initiator Factor 2
GEF	Guanine Exchange Factor
GSH	Reduced Glutathione
GSH-Px	Glutathione Peroxidase
GR	Glutathione Reductase
GSSG	Oxidized Glutathione
HCI	Haem Controlled Inhibitor
hpO_2I	High Partial Pressure Activated Inhibitor
I(GSSG)	Oxidized Glutathione-induced Inhibitor
K_b	Fractional Rate of Protein Breakdown
K_g	Fractional Rate of Growth
K_s	Fractional Rate of Protein Synthesis
Met-RNA_f	Initiator Methionyl-tRNA
m^7GTP	7-Methylguanosine-5'-Triphosphate
PC	Phosphatidylcholine
POPC	Palmitoyl Oleoyl Phosphatidylcholine
RDS	Respiratory Distress Syndrome
SOD	Superoxide Dismutase

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

DEPARTMENT OF HUMAN NUTRITION

Doctor of Philosophy

The Influence of Hyperoxia and Dexamethasone on Pulmonary Protein Synthesis

by Julia Fussell

The structural and biochemical components of the human lung, which are vital for normal respiration, develop during the latter part of gestation. Before approximately 32 weeks gestation (full term is 40 weeks) the extent of pulmonary immaturity is such, that an infant born at this stage of development will succumb to life threatening disorders. The survival of preterm babies is dependent upon the provision of supplementary oxygen, while steroid therapy is employed pre- and postnatally to alleviate the acute and chronic conditions associated with prematurity. This thesis has utilized animal models to study the effects of hyperoxia and dexamethasone on lung growth, and thereby has assessed their potential in contributing towards the poor pulmonary growth experienced by infants with chronic respiratory problems.

Following a 24 hour exposure to ≥95% oxygen, the rate of protein synthesis and breakdown were depressed (12-15%) in the lung of neonatal guinea pigs. As a consequence, pulmonary growth was unaffected. Extending the period of hyperoxia to 72 hours had no greater inhibitory effect. Adolescent guinea pigs did not show any difference in lung protein turnover following hyperoxia. The overall reduction in ribosomal efficiency in the lungs of oxygen exposed animals was not defined to a specific translational step, however the unchanged polysome and monomer pools would suggest a block occurring at both peptide chain initiation and elongation. Further investigations were conflicting in their support that oxidized glutathione (GSSG) was involved in the inhibitory activity of hyperoxia on pulmonary protein synthesis. Although GSSG inhibited peptide chain initiation in a cell-free system derived from the mammalian lung, the concentration (0.2-0.5mM) required to produce such an effect could not be detected in the lung of animals following hyperoxia.

The potential benefits and risks associated with antenatal glucocorticoid therapy were investigated by assessing indices of biochemical maturation and growth in the lung of the fetal guinea pig. The administration of dexamethasone (2.0mg/Kg/day) from day 55 to day 60 of gestation had no effect on protein metabolism in the lung of the 61 day fetus. Furthermore, fetal pulmonary antioxidant and surfactant systems were unresponsive to maternal steroid treatment. Possible explanations regarding the insensitivity of the fetal guinea pig lung to the steroid regime employed are discussed.

The influence of postnatal glucocorticoid treatment on pulmonary protein turnover was examined in young rats. Within 24 hours, dexamethasone (2.5mg/Kg/day) completely inhibited lung growth. This rapid response was due to a 38% fall in the pulmonary protein synthesis which was associated with a reduced (44%) ribosomal efficiency. Extending dexamethasone treatment to 5 days resulted in decreases in both ribosomal efficiency (35%) and capacity (28%) explaining the greater inhibition (53%) of lung protein synthesis at this time. Following both the acute and chronic steroid regimes, the decreased rates of pulmonary protein synthesis were accompanied by a loss of polysomes and an elevated ribosomal monomer pool, indicating that dexamethasone blocked translation at the site of peptide chain initiation.

CHAPTER 1. INTRODUCTION

1. Lung Growth and Development

An infant who is born prematurely before approximately 32 weeks of gestation (full-term is 40 weeks) will experience respiratory problems as a consequence of pulmonary immaturity. While supplementary oxygen and mechanical ventilation are provided to maintain very premature babies, pre- and postnatal steroid administration are employed as possible preventive and therapeutic agents respectively. One of the numerous problems experienced by premature infants who undergo prolonged periods of intensive neonatal care is poor whole body growth. Moreover, indirect evidence suggests suboptimal pulmonary growth and development in these individuals. The focus of this thesis is an investigation into the possible role that hyperoxia and the synthetic corticosteroid dexamethasone have in compromising growth of the immature lung. Before discussing the problems that premature infants develop as a consequence of pulmonary immaturity and the provision of intensive neonatal care, it is essential to understand the series of events which constitute normal lung development in a healthy individual. The growth and development of the human lung should not be sharply divided into fetal and postnatal stages, but considered as a continuum of events which extends throughout gestation, infancy and early childhood (Thurlbeck, 1975; Langston *et al.*, 1984).

1.1 Growth and Morphological Development of the Lung

The human lung develops during the fourth week of gestation from a laryngotracheal groove in the endodermal tube which evaginates to form the lung bud. The initial endodermal structure undergoes a series of branching to form the major bronchi (Charnock & Doershuk, 1973). This early phase of lung development is termed the embryonic period and is completed by the sixth week of gestation. During the pseudoglandular phase which extends from the 6th to the 16th week, the bronchi continue to divide; 65 to 75% of bronchial branching occurs between

the 10th and 14th week, and by the 16th week, the conducting airways are fully formed (Bucher & Reid, 1961). At this stage, the airways of the lung are lined with low columnar epithelial cells. The pseudoglandular phase is succeeded by the canalicular phase which lasts from the 16th to the 28th week, and is characterized by airway dilation and the thinning out of the epithelium into a simple cuboidal form. At the same time, capillaries become intimately associated with the epithelium giving rise to thin blood-air barriers and elastin appears within the airways. During the saccular phase of pulmonary development, which extends from the 28th to the 36th week, a dramatic change in the appearance of the lung is seen. This is manifested by a decrease in interstitium thickness, further thinning of the epithelium and the appearance of secondary crests which divide the distal air-space structures, the saccules, into smaller units. Blood vessels are now well orientated to the epithelium and protrude to the air spaces, forming many thin air-blood interfaces. Although the acquisition of alveoli has been reported as early as the 29-30th gestational week (Langston *et al.*, 1984; Hislop *et al.*, 1986), the alveolar phase of lung development is classically defined as commencing on the 36th week of gestation (Langston et al., 1984). The increased saccule complexity and the appearance of alveoli occurring in the last trimester is reflected in an abrupt increase in lung volume and alveolar surface area, at the expense of air-space wall thickness. It should be borne in mind that the four stages of intrauterine lung development described above are not distinct, but overlap, and will vary in onset among individuals.

At full-term, the alveolar surface area is approximately 3.0 to 5.0m^2 (Langston *et al.*, 1984; Hislop *et al.*, 1986). Considerable variation exists however in the number of alveoli observed at birth both between groups and within individual studies. Langston *et al.* (1984) documented a mean value of $53x10^6$ within a range of 9.6 to $148x10^6$, while Hislop *et al.* (1986) reported an average of $150x10^6$ within a range of 110 to $174x10^6$. This wide range probably reflects individual variability in the onset of alveolar formation and rate of alveolar acquisition, in addition to the technical difficulties in identifying alveoli at this stage.

The postnatal growth and development of the human lung has been examined by both morphometric and morphological studies. Morphometric analysis

characterizes two phases of lung growth and development. The first stage lasts from birth to 18 months and results in a disproportionate volume increase in the oxygen-transporting media, i.e. the air and blood at the expense of the parenchymal tissue components. During the second stage which extends into adulthood, there is a proportional growth of all lung compartments (Zeltner *et al.*, 1987). Morphologically, postnatal growth of the human lung involves extensive and rapid restructuring of the parenchyma due to bulk alveolar formation and the remodelling of septal morphology (Zeltner & Burri, 1987). There is considerable debate in the literature over the time at which alveolar multiplication ceases. Originally it was accepted that the process was complete by 8 years of age (Thurlbeck, 1975). Recent studies indicate however that the majority of alveolar formation occurs within the first postnatal year and is complete within 2 to 4 years of age (Thurlbeck, 1982). The total number of alveoli in the fully developed adult lung is believed to be approximately 375×10^6 (Angus & Thurlbeck, 1972). Septal remodelling extends from the first few months of life to 2 to 3 years of age. During this period the immature parenchymal septa, characterized by a double capillary network and a central sheet of connective tissue, is transformed, producing a single capillary network interwoven with connective tissue strands which stabilize the interalveolar wall.

The phases of human lung growth detailed above are essentially similar for small laboratory animals, but will vary in onset between species. Considerable inter-animal variation exists in the state of overall body development at birth. For example, newly born small laboratory animals are far more immature than the human infant. In parallel, the gas-exchanging region of the newborn rat is composed of large, thick-walled structures termed saccules rather than alveoli (Burri, 1974; Burri *et al.*, 1974). The subdivision of the saccules into smaller compartments and thinning of the developing alveolar wall does not begin until the 4th to 5th postnatal day. This corresponds to the saccular phase of intrauterine development in the human. Moreover, it is not clear whether alveoli continue to multiply throughout life of the rat, or whether multiplication is complete by 10 weeks of age (Thurlbeck, 1975). The formation of alveoli is also a postnatal process in the mouse (Amy *et al.*, 1977), rabbit (Kikkawa *et al.*, 1968) and hamster (Crocker *et al.*, 1970). Compared with other small laboratory animals, the guinea pig has a

long gestational period (68 days) and this is associated with a greater degree of physical and functional maturity at birth. Concomitant with this, alveolar formation takes place in utero, giving rise to advanced morphologic lung development in the newborn (Lechner & Banchero, 1982). This is more representative of human lung development. The close relationship cited above between overall maturity and maturity of the lung supports an original hypothesis by Engel (1953), that pulmonary development parallels general body development at birth.

1.2 Biochemical Development of the Lung

Accompanying the late structural changes in pulmonary development, biochemically, the lung develops in the latter part of gestation. The surfactant and antioxidant systems constitute important biochemical components of lung maturation. The adequate development of both systems at birth is critical to the survival of the newborn.

1.2.1 Pulmonary Surfactant System

Lung surfactant is a lipoprotein-rich material which lines the inner surface of the alveoli and the respiratory passages (Rooney, 1979). The surface tension lowering capacity of surfactant reduces the work of breathing and prevents lung collapse at the end of expiration. The type II alveolar epithelial cell is the site of surfactant production (Van Golde, 1976). The lamellar inclusion bodies of these cells are responsible for the storage of surfactant and promote its release into the alveoli (Shapiro *et al.*, 1978). Surfactant consists largely of a phospholipid mixture, of which phosphatidylcholine (PC) accounts for 80-90%. The major surface active component of surfactant is dipalmitoyl phosphatidylcholine (DPPC), while phosphatidylglycerol (PG) is the next most abundant phospholipid. The protein component of surfactant comprises only 20% by weight and can be divided into a 30 to 35 kDa glycoprotein group, surfactant protein A (SP-A) (Floros *et al.*, 1985), and a group of hydrophobic, low molecular weight proteins, surfactant protein B (SP-B) and surfactant protein C (SP-C) (Whitsett *et al.*, 1986). SP-A is thought to

enhance the rate of adsorption of phospholipids to the air-liquid interface and augment the stability of the surfactant lining layer (Wright & Clements, 1987). SP-B and SP-C are believed to have a significant role in the surface activity of surfactant (Whitsett *et al.*, 1986).

The late gestational development of surfactant has been documented in a number of species. The amount of PC in lung lavage obtained from the fetal rabbit (Rooney *et al.*, 1976), rat (Maniscalco *et al.*, 1978) and the rhesus monkey (Epstein *et al.*, 1976) increases significantly in the final 10% of gestation. In addition, increases in lung tissue surfactant have been reported during the final period of gestation in the fetal rabbit (Khosla *et al.*, 1983), rat (Sosenko *et al.*, 1986) and guinea pig (Kelly *et al.*, 1990). Other developmental studies have made further correlations between the increased amount of surfactant in the lung and the rate of PC synthesis. Late gestational increments have been reported in both the rate of choline incorporation into PC and in the activity of cholinephosphate cytidyl-transferase in lung slices (Epstein *et al.*, 1976; Rooney *et al.*, 1976; Rooney *et al.*, 1977; Maniscalco *et al.*, 1978; Rooney *et al.*, 1979). This enzyme catalyzes the conversion of choline phosphate into CDP-choline, the rate limiting reaction of PC synthesis. The gestational profile of pulmonary PC follows a more gradual course in the human fetus, total lung PC increases slowly from week 20 of gestation and peaks at term (Adams *et al.*, 1970). In parallel with the lipid component of surfactant, increases in the SP-A content of human amniotic fluid and rat lung tissue have been reported to occur late in gestation (Katyal & Singh, 1983; Katyal *et al.*, 1984)

1.2.2 Pulmonary Antioxidant System

The antioxidant system consists of a number of enzymatic and non-enzymatic defences which protect the cell against oxygen free radicals. An oxygen free radical is defined as any species, atom or molecule, which possesses one or more unpaired electrons. The high reactivity of these species (by virtue of their unpaired electron) makes them capable of widespread toxic activities. The types of damage caused by oxygen free radicals include lipid peroxidation and the oxidation

of proteins and nucleic acids (Halliwell & Gutteridge, 1984; Slater, 1984). As a consequence, these species can induce membrane damage, enzymatic inactivation and mutagenesis. Dioxygen itself is an oxygen free radical, having two unpaired electrons in its outer shell. Dioxygen does not however react readily with other molecules because the two unpaired electrons have parallel spins. This arrangement prohibits the insertion of a pair of electrons from another molecule because this would force two electrons with parallel spin to occupy the same orbit (Halliwell & Gutteridge, 1984).

Oxygen free radicals are generated in the cytoplasm, in organelles and in association with the plasma membrane as a result of normal metabolism. More than 95% of molecular oxygen produced at the end of the electron transport chain, undergoes a four electron reduction by mitochondrial cytochrome oxidase. The end product of this reaction is water.

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

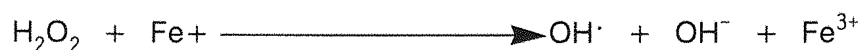
Single electrons may however "leak" at sites of transfer, permitting the inappropriate single electron reduction of oxygen to produce the superoxide ion ($O_2^{-\cdot}$).

$$O_2 + e^- \longrightarrow O_2^{-\cdot}$$

This species is very reactive, and by extracting an electron from another molecule, in the presence of hydrogen, can produce hydrogen peroxide.

$$O_2^{-\cdot} + e^- + 2H^+ \longrightarrow H_2O_2$$

Alternatively, hydrogen peroxide will be produced if two superoxide ions react together, or if molecular oxygen undergoes a two electron reduction. In the presence of transition metals ions, hydrogen peroxide reactivity is greatly increased, decomposing to form the hydroxyl radical ($OH^\cdot$) by the Fenton reaction.

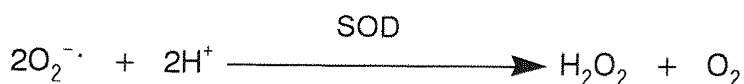


$$H_2O_2 + Fe+ \longrightarrow OH^\cdot + OH^- + Fe^{3+}$$

The latter can react rapidly with most biological molecules, hence has the capacity to cause widespread damage to cell constituents. It is the hydroxyl radical that has particular affinity for the polyunsaturated fatty acid side chain of membrane lipids. As a consequence, lipid peroxidation occurs, leading to membrane dysfunction and the generation of toxic lipid hydroperoxides.

Enzymatic antioxidant defences which have evolved to eliminate oxygen free radicals are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (Halliwell & Gutteridge, 1984; Slater, 1984).

SOD exists in two forms in eukaryotic cells, both of which are metalloenzymes. Cu/Zn-SOD is located primarily in the cytoplasm, while Mn-SOD is found in the mitochondria. SOD appears to be the first line of defence against oxidant damage, catalyzing the dismutation of the superoxide ion.



$$2O_2^{-\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Hydrogen peroxide is removed by either CAT or GSH-Px. CAT is located almost exclusively in the peroxisome and is thought to remove high concentrations or bursts of hydrogen peroxide.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

GSH-Px reacts on relatively low concentrations of hydrogen peroxide and other organic peroxides produced in the cytoplasm. Glutathione (GSH) acts as a reductant in this reaction (see section 7.2).

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

GSH-Px

$$2GSH + ROOH \longrightarrow GSSG + ROH + H_2O$$

Non-enzymatic antioxidant defences include alpha-tocopherol (vitamin E), ascorbic acid (vitamin C), niacin (vitamin B_6), riboflavin (vitamin B_2), protein

sulphydryl groups and polyunsaturated fatty acids.

The developmental profile of the pulmonary antioxidant enzyme system has only recently been explored by assessing the activity of these enzymes in fetal lung throughout gestation. Elevations in pulmonary SOD, CAT and GSH-Px have been reported during the final 10 to 15% of gestation in the rabbit (Frank & Groseclose, 1984), rat (Tanswell & Freeman, 1984; Gerdin *et al.*, 1985; Kelly & Rickett, 1987) and guinea pig (Sosenko & Frank, 1987b; Rickett & Kelly, 1990). In the human fetal lung, while SOD and GSH-Px activities do not change appreciably throughout gestation, an approximate three fold increase in pulmonary CAT activity occurs from fifteen weeks of gestation to term (McElroy *et al.*, 1990)

2. Acute Respiratory Problems of the Newborn

As a consequence of the continuation of pulmonary development late into gestation, infants born prematurely will possess lungs which are both morphologically and biochemically immature. Before approximately 32 weeks of gestation, the degree of pulmonary immaturity is such that a baby is unable to support respiration in the outside world. Such a condition manifests in the development of an acute respiratory disorder, respiratory distress syndrome (RDS), also referred to as hyaline membrane disease (Farrell, 1982). The characteristic features of respiratory distress, including tachypnea, hypoxia and progressive hypercarbia are generally present in the first few hours of life. This necessitates the early provision of assisted ventilation and oxygen supplementation in an attempt to open up the airways of the lung and ensure oxygenation of the body. Chest X-rays which are employed in the monitoring of RDS, reveal poorly inflated lungs with reticulogranular opacities. Pathologically, collapsed peripheral air-spaces and dilated respiratory bronchioles are present. Damage to the bronchial epithelium and exudation of plasma constituents leads to the formation of an eosinophilic material (hyaline membranes) which lines the terminal air-spaces. The principal factor responsible for initiating RDS was first recognised by Avery and Mead (1959), who observed that the lungs of infants succumbing to the disorder were deficient in pulmonary surfactant. More recent work has identified specific decreases in lung

PC in individuals suffering from RDS (Adams et al., 1970). It is now universally accepted that the inability of a premature lung to synthesize and/or secrete surfactant in amounts sufficient for neonatal respiratory adaptation, is the cause of acute lung disease. As a consequence, the severity of RDS is most strongly influenced by the degree of prematurity.

Despite considerable variation in the progress made by babies with RDS, a characteristic "clinical course" can been described. In addition to the early postnatal onset of respiratory distress, there is a tendency for pulmonary dysfunction to increase in severity for approximately the first 24 to 48 hours of life. During this period, ventilatory support and oxygen supplementation is maintained. Infants who experience uncomplicated RDS (generally those born after 32 weeks of gestation) undergo an improvement of lung function after 48 to 72 hours of life and require minimal or no respiratory support at one week of age (Farrell, 1982; O'Brodovich & Mellins, 1985).

3. Secondary Complications of Respiratory Distress Syndrome

The outcome of infants born before 32 weeks of gestation is less favourable. Before the technological developments in neonatal care, there was a high mortality rate amongst very premature infants with acute lung disease. Now individuals born as early as 24 weeks gestation can be maintained in highly advanced special care baby units. Unfortunately, the invasive nature of the intensive neonatal care, may lead to a number of secondary complications, including retinopathy of prematurity, intraventricular haemorrhage and chronic lung disease. These conditions at best result in a complicated clinical course, characterized by an incomplete or very slow recovery, and may be fatal.

3.1 Retinopathy of Prematurity

Retinopathy of prematurity is a disease of the retina experienced by premature infants exposed to high concentrations of oxygen (James & Lanman,

1976). The hyperoxic environment inhibits the development of the retinal blood vessels. When the oxygen treatment is reduced and ultimately stopped, the tissue becomes hypoxic and this serves as a stimulus for the development of additional blood vessels. The rapid outgrowth of the new blood vessels can penetrate the vitreous body and result in the detachment of the retina and ultimate blindness. Fortunately, more careful monitoring of oxygen therapy has led to a diminished incidence of retinopathy of prematurity in the U.K. (Farrell, 1982).

3.2 Intraventricular Haemorrhage

Intraventricular haemorrhage appears to occur in as many as half of the premature infants who develop RDS and is most common in babies who die within the first week of life (Pape & Wigglesworth, 1979; Farrell, 1982). Damage to the delicate vasculature of the brain, as a result of oxygen toxicity, produces bleeding from the thin walled capillaries in the matrix of the subendymal layer of the brain. Haemorrhage occurs if the bleeding becomes extensive and ruptures the ventricles. Reassuringly however, recent follow-up studies have suggested that except for severe haemorrhages, long-term consequences of this complaint are not often seen (Krishnamoorthy *et al.*, 1979).

3.3 Chronic Lung Disease

In the late 1960's it was observed that infants with more severe degrees of respiratory distress did not recover within the first week of life. Northway and colleagues (1967) first recognised this major respiratory problem and gave it the name of bronchopulmonary dysplasia (BPD). In this thesis, the currently accepted term of chronic lung disease (CLD) will be used in preference to bronchopulmonary dysplasia.

The incidence of CLD is markedly dependent upon birth weight and gestational age. A large number of retrospective studies have reported that between 2% to 68% of infants requiring assisted ventilation and oxygen therapy, develop

CLD (Harrod *et al.*, 1974; Mayes *et al.*, 1983). This variable incidence probably reflects different patient populations, changes in survival rates and differences in the clinical management of the premature infants with respiratory distress (Wung *et al.*, 1979; O'Brodovich & Mellins, 1985)

An infant with CLD is clinically defined as being oxygen dependent at 28 days of life and having a characteristic abnormal chest X-ray (O'Brodovich & Mellins, 1985). Pathological studies of CLD reveal an initial stage characterized by alveolar and airway damage, followed by a regenerative stage with repair (Taghizadeh & Reynolds, 1976). Characteristic features include oedema and fibroblastic proliferation in the vicinity of the bronchioles and saccular septa. Terminal airways and alveolar duct regions are overexpanded and are lined with persistent hyaline membranes. There is often hyperplasia and squamous metaplasia of the bronchial epithelium. At a later stage, the lung has an irregular appearance, with over-distended regions interspersed with areas of collapse. The oedematous areas around the bronchioles and in the interstitium become replaced by patchy fibrosis and bronchiole walls may show muscular hypertrophy (Taghizadeh & Reynolds, 1976). It should be borne in mind that these pathological abnormalities are based on autopsy data and therefore represent the most severe end of the CLD spectrum.

It is unlikely that a single mechanism is solely responsible for the emergence of CLD. The major causative factor is the immaturity of the lung, while the pathologic effects of the intensive neonatal therapy will complicate and intensify the condition. Inhalation of pure oxygen is toxic and ultimately lethal to animals, as a consequence of free radical generation. (Freeman & Crapo, 1982). Animal studies have extensively characterized the progressive lung changes that occur during prolonged exposure to hyperoxia (Clark & Lambertsen, 1971). The acute phase of oxygen toxicity is associated with interstitial, perivascular and intra-alveolar oedema, alveolar haemorrhage, extensive necrosis of the pulmonary endothelium and type I epithelial cells and the influx of inflammatory cells into the lung. The chronic or proliferative phase is characterized by hyperplasia of the interstitial cells and the alveolar type II epithelial cells. These changes, together with the deposition of collagen and elastin fibres within the interstitium, manifests in a thickened

membrane for gaseous exchange. This sequence of pathologic events is very similar to that observed in the human infant during acute and chronic respiratory illness. Oxygen, at high concentrations, would appear therefore to play an important role in the development of neonatal lung disease. Moreover, the endotracheal tube which administers ventilatory support can cause direct mechanical damage to the tracheal epithelium (Lee & O'Brodovich, 1988). As the degree of prematurity increases, the pulmonary surfactant and antioxidant enzyme systems of the infant will be more immature. As a consequence, mechanical ventilation and oxygen support will be required for a longer duration. This will lead to a greater likelihood for the patient to succumb to serious oxygen toxicity and barotrauma. There progressive development of CLD following a premature birth and the provision of respiratory support is diagrammatically summarized in Fig. 1.

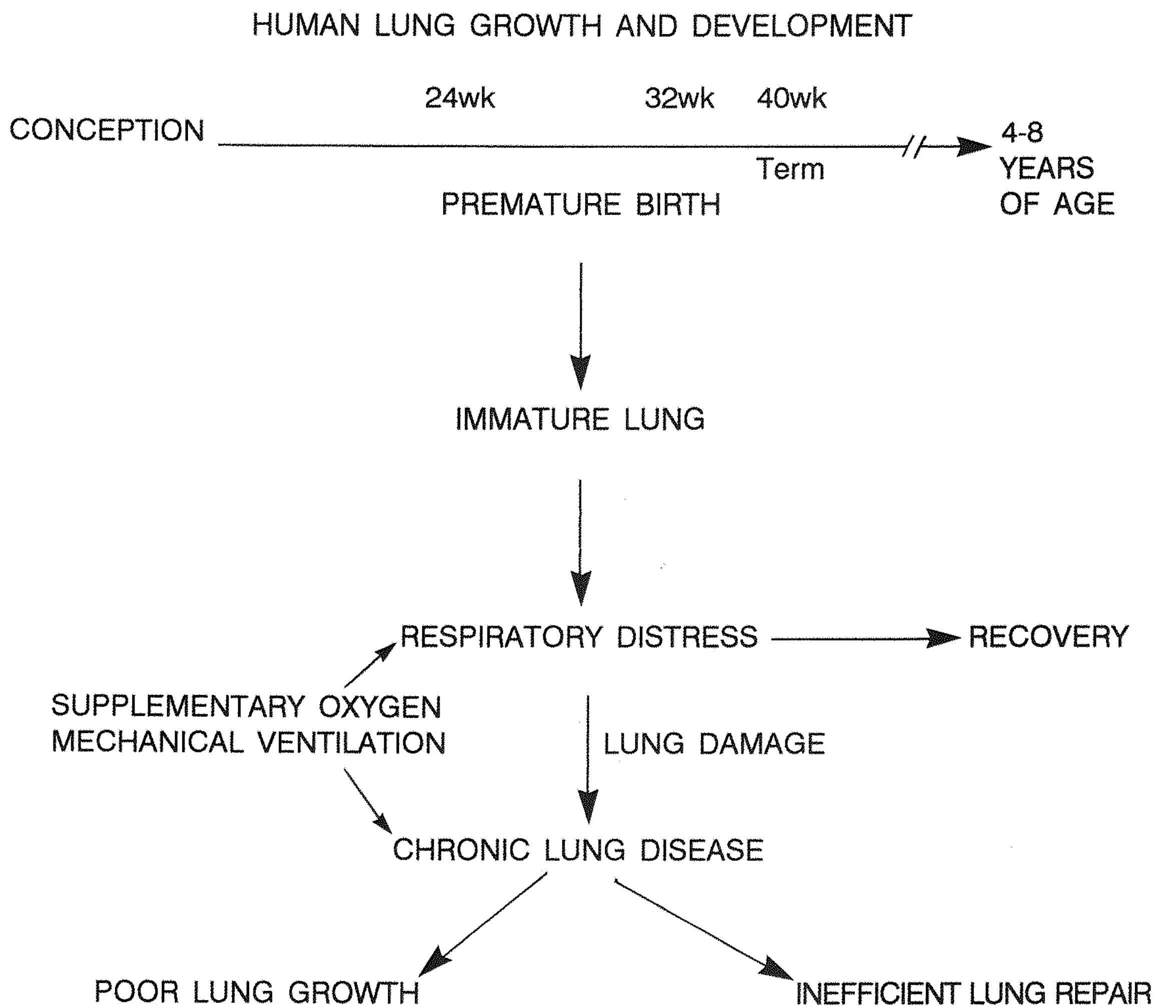


Figure 1. The Development of Acute and Chronic Lung Disease in the Preterm Infant

Premature infants born before approximately 26 weeks of gestation possess meagre caloric reserves and minimal stores of nutrients required for lung defence and repair (Walravens, 1980; American Academy of Pediatrics, Committee on Nutrition, 1985; Shenai *et al.*, 1985; Girard, 1985). The provision of adequate nutrition is fundamental to maintain the individual as well as to promote tissue growth and repair. Adequate lung growth together with a favourable balance between lung injury and repair, will dictate the occurrence of CLD and the outcome of those who succumb to the disease (Fig. 2). In view of the inherent difficulties in the nutritional management of very low birth weight infants, the role of under

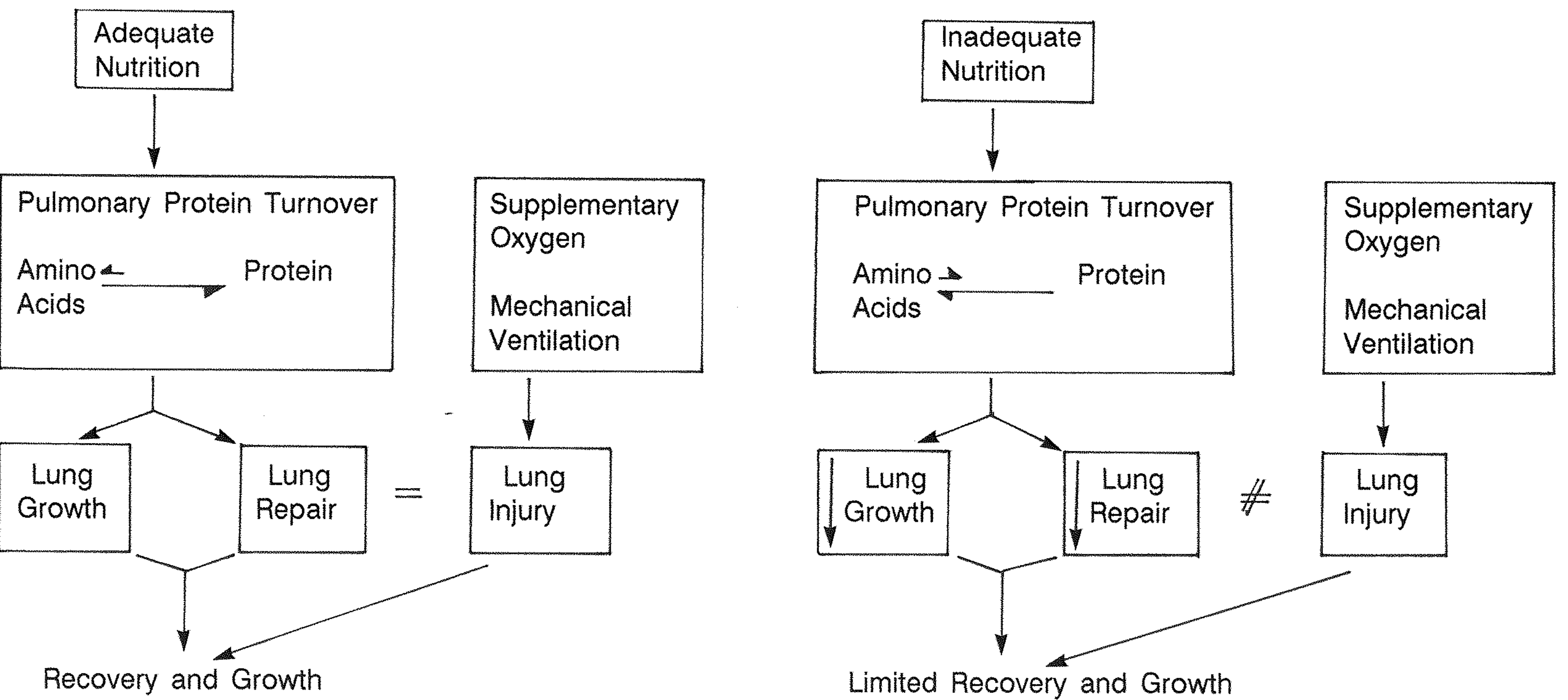


Figure 2. Nutrition as a Contributory Factor in the Outcome of Chronic Lung Disease

The meagre nutritional status of a premature infant necessitates an adequate nutrient supply to maintain optimal protein synthesis in the lung, hence the deposition of pulmonary protein. The net accumulation of protein in the immature and damaged lung constitutes tissue growth and repair, which in turn are essential for successful recovery from acute and chronic lung disease

nutrition in the development of CLD has been recognised (Edelman *et al.*, 1986; Frank & Sosenko, 1988).

The mortality rate amongst individuals with CLD is high (39 to 54%) during the initial hospitalization and falls to approximately 8 to 22% following hospital discharge (Edwards *et al.*, 1977; Northway, 1979; Szego *et al.*, 1980). Infants who survive this debilitating condition experience a slow recovery. The length of hospitalization and duration of supplementary oxygen therapy varies with the severity of the disease. In recent years it is not uncommon for infants to have remained in Southampton's Special Care Baby Unit for up to one year, at which time they are discharged while still requiring oxygen therapy. Furthermore, rehospitalisation of infants who experience CLD is not uncommon as a consequence of their apparent susceptibility to respiratory tract infections and the development of hyperactive airway disease (Markestad & Fitzhardinge, 1981; Smyth *et al.*, 1981; Vohr *et al.*, 1982; Bertrand *et al.*, 1985).

3.3.1 Chronic Lung Disease and Whole Body Growth

Despite the recognition that CLD is a serious postnatal respiratory complication, until recently, very little information was available regarding the developmental sequelae of this severe neonatal illness. As a result of a number of investigations into the progress of survivors of CLD, growth failure in these infants is now recognised as a major clinical problem.

Markestad and Fitzhardinge (1981) observed that the growth deficit (body weight and height below an average percentile for age) in children recovering from CLD was associated with prolonged respiratory dysfunction. Conversely, all infants exhibited a growth spurt after their respiratory status had improved. Following this initial investigation into the relationship between CLD and growth, the subject has attracted considerable research (Vohr *et al.*, 1982; Sauve & Singhal, 1985; Meisels *et al.*, 1986; Kurzner *et al.*, 1988). When investigating the failure of infants with CLD to thrive, Meisels *et al.* (1986) selected a comparison group of infants with RDS.

It became clear that infants with CLD were at a greater risk for growth retardation than those who recovered after RDS. Growth failure amongst infants with CLD is often seen in a sub-group of the population, relating to the severity of the condition (Markestad & Fitzhardinge, 1981; Kurzner *et al.*, 1988). Infants with a lower birth weight, lower gestational age and a greater number of days in supplementary oxygen and mechanical ventilation are those in which a growth retardation is most likely apparent.

It is questionable whether a failure of infants with CLD to thrive early on in life results in a permanent growth deficit. Vohr *et al.* (1982) reported that by 24 to 36 months of age, previously found differences between infants who had experienced CLD and those considered controls, had disappeared. In contrast, others have observed that catch up growth is not achieved at one and two years, again in a sub-group of infants with CLD (Yu *et al.*, 1983; Meisels *et al.*, 1986). We may conclude from these studies, that the severity of CLD dictates the extent to which infants experience poor growth in the early neonatal period and fail to achieve catch up growth within two years of life.

3.3.2 Influence of Acute and Chronic Lung Disease on Pulmonary Growth and Development

A major proportion of pulmonary growth occurs late in gestation and postnatally in humans (see Section 1.1). Optimal pulmonary growth and repair in premature babies who undergo respiratory support is essential if the lung is to develop normally and therefore function independently. While it is relatively straight forward to monitor whole body growth patterns in children who experience RDS and CLD, investigation into the growth and development of individual organs obviously presents a greater problem. Improvements in pulmonary function in subjects with lung disease is dependent upon adequate tissue growth. Lung function tests performed in survivors of RDS and CLD may therefore provide some information regarding pulmonary growth in these individuals.

It appears that uncomplicated RDS is not associated with abnormalities of

lung function in childhood. Pulmonary dysfunction was not detected in follow-up studies on children aged seven to ten years who were treated for respiratory distress at birth, when compared to subjects born at term (Stahlman *et al.*, 1982; MacLusky *et al.*,1986). Unfortunately an evaluation of lung function in infants with CLD and in survivors of the condition, suggests a less favourable outcome. Serial measurements of pulmonary function performed on infants with CLD during their first year of life have shown marked abnormalities and indicate poor growth of the airways (Bryan *et al.*, 1973; Tepper *et al.*, 1986). The long-term consequences of CLD has been investigated in children aged six to ten years who had experienced either CLD, RDS or a premature birth without an associated lung disease (Wheeler *et al.*, 1984). Persistent lung function abnormalities later in childhood were observed following a complicated neonatal course, while in agreement with others, pulmonary dysfunction was not found in children who had suffered from RDS alone and those associated with an uncomplicated premature delivery.

Further information regarding the status of lung growth following RDS and CLD has been obtained from anatomical studies. Detailed morphometric analysis of one patient who had suffered severe neonatal lung injury has been reported along with three control infants (Sobonya *et al.*, 1982). The patient was born prematurely at 30 weeks gestation, developed RDS and subsequently succumbed to CLD. Throughout infancy the child was persistently oxygen dependent and died at 33 months of age of pulmonary insufficiency and congestive heart disease. Total alveolar number was 19×10^6, only 10% of the value in control subjects and comparable to the number usually present at term. Alveolar surface area was $8.4m^2$ compared to 15.3 to $27.8m^2$ for the age-matched controls. Further studies have investigated the effect of a premature birth alone on pulmonary growth. The lungs of prematurely born infants who died at four to sixteen weeks of age without having received mechanical ventilation, showed a normal alveolar number and alveolar surface area. In contrast, infants treated with mechanical ventilation for RDS who died at one week up to fourteen months, exhibited a low alveolar number and surface area (Hislop *et al.*, 1987). We can conclude from this data that premature birth alone does not adversely affect lung development. Conditions necessitating mechanical ventilation may, however, lead to an impaired alveolar development. These morphometric analyses represent the severe end of the spectrum as all the

infants have died and therefore should be extrapolated to other patients with CLD with caution. The findings do, however, when reinforced with the pulmonary function studies, emphasise the lack of alveolar growth that may occur during severe neonatal lung disease. Of greater concern, functional abnormalities later in childhood, imply a long-term deficit in pulmonary growth following CLD.

3.3.3 Factor(s) Involved in the Growth Failure Experienced by Infants with Chronic Lung Disease

The mechanism responsible for the failure of infants with CLD to thrive is not understood. Several hypotheses have been proposed, including chronic hypoxia and the emotional disruption and deprivation associated with prolonged hospitalization (Vohr *et al.*, 1982; Yu *et al.*, 1983). In addition, a 25% higher energy expenditure has been recorded in infants with CLD probably as a consequence of the greater work of breathing associated with their respiratory distress (Yu *et al*, 1983; Kurzner *et al.*, 1988). This implies that more energy is required to maintain these individuals in the resting state, and in turn, less will be available for growth. Energy expenditure measurements in infants with CLD should, however, be treated with caution. Substantial errors in indirect respiratory calorimetry measurements are likely in situations when supplementary oxygen has been employed (Kalhan & Denne, 1990). In addition, the nutritional status of the patient during CLD will undoubtedly contribute to the deficit in whole body and tissue growth. An elevated expenditure, in addition to meagre nutritional reserves will necessitate the provision of an adequate caloric intake to promote growth. Unfortunately, successful nutritional support is often fraught with difficulties, owing to the immaturity of the gastrointestinal tract, leaving the infant undernourished during periods of chronic respiratory illness.

4. Effect of Hyperoxia on Lung Growth

Infants with CLD must be provided with increased oxygen to prevent hypoxic vasoconstriction of the pulmonary vascular bed and to decrease the work of

breathing. This will enable more calories to be available for growth. Ironically, at the same time, hyperoxic therapy administered for prolonged periods has the potential to contribute to a deficit in whole body and pulmonary growth. Exposure of laboratory animals to elevated inspired oxygen concentrations leads to a severe inhibition of normal lung development (Bartlett, 1970; Bucher & Roberts, 1981; Roberts *et al.*, 1983). The lower lung weight, lung volume, alveolar number and alveolar surface area reported in newborn rats following hyperoxia is due to a delay in secondary septal development i.e. the conversion of large saccular air-spaces into mature alveoli with a smaller diameter. This data strongly suggests that the lack of alveolar growth in an infant who died of CLD (Sobonya *et al.*, 1982) can be attributed to the lengthy periods of oxygen therapy required by the patient during the complicated neonatal course. Oxygen induced inhibition of lung growth will impede gaseous exchange, which in turn may explain the general somatic growth failure experienced by infants with CLD.

4.1 Mechanisms by which Hyperoxia may Inhibit Lung Growth

The powerful growth inhibitory effect of high concentrations of oxygen on non-pulmonary cultured cells has been recognised for some time (Reuckert & Mueller, 1960; Brosemer & Rutter, 1961; Balin *et al.*, 1976). Before considering the ways in which hyperoxia could inhibit growth, the latter should be defined. Growth is the accumulation of protein. Protein is continually synthesized and broken down, a process termed protein turnover. For growth to occur, the rate of protein synthesis must exceed that of protein degradation. Protein synthesis and its regulation are usually considered in two stages, transcription and translation. Transcription involves the gene directed synthesis of messenger RNA (mRNA) from DNA in the nucleus. After a variety of processing steps, the message moves into the cytoplasm where translation takes place on ribosomes. Translation gives rise to the completed polypeptide through the assembly of amino acids in a stepwise manner, according to the sequence of bases in the mRNA.

4.1.2 Effect of Hyperoxia on DNA Synthesis

The inhibitory effect of hyperoxia at the level of transcription has been demonstrated both *in vitro* and *in vivo*. Decreased lung growth in the newborn mouse exposed to 96 to 100% oxygen for 72 hours is accompanied by a reduction in DNA synthesis and cell replication. (Northway *et al.*, 1972,1976). This is supported by earlier work which demonstrated that DNA synthesis in HeLa cells is severely reduced in a hyperoxic environment (Reuckurt & Mueller, 1960).

Endothelial cells are particulary sensitive to the effects of high oxygen concentrations and have been employed to investigate the step at which hyperoxia may exert its inhibitory effect on DNA synthesis (Clément *et al.*, 1985). An effect at the membrane level would impair nucleoside transport into the cell. Alternatively, abnormalities in the metabolism of the nucleoside could occur, especially phosphorylation by kinases which produce the nucleotide. The final step, namely the incorporation of the nucleotide into DNA has also been considered a likely target of oxygen toxicity. The decreased incorporation of thymidine into DNA in cells exposed to 95% oxygen follows a time course comparable with that observed for the fall in thymidine kinase activity. It is therefore believed that the inhibitory effect of hyperoxia on DNA synthesis is primarily exerted at the step involving the phosphorylation of thymidine and the formation of thymidine 5' phosphate. In addition, an increased activity of DNA ß-polymerase, an enzyme involved in DNA repair, was found in the endothelial cells following oxidative stress. This supports the concept that through the generation of oxygen free radicals, hyperoxia oxidizes nucleotides and thereby damages DNA (Halliwell & Gutteridge, 1984, Slater, 1984).

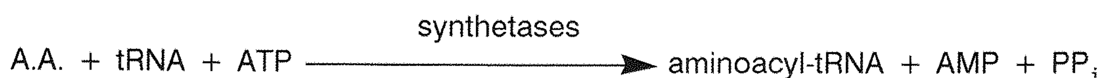
5. Protein Synthesis in Mammalian Cells

A short account of the mechanism and regulation of translation is necessary before presenting evidence which highlights the potential of hyperoxia to influence pulmonary protein synthesis.

Translation takes place in the cytoplasm of the cell and involves the interplay between many macromolecules including mRNA, transfer RNA (tRNA), ribosomes, amino acids, ATP, GTP and various enzymes. mRNA carries the genetic code from DNA specifying the order in which the amino acids are incorporated into proteins. Ribosomes form a matrix on which protein synthesis takes place and consist of ribosomal RNA (rRNA) and proteins. Eukaryotic ribosomes consist of two species, the 40 S and 60 S subunits, which are classified according to their sedimentation behaviour on sucrose density gradients. At most stages in protein synthesis, these subunits are associated into a monomeric (80 S) ribosome. tRNA carries the amino acid in an activated form to the ribosome for peptide bond formation, in a sequence determined by the mRNA template.

5.1 Reactions Involved in Protein Synthesis

Translation can be divided into three phases, initiation, elongation and termination. Initiation involves the binding of a ribosome to the initiator site of the mRNA, forming a complex which is primed for the synthesis of the first peptide bond. To enable a stretch of mRNA to be utilized efficiently, more than one ribosomal pair (40 S and 60 S) is recruited onto the message. A group of ribosomes bound to the message on which protein synthesis is occurring is termed a polysome. During elongation, the ribosome moves along the mRNA as it incorporates amino acids into the growing polypeptide chain. At termination, the newly synthesized peptide chain together with the ribosome are released from the message. The three steps of translation are not discrete, but together they constitute a cyclic series of events. In addition, each amino acid has first to become bound to a particular species of tRNA. This is an energy requiring process brought about by aminoacyl-tRNA synthetases:



$$\text{A.A.} + \text{tRNA} + \text{ATP} \xrightarrow{\text{synthetases}} \text{aminoacyl-tRNA} + \text{AMP} + \text{PP}_i$$

5.2 The Mechanism of Peptide Chain Initiation

Mammalian protein synthesis has been investigated widely with respect to nutritional and hormonal control. In many situations, the overall rate of translation is controlled at peptide chain initiation (Rannels *et al.*, 1978 a,b; Flaim *et al.*, 1982; Kelly & Jefferson, 1985).

Peptide chain initiation has been described in detail in a number of recent reviews (Ochoa, 1983; Moldave, 1985; Pain, 1986). The overall process shown in Fig. 3, is regulated by at least ten initiation factors and can be divided into 4 stages.

5.2.1 Formation of the Ternary Complex, [eIF-2.GTP.Met-tRNA$_f$]

The first step of peptide chain initiation is the formation of a ternary complex between eukaryotic initiation factor 2 (eIF-2), initiator methionyl-tRNA (Met-tRNA$_f$) and GTP. eIF-2 is a trimeric protein composed of 3 non-identical subunits, α, β, and γ with molecular weights of 32,000, 35,000 and 55,000 Da respectively (Lloyd *et al.*, 1980). A binary complex between eIF-2 and GTP is believed to take place first, followed by the addition of Met-tRNA$_f$ which binds to the β-subunit of eIF-2 (Nygård *et al.*, 1980).

5.2.2 Formation of the 43 S Preinitiation Complex

Stage two involves the binding of the ternary complex to a 43 S ribosomal complex to form the 43 S preinitiation complex. The formation of the 43 S ribosomal complex requires the dissociation of an 80 S ribosome into its constituent subunits. The 40 S subunit binds to the eukaryotic initiation factor eIF-3 and possibly eIF-4C. eIF-3 is a peptide composed of nine to eleven subunits with a total molecular weight of 700,000 Da (Benne & Hershey, 1976,1978; Benne *et al.*, 1978). Through binding to the 40 S subunit, eIF-3 acts as a dissociation factor to

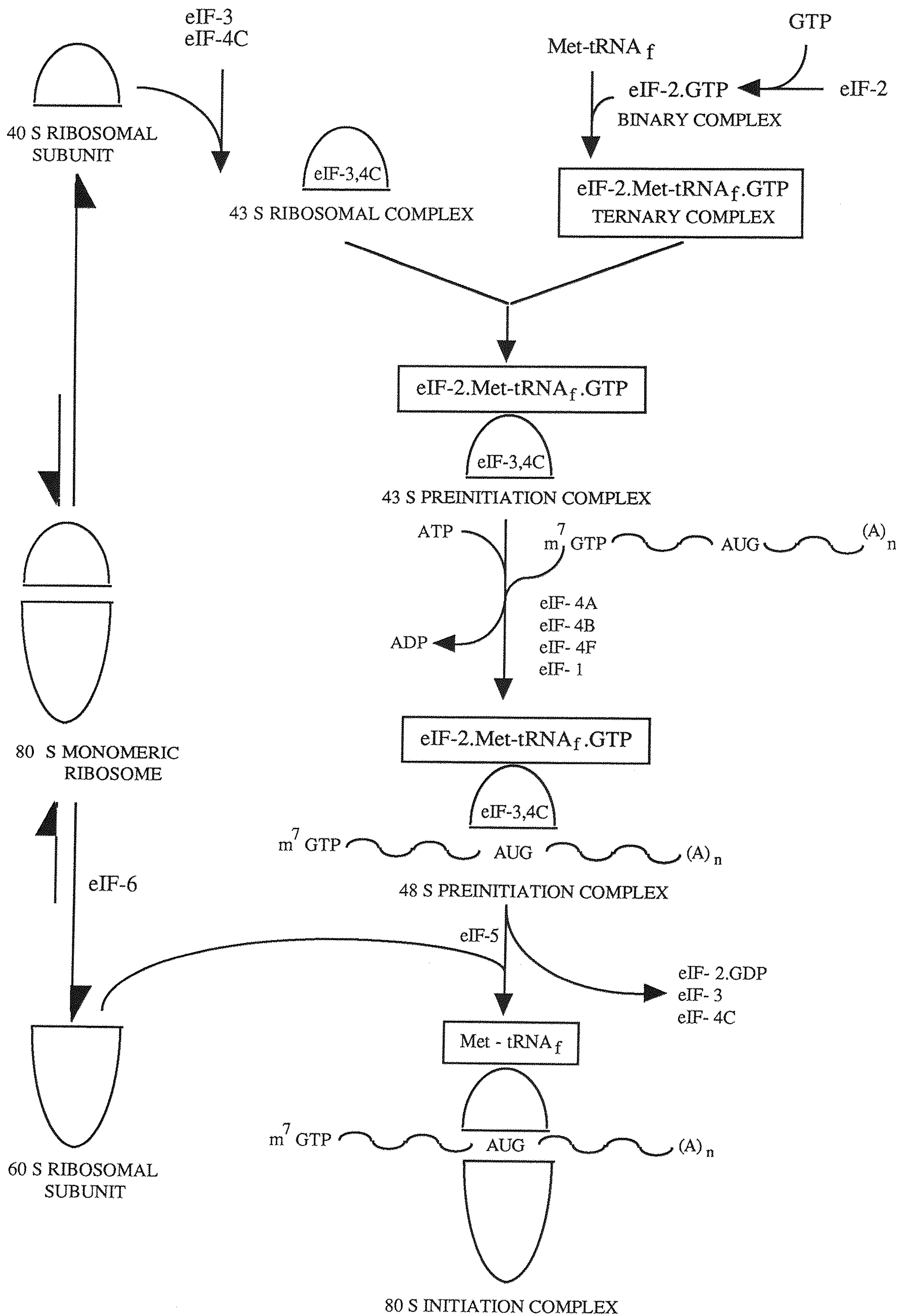


Figure 3. Initiation of Protein Synthesis in Eukaryotic Cells

prevent reassociation between the 40 S and 60 S subunit. eIF-4C, a single peptide with a molecular weight of 17,500 Da, prevents dimerisation of 40 S subunits as well as playing an accessory role to eIF-3 (Benne *et al.*, 1978; Goumans *et al.*, 1980). Another protein with ribosome dissociation properties is eukaryotic initiation factor 6 (eIF-6) (Raychaudhuri *et al.*, 1984). eIF-6 has a molecular weight of approximately 25,000 Da and is thought to achieve its effect through its binding to the 60 S ribosomal subunit.

5.2.3 Binding of the 43 S Preinitiation Complex to mRNA

The binding of mRNA to the 43 S preinitiation complex is an elaborate reaction, requiring the hydrolysis of ATP and the initiation factors designated eukaryotic initiation factor 4A (eIF-4A), eukaryotic initiation factor 4B (eIF-4B), eukaryotic initiation factor 4F (eIF-4F), eukaryotic initiation factor 1 (eIF-1) in addition to eIF-3 already associated with the 40 S ribosomal subunit. eIF-4F consists of at least three polypeptides of molecular weights 220,000, 46,000 and 24,000-26,000 Da (Grifo *et al.*, 1983). It is the 24,000 Da component of eIF-4F that recognises the cap structure, $M^7G(5')ppp(5')N$, at the 5'terminus of mRNA. eIF-4A and eIF-4B are both single polypeptides having molecular weights of around 45,000 and 80,000 Da respectively (Trachsel *et al.*, 1977; Grifo *et al.*, 1982). eIF-4A is believed to be intimately involved in the ATP-dependent unwinding of the mRNA structure, an activity which is probably augmented by eIF-4B. The unwinding of the mRNA allows the 43 preinitiation complex to bind. The complex then moves along the message until it reaches the AUG codon constituting the correct site for initiation to proceed. eIF-1, a polypeptide of molecular weight 15,000 Da (Benne *et al.*, 1978), is believed to stabilise Met-$tRNA_f$ binding to mRNA. This has been suggested to occur via an eIF-1 induced re-positioning of Met-$tRNA_f$ thereby facilitating the codon-anticodon interaction (Thomas *et al.*, 1980).

5.2.4 Binding of the 60 S Subunit to Form an 80 S Initiation Complex

The final stage of initiation involves the binding of the 60 S ribosomal

subunit to produce the 80 S initiation complex together with the release of the initiation factors eIF-2, eIF-3 and eIF-4C. Eukaryotic initiation factor 5 (eIF-5), a single polypeptide of 125,000, is responsible for GTP hydrolysis in this final step which is required for 60 S binding and for the release of the initiation factors (Trachsel *et al.*, 1977; Benne *et al.*, 1978; Peterson *et al.*, 1979a,b).

In view of the high affinity of eIF-2 for GDP, it is likely that this factor is released at the end of initiation as a binary complex with the GDP produced during hydrolysis (Raychaudhuri *et al.*, 1985). Before eIF-2 can participate in a new round of initiation, GDP must be replaced by GTP. This reaction is catalyzed by guanine exchange factor (GEF), a multipolypeptide initiation factor, which has major protein subunits of approximately 85,000, 67,000, 37,000 and 27,000 Da (Siekierka *et al.*, 1982; Panniers & Henshaw, 1983).

5.3 Regulation of Protein Synthesis

The overall rate of protein synthesis in the cell is regulated by two types of translational control. The first is the rate at which a given number of ribosomes are able to make protein, this is termed ribosomal activity or efficiency. Secondly, the ribosomal content of the cell will dictate the rate of protein synthesis and this is termed ribosomal capacity. Since approximately 80% of total cellular RNA is rRNA, the rate of protein synthesis per unit RNA and the RNA/protein ratio are the indices used to represent ribosomal efficiency and capacity respectively (Henshaw *et al.*, 1971; Millward *et al.*, 1973). Short term changes in the rate of protein synthesis are often initiated by an alteration in translational efficiency while changes in the capacity for protein synthesis subsequently follow (Millward et al., 1973; Pain *et al.*, 1983; Fussell & Kelly, 1990).

5.3.1 Regulation of the Efficiency of Translation

A change in translational efficiency may reflect an alteration in the rate of initiation, elongation, termination or a combination of these steps. Analysis of

ribosomal aggregation in a tissue in which the overall rate of protein synthesis has changed, enables us to detect relative alterations in the translational steps. The different states of ribosomal aggregation which exist during translation are summarised in Fig. 4. Ribosomes consist of three populations, the native subunits, ribosomes in polysomes and the inactive 80 S monomers. The pool of native ribosomal subunits constitutes a very small proportion of the total ribosome content of the cell. When the rate of protein synthesis varies, the size of this pool remains constant (Hogan & Korner, 1968; Hirsch *et al.*, 1973), and is probably set by the concentration of eIF-3 in the cell (Hunt, 1980). In contrast, the size of the 80 S monomer pool is sizable and can vary (Hunt, 1980). The dissociation of 80 S monomers is a prerequisite for the first step of initiation (Sabol & Ochoa, 1971). Changes in the rate of initiation relative to elongation and termination will therefore cause fluctuations in the 80 S monomer pool size. If the rate of initiation falls relative to elongation, two changes in ribosomal aggregation will be seen. The pool of free monomeric ribosomes will increase as less ribosomes become involved in translation. Subsequently less ribosomes will be recruited onto the message resulting in a fall in the average size of the polysomes. Alternatively, a profile exhibiting heavier polysomes and a diminished 80 S monomer pool characterizes a fall in the rate of elongation and termination relative to initiation. The relative proportions of native subunits, monomers and polysomes in a system in which protein synthesis is being studied is routinely determined utilizing sucrose density gradient centrifugation.

5.3.2 Regulation of Peptide Chain Initiation

The considerable progress made in our understanding of both the mechanism of initiation and its regulation in eukaryotic cells has arisen primarily from studies with the immature red blood cell, the reticulocyte. The lysate which can be derived from reticulocytes is the most widely used cell-free system owing to its high translatable activity (equivalent to 100% of the intact cell) and its capacity to exhibit the control mechanisms of the intact cell (Clemens, 1984).

Protein synthesis in the reticulocyte lysate is almost entirely dedicated to the

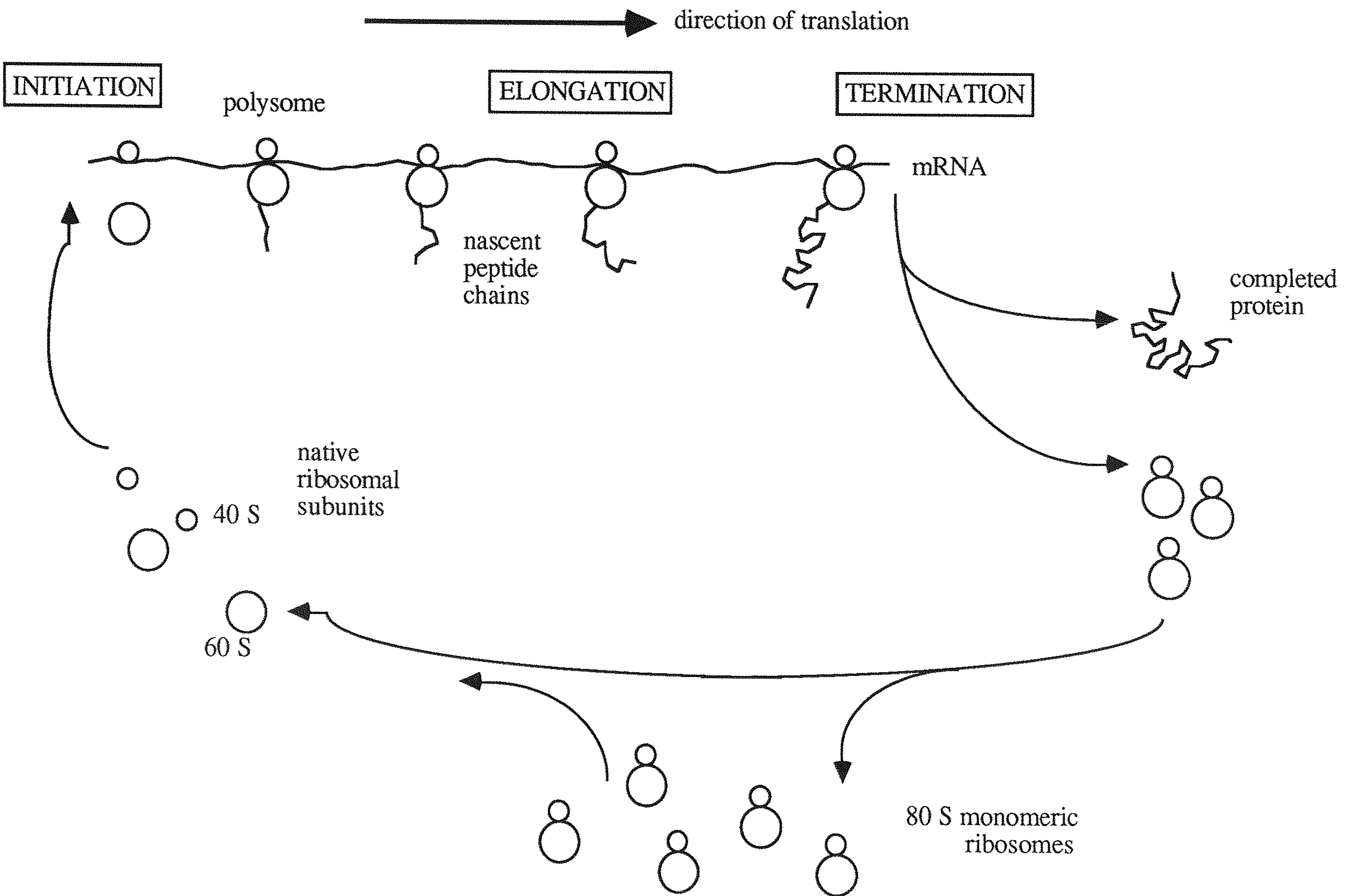


Figure 4. Diagram Depicting the Distribution of Ribosomes Between Subunits, Polysomes and Monomers

synthesis of globin and is regulated by haem. In the absence of haem, protein synthesis proceeds at the initial rate for several minutes and then declines abruptly. The cessation of protein synthesis is accompanied by polysome disaggregation with a concomitant increase in 80 S ribosomes, indicating a block at peptide chain initiation (Zucker & Schulman, 1968). The inhibition is reversed by eIF-2 (Beuzard & London, 1974), cAMP (Legon *et al.*, 1974), GTP (Ernst *et al.*, 1976) and is potentiated by ATP (Ernst *et al.*, 1976). The inhibition of initiation in haem-deficient lysates is due to the activation of a translational inhibitor termed the haem controlled inhibitor (HCI) or alternatively, the haem controlled repressor (HCR). HCI is a cAMP-independent protein kinase that specifically phosphorylates the α-subunit of eIF-2 (Farrell *et al.*, 1977) (Fig. 5). It is generally accepted that the activation of HCI involves the oxidation of labile SH groups present in the proinhibitor (proHCI) or other lysate proteins (Jackson *et al.*, 1983).

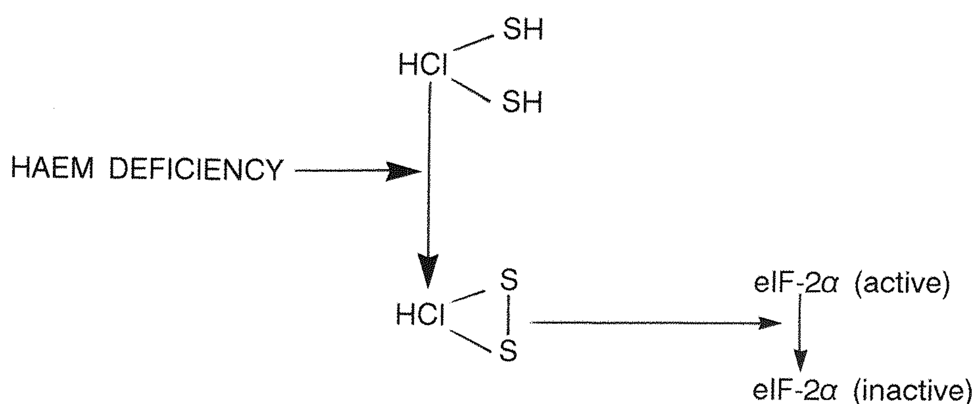


Figure 5. Mechanism of Protein Synthesis Inhibition in the Reticulocyte Lysate in the Absence of Haem

At the end of an initiation cycle, a phosphorylated binary complex, eIF-2(αP).GDP will bind tightly to GEF producing GEF.eIF-2(αP).GDP, a 15 S complex, in which GEF is not functional. Since there is relatively more eIF-2 in the cell than GEF, the phosphorylation of only a fraction of eIF-2 sequesters all the GEF into the 15 S complex (Thomas *et al.*, 1984; Matts & London, 1984). GEF is inhibited from interacting with unphosphorylated eIF-2.GDP and therefore the exchange of GDP for GTP is prevented. If GTP cannot bind eIF-2, Met-tRNA$_f$ does not form a ternary complex with eIF-2.GTP, and as a consequence, 43 S preinitiation complex

formation is prevented. When haem is added to a deficient lysate, HCl activity is blocked, an endogenous phosphatase dephosphorylates the 15 S complex and functional GEF is released enabling protein synthesis to be resumed (Matts *et al.*, 1986). This mechanism of inhibition is also induced in haem supplemented lysates treated with double stranded RNA and oxidized glutathione (see section 7.3). Furthermore, the inhibition of peptide chain initiation in extracts prepared from amino acid deficient Ehrlich ascites tumour cells and rat hepatocytes is characterized by a reduction in 43 S preinitiation complex formation which is overcome with the addition of eIF-2 (Pain *et al.*, 1980; Kimball *et al.*, 1989). These observations from other mammalian systems support the concept that the mechanism which regulates initiation in the reticulocyte, represents a common mechanism of control in eukaryotic protein synthesis.

6. Effect of Hyperoxia on Protein Synthesis

The deleterious effect induced by hyperoxia on protein synthesis is well documented in cultured cells (Reuckert & Mueller, 1960; Hollenberg, 1971; Goetz, 1975; Jornot *et al.*, 1987). It has also been observed that protein synthesis in whole reticulocytes and their lysates is compromised upon exposure to high partial pressures of oxygen (Almis-Kanigür *et al.*, 1982,1983).

A fall in pulmonary protein synthesis following hyperoxia was first suggested by Gacad and Massaro (1973). After exposing rats to $\geq$98% oxygen for 24 hours, protein synthesis, measured by monitoring the incorporation of ^{14}C-leucine into lung slices was significantly reduced. This inhibition was further increased after a 48 hour exposure. The major short coming of this study was the failure to measure the specific activity of tissue unbound label. As a consequence, the result obtained may reflect an effect by hyperoxia on parameters which influence the precursor pool for protein synthesis, rather than an effect on protein synthesis per se. The potential that hyperoxia has to inhibit pulmonary protein synthetic activity has since been readdressed by exposing rats to $\geq$95% O_2 for either 6 or 24 hours (Kelly, 1988). The reliability of the methodology employed (Garlick *et al.*, 1980), enabled an accurate measurement of *in vivo* tissue protein synthesis to be made. An acute

(6 h) hyperoxic exposure led to a 20% fall in the rate of pulmonary protein synthesis. On extending the period of hyperoxia to 24 hours, protein synthesis in the lung was further depressed and significantly lower than control lungs. The decline in pulmonary protein synthesis was related to a fall in translational efficiency, while synthetic capacity was unaltered. Hyperoxia therefore not only damages the lung, but by inhibiting pulmonary protein synthesis, is capable of retarding lung growth and repair. Supplementary oxygen administered to premature babies is thus an obvious candidate for compromising growth and development of the immature lung.

6.1 Mechanism of Hyperoxic-induced Inhibition of Protein Synthesis

The precise mechanism by which hyperoxia inhibits protein synthesis has not, up until now, been investigated. The direct inhibitory effect of hyperoxia on DNA and RNA synthesis (Reuckert & Mueller, 1960; Northway *et al.*, 1972,1976; Clément *et al.*, 1985) suggests an effect of oxygen on the nucleus or the cytoplasm or both. I will be focusing specifically on how an overall fall in the rate of tissue protein synthesis may be related to an action of hyperoxia at the level of translation.

It is highly likely that hyperoxia inhibits protein synthesis through the generation of oxygen free radicals. Primary radicals and their secondary lipid radical intermediates are capable of amino acid residue modification and the covalent crosslinking and fragmentation of proteins (Levine, 1983; Fucci *et al.*, 1983; Wolff & Dean, 1986; Prinsze *et al.*, 1990). The outcome of radical induced protein damage includes enzyme inactivation and their increased susceptibility to degradation (Davies & Goldberg, 1987a,b; Dean, 1987; Prinsze *et al.*, 1990). Of particular relevance is the sensitivity of sulphydryl groups to oxidation (Jamieson *et al.*, 1963; Halliwell & Gutteridge, 1984; Slater, 1984). Some ribosomal binding sites (McAllister & Schweet, 1968; Bermek *et al.*, 1971) and initiation factors (Miller & Schweet, 1968; Leader *et al.*, 1970) contain sulphydryl sensitive groups, while the translocation step involving eEF-2 also relies upon the maintenance of reduced thiol groups for maximal activity (Sutter & Moldave, 1966; Bermek *et al.*, 1970; Baliga &

Munro, 1971). Conversely, the endogenous inhibitor of protein synthesis in the reticulocyte lysate, HCI can become activated by the oxidation of its thiol groups by the superoxide ion and other reactive oxygen species (Palomo *et al*, 1985; Savolainen *et al.*, 1985) (Fig. 6). It should be appreciated therefore that there are a variety of components and reactions in the pathway of protein synthesis which upon oxidation, could contribute towards the hyperoxic-induced decrease in protein synthetic activity.

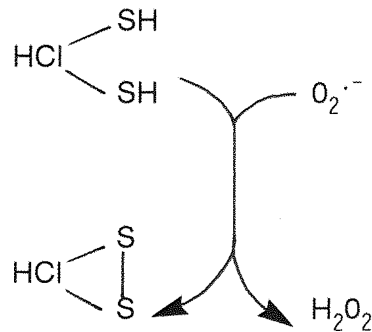
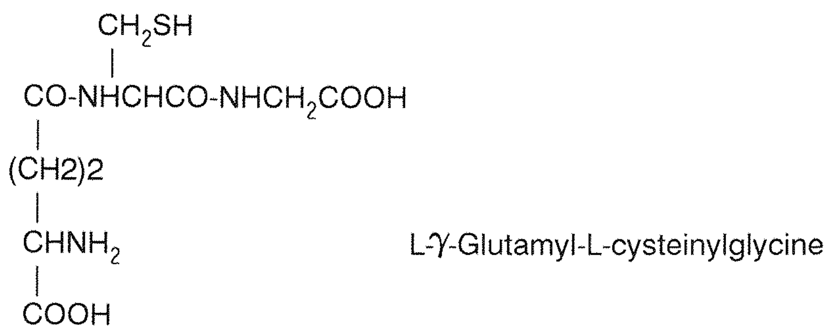


Figure 6. Activation of the proHCI in the Reticulocyte by the Superoxide Ion

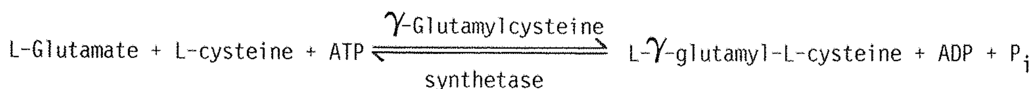
Under hyperoxic conditions, the increased production and subsequent removal of oxygen free radicals will increases the utilization of cellular glutathione. There are several lines of evidence which suggest that a perturbation in the glutathione status of the cell, more specifically an accumulation of the oxidized species (GSSG), may mediate a decreased rate of pulmonary protein synthesis following hyperoxia.

7. Introduction to Glutathione

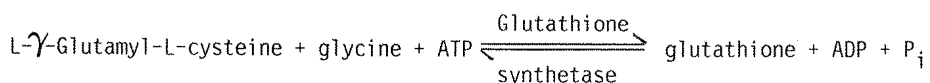
Reduced glutathione (L-γ-glutamyl-L-cysteinylglycine) is the major cellular thiol and the most abundant low molecular weight peptide present in cells. The various aspects of glutathione biochemistry are the subject of numerous reviews (Kosower & Kosower, 1978; Meister & Anderson, 1983; Meister, 1984).



The synthesis of glutathione is a two-step reaction utilizing ATP and two enzymes (Meister, 1974). In the first step, γ-glutamylcysteine synthetase catalyzes the peptide bond formation between L-glutamate and L-cysteine.



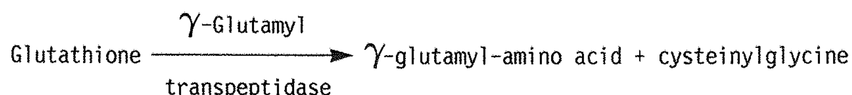
The second enzyme, glutathione synthetase, catalyzes the formation of the tripeptide with the addition of a glycine residue.



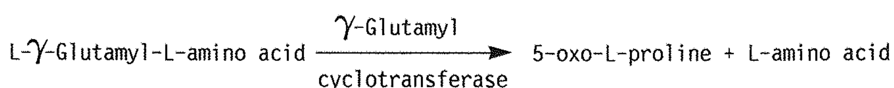
Both reactions take place by mechanisms involving the intermediate formation of enzyme-bound acyl phosphates. Under normal conditions, the activity of γ-glutamylcysteine synthetase is less than maximal since this enzyme is feedback-inhibited by glutathione. In this way, the tissue content of glutathione plays a

significant role in the regulation of glutathione synthesis.

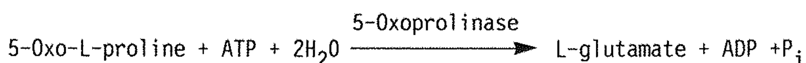
The first step in the breakdown of glutathione is catalyzed by γ-glutamyl transpeptidase (Meister *et al.*, 1976). This is a membrane bound enzyme that catalyzes the transfer of the γ-glutamyl moiety of extracelluar glutathione to yield γ-glutamyl amino acids and cysteinylglycine.



Cysteinylglycine is cleaved to cysteine and glycine by the action of a dipeptidase, present on the cell membrane and intracellularly. Cysteine is the most active acceptor of the γ-glutamyl group, although glutamine, methionine, alanine and other neutral amino acids are also good acceptors (Thompson & Meister, 1975). The glutamyl-amino acid is formed in close association with the cell membrane and is translocated into the cell. This is the basis of the amino acid transport system provided by glutathione. Intracellularly, γ-glutamyl cyclotransferase converts the glutamyl amino acids into the corresponding free amino acids and 5-oxoproline (Taniguchi & Meister, 1978).



5-oxoproline is then converted to glutamate in a reaction catalyzed by 5-oxoprolinase which utilizes ATP (Van Der Werf *et al.*, 1971).



7.1 Functions of Glutathione

Glutathione has many indispensable functions, all of which are concerned with maintaining the structural integrity of the cell. The thiol transfer reactions of glutathione (a) promote thiol-disulphide exchange necessary for protein assembly and degradation (b) provide reducing capacity for a number of reactions including DNA synthesis and (c) detoxify free radicals, organic peroxides and foreign compounds before the latter interact with more critical cellular constituents. Glutathione is also an essential cofactor for many enzymes and functions in the transport of amino acids. The important role played by pulmonary glutathione under normal conditions is clearly evident from the significant cellular damage to the lung following the treatment of mice with the glutathione synthesis inhibitor buthionine sulfoximinie (Mårtensson *et al.*, 1989).

7.2 The Glutathione Status of the Cell

The glutathione status of the cell can be defined by the total cellular concentration of glutathione and the nature of the possible forms in which glutathione can occur in cells. The most important forms of this compound include reduced glutathione (GSH), oxidized glutathione (GSSG), mixed disulphides, thiol esters and glutathione derivatives bound through linkages other than sulphur. The glutathione status of the cell is a dynamic system. Perturbations in the cell, for example increased oxidation, will shift the equilibrium between the various forms.

The majority of intracellular glutathione exists as the reduced form in concentrations ranging from 0.5 to 10mM depending upon the individual tissue type. Many tissues, including the lung, contain 2-5mM glutathione (Kosower & Kosower, 1978; Hill *et al.*, 1987; Jenkinson *et al.*, 1988a). The concentration of glutathione in the plasma is lower and has been reported as 3μM in humans (Cantin *et al.*, 1987) and 11-20μM in rats (Hill *et al.*, 1987).

Under normal conditions, in mammalian systems including the human and

rat erythrocyte (Srivastava & Beutler, 1968) and the rat liver and kidney (Tietze, 1969), GSSG represents 0.2 to 5.0% of total glutathione. Concentrations of the oxidized and reduced forms depend on the redox status of the cell which is regulated by a series of reactions shown in Fig. 7.

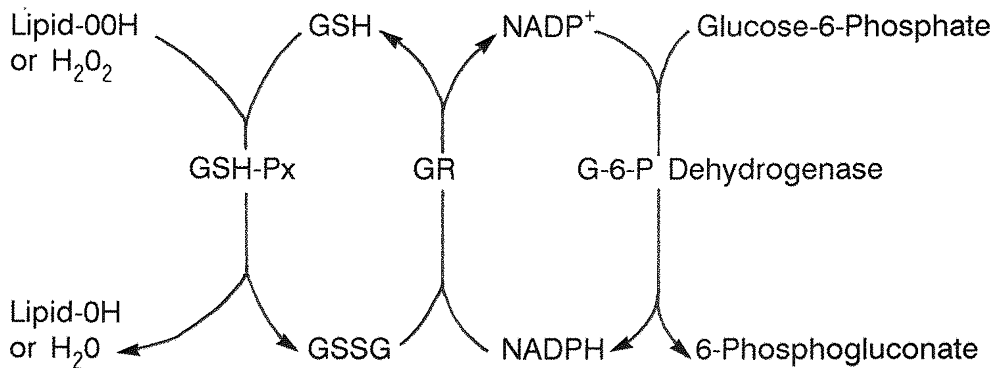
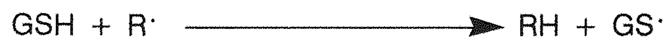


Figure 7. Oxidation and Reduction of Glutathione

The antioxidant capacity of glutathione involves its reaction with hydrogen peroxide and lipid hydroperoxides thereby preventing radical-induced tissue injury. This is catalyzed by GSH-Px. The interaction of glutathione with the oxidant initially produces the GS· free radical which is stable and survives until another GS· appears. The dimerisation between the two radicals produce one molecule of GSSG.



$$GSH + R^{\cdot} \longrightarrow RH + GS^{\cdot}$$

$$GS^{\cdot} + GS^{\cdot} \longrightarrow GSSG$$

Cells are able to regenerate GSH from GSSG via the NADPH dependent enzyme glutathione reductase (GR). The re-reduction of $NADP^+$ utilizes one of a number of enzymes including glucose-6-phosphate dehydrogenase. The steady state production of NADPH depends upon the presence of glucose and its flux through the pentose phosphate pathway. Glucose-6-phosphate dehydrogenase is inhibited by NADPH, while its inhibition is alleviated by GSSG. Under normal conditions the recycling of glutathione is such as to maintain a high cellular

GSH/GSSG ratio. Oxidative stress, in generating an increased production of free radicals will lead to a greater utilization of glutathione. Under such conditions, the rate of GSSG production will exceed that of its reduction and ultimately, GSSG will accumulate in the cell. Changes in the glutathione status of the lung have been demonstrated in response to various forms of oxidative stress. Significant increases in the GSSG content of the isolated perfused lung are found following *tert*-butyl hydroperoxide infusion (Jenkinson *et al.*, 1987) and reoxygenation of the lung following a period of hypoxia (Jenkinson *et al.*, 1988b). Elevations in pulmonary GSSG has been reported *in vivo* upon exposing adult rats to hyperoxia (>99% O_2) for 48 to 52 hours (White et al., 1986, 1987).

7.3 Inhibition of Protein Synthesis by Oxidized Glutathione

The increased concentration of GSSG reported in the lung under conditions of oxidative stress has the potential to mediate the hyperoxic-induced fall in pulmonary protein synthesis. The addition of GSSG (0.05 to 2.0mM) to haem supplemented reticulocyte lysates results in an inhibition of protein synthesis (Kosower *et al.*, 1971,1972). The characteristics and mechanism of this inhibition have been extensively investigated and are identical to that described for haem deficiency. Following the addition of GSSG to the lysate, protein synthesis proceeds at a linear rate for several minutes. This is followed by a complete cessation of protein-synthesizing activity which is accompanied by polysome disaggregation, an accumulation of 80 S monomers (Kosower *et al.*, 1972) and a depletion of 43 S preinitiation complexes (Legon *et al.*, 1974). Furthermore, the inhibition is reversed by eIF-2 (Clemens *et al.*, 1975), cAMP (2 to 20mM) (Legon *et al.*, 1974) and is potentiated by ATP (2mM) (Ernst *et al.*, 1978a). GSSG achieves its inhibitory effect by activating a protein kinase, termed the GSSG-induced inhibitor [I(GSSG)]. The activation of I(GSSG) is accompanied by an increased cyclic AMP-independent protein kinase activity which phosphorylates the α subunit of eIF-2 (Ernst *et al.*, 1978a). The fact that an antibody against HCI strongly inhibits the GSSG-activated kinase suggests that the inhibitor that is activated by GSSG is HCI itself (London *et al*, 1981). More recent work has studied the role of GEF in the inhibition of peptide chain initiation by GSSG (Kan *et al.*, 1988). In parallel to haem deficient

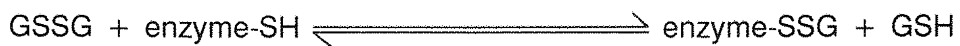
lysates, GSSG leads to the sequestration of GEF in a phosphorylated 15 S complex, however GEF is more profoundly inhibited in GSSG treated lysates than in haem deficient lysates. Isolated GEF contains bound NADPH and under appropriate conditions, NAD^+ and $NADP^+$ inhibit GEF activity (Dholkia & Wahba, 1989). NADPH is hypothesized to maintain GEF activity by preventing or reversing the formation of GEF disulphide complexes. The inhibition of peptide chain initiation in GSSG-treated reticulocytes is believed to result from the oxidation of the sulphydryl groups required for functional GEF activity as well as the sequestration of GEF in the 15 S complex.

The mechanism by which GSSG activates the protein kinase is not understood but several possibilities may be considered. GSSG could oxidize the sulphydryl groups in the inhibitor in a manner analogous to that observed on treating reticulocytes with N-ethylmaleimide (Gross & Rabinovitz, 1972). It is unlikely that GSSG oxidises the proinhibitor directly because it has no effect on partially purified HCI (Vicente, 1983). The sulphydryl groups could however be part of another component necessary to maintain the proinhibitor in an inactive form. It is believed that the disulphide bonds formed in the inhibitor by oxidation are reduced by NADPH via the thioredoxin-thioredoxin reductase system (Jackson *et al.*, 1983). NADPH would then become a prerequisite to maintain the inhibitor in its inactive form. It is feasible therefore that GSSG, through its reduction by NADPH, catalyzed by glutathione reductase, can activate the proinhibitor by way of NADPH depletion. However GSSG still causes translational inhibition in lysates depleted of glutathione reductase by affinity chromatography (Jackson, 1982) or by the use of an antibody against glutathione reductase (Palomo & Sierra, 1988). This suggests that GSSG activates the proinhibitor through a more direct mechanism.

Further evidence that the hyperoxic-induced decrease in protein synthesis is mediated through GSSG, comes from the characteristics of protein synthesis inhibition in the reticulocyte lysate exposed to a high partial pressure of oxygen (50% 0_2) (Almis-Kanigür *et al.*, 1982,1983). The decrease in protein synthetic activity is accompanied by the activation of an inhibitor termed the high partial pressure activated inhibitor ($hp0_2I$). This inhibitor shows some of the common features of other translational inhibitors from reticulocytes. These include biphasic kinetics of

inhibition which can be potentiated by ATP and prevented by cAMP and glucose-6-phosphate (Almis-Kanigür *et al.*, 1982). Further studies showed that the hpO_2I was activated through the oxidation of SH-groups and could also be activated by the treatment of lysates with GSSG (Almis-Kanigür *et al.*, 1983). These investigations suggest that under conditions of high oxygen concentrations, the formation of GSSG in the reticulocyte oxidizes the SH-groups of an inhibitor, hpO_2I/I(GSSG) which shuts off protein synthesis. This work did not specify however the translational step which was blocked in the hyperoxic-exposed reticulocyte.

In addition to the well documented effect of GSSG on peptide chain initiation in the reticulocyte, early work reported the detrimental effect of the disulphide on peptide chain elongation in other mammalian systems. Phenylalanine incorporation into protein or a synthetic peptide, polyphenylalanine is almost completely inhibited in ribosomal preparations from rat brain in the presence of 8mM GSSG (Murthy, 1966). Partial reversal of the inhibition was possible by equimolar additions of GSH. It was suggested that GSSG acted by oxidizing the SH-groups of enzyme(s) involved in the incorporation of phenylalanine into protein, and that this reaction was reversible.



$$\text{GSSG} + \text{enzyme-SH} \rightleftharpoons \text{enzyme-SSG} + \text{GSH}$$

Kinetic studies into the aminoacyl transfer from aminoacyl soluble RNA into ribosomal protein in rat liver preparations provided evidence for the participation of GTP and sulphydryl compounds in this reaction (Hoagland *et al.*, 1958; Hülsmann & Lipmann, 1960). The use of microsomes isolated from rat liver demonstrated that the addition of GSSG (0.1mM) led to an 80% reduction in the incorporation of ^{14}C-aminoacyl-tRNA into protein (Nolan & Hoagland, 1971). These workers hypothesized that a factor X, associated with the membrane component of the microsome could exist in an oxidized or reduced form. X must bind GTP for maximal activity and for binding, X must be in the reduced form, XSH. The addition of GSSG to the system would oxidize X leading to a decrease in X(GTP) complex formation and a fall in amino acid incorporation into protein.

8. Glucocorticoids and Respiratory Distress Syndrome

A premature infant who succumbs to CLD is in the unfortunate position of being critically dependent upon supplementary oxygen and mechanical ventilation, the very agents which contributed towards the occurrence of the disease. In an attempt to prevent such a vicious circle developing in situations when a premature birth is imminent, attempts have been made to accelerate fetal lung maturation by antenatal glucocorticoid administration.

8.1 Induction of Fetal Lung Maturation by Glucocorticoids

An acceleration of fetal lung maturation by glucocorticoids was first suggested by Liggins (1969) while studying the effects of glucocorticoids as a means of inducing premature delivery. When dexamethasone was infused into fetal lambs (0.25 to 2.0mg/Kg fetal body weight/24 hours) between day 117 to 128 of gestation (term is 150 days), a significant proportion of lambs born prematurely possessed lungs showing partial aeration. In the majority of cases, the aeration consisted of patchy expansion in the upper lobes, but in one lamb delivered at 123 days of gestation, the lower lobes were also expanded. It was postulated that dexamethasone was inducing lung maturation and causing the appearance of surfactant in the alveolar spaces. Subsequent studies have substantiated this earlier work demonstrating precocious lung maturation in the fetal lamb (Platzker *et al.*, 1975), rabbit (Kotas & Avery, 1971; Wang *et al.*, 1971; Motoyama *et al.*, 1971), monkey (Kling & Kotas, 1975), rat (Frank *et al.*, 1985) and guinea pig (Lechner, 1987) upon prenatal glucocorticoid administration. In general the effect elicited by the hormone is initially detected 24 to 48 hours after drug administration. The indices of a glucocorticoid-induced lung maturation reported in these *in vivo* studies include an accelerated differentiation of alveolar type II epithelial cells, a greater alveolar air-space volume and an increase in pulmonary surfactant. In addition, the synthesis of the surfactant-associated proteins, SP-A, SP-B and SP-C have been shown to be enhanced by glucocorticoids during fetal development (Mendelson *et al.*, 1986; Whitsett *et al.*, 1987.

8.2 The Mechanism of Glucocorticoid Acceleration of Fetal Lung Maturation

A direct action of glucocorticoids on a tissue almost exclusively involves the interaction of the steroid with specific glucocorticoid receptors in the cell cytoplasm. The receptor-steroid complex then migrates to the nucleus where it presumably attaches with high affinity to a component of the chromatin structure, influencing the synthesis of mRNA. The message leaves the nucleus and directs the synthesis of specific proteins, the production of which ultimately leads to the response of the cell to the steroid.

Cytoplasmic glucocorticoid receptors have been detected in the fetal lung of various species over the latter part of gestation (Giannopoulos, 1974). Dexamethasone receptors have been demonstrated in the lungs of fetal rabbits (term =31d) between day 21 of gestation and birth, fetal rats (term =22d) during the last 3 days of gestation and in the human fetal lung during the 9th to 17th gestational week. In both the human and rabbit fetus, lung tissue contains a higher concentration of dexamethasone binding sites than all other fetal tissues analyzed (Ballard & Ballard, 1972, 1974), thus making the lung a potential target for steroid action. Further studies detected specific glucocorticoid binding in the cytoplasmic and nuclear fractions of cultures derived from fetal rat lung containing primarily type II cells (Ballard *et al.*, 1978). This is consistent with a direct action of glucocorticoids on the lung, mediated by the receptor system of the surfactant producing type II epithelial cells.

The close relationship between binding of dexamethasone to receptors and an increase in the PC synthesis by explants of fetal lung has been demonstrated following glucocorticoid exposure (Gross *et al.,* 1983). This investigation also reported that the steroid-induced increase in PC synthesis could be blocked if actinomycin D was added at the initiation of hormone exposure, while cycloheximide was inhibitory when introduced 24 to 48 hours after dexamethasone treatment. These findings support the view that glucocorticoid stimulation of PC synthesis in the fetal lung is mediated via the classical mechanism, involving *de novo* synthesis of RNA and subsequently the synthesis of new proteins.

At the cellular level, glucocorticoids stimulate choline-phosphate cytidyltransferase activity in fetal lung type II cells (Rooney *et al.*, 1976; Post, 1987). This enzyme catalyzes the rate-limiting reaction in the synthesis of PC. Further investigations support the concept that the action of glucocorticoids on type II cells is mediated by fibroblast-pneumonocyte factor which in turn stimulates the activity of choline-phosphate cytidyltransferase (Post *et al.*, 1986). In addition, glucocorticoids may accelerate surfactant synthesis by providing an adequate supply of fatty acids, required for PC production. In support of this, an enhanced activity of fatty acid synthetase and an associated increase in fatty acid synthesis has been demonstrated in the fetal lung both *in vivo* and *in vitro* following glucocorticoid exposure (Rooney *et al.*, 1986; Pope & Rooney, 1987). Finally, steroid hormones increase the number of ß-adrenergic receptors in the fetal lung. Glucocorticoids therefore have the potential to regulate the secretion of surfactant, since the stimulation of these receptors mediate the release of surfactant from the type II cells into the alveoli lining fluid (Barnes *et al.*, 1984).

8.3 Antenatal Glucocorticoid Treatment for the Prevention of Respiratory Distress Syndrome

The experimental evidence derived from animal studies that glucocorticoids induce fetal lung maturation suggested a role for these hormones as a therapy for the prevention of RDS. The efficacy of antenatal glucocorticoids in lowering the incidence of this acute neonatal disorder was initially demonstrated by Liggins and Howie (1972). Prenatal administration of betamethasone (6mg every 12 hours for a maximum of two days) at least 24 hours before delivery significantly reduced the incidence of RDS in premature infants delivered between 24 and 34 weeks of gestation. In the majority of cases, subsequent studies have supported the advantages of steroid treatment (Block *et al.*, 1977; Papageorgiou *et al.*, 1979; Collaborative Group, 1981; Doyle *et al.*, 1986). Established benefits include a decreased mortality rate, a lowered incidence of RDS, the requirement of less ventilatory support, fewer days in oxygen for those who succumb to RDS and a decreased incidence of CLD. The shortcomings of this therapy include the reports

of a poor response to steroids in multiple pregnancies. In addition there appears to be an interaction of sex and race with the treatment, such that antenatal glucocorticoid therapy has a greater efficacy in the female, black population compared to the male, non-black population (Ballard *et al.*, 1980; Collaborative Group, 1981).

Since the initiation of these studies, limited data suggests that glucocorticoids may have dual benefits when employed clinically, by stimulating the development of the pulmonary antioxidant system in parallel to the surfactant system. Significant increases in the activities of pulmonary SOD, CAT and GSH-Px have been reported in the fetal rat following the administration of dexamethasone to the pregnant dam (Frank *et al.*, 1985). A direct action of glucocorticoids on this biochemical component of lung maturation is supported by the elevated antioxidant enzyme activities in both fetal lung explants and fetal lung fibroblasts in response to dexamethasone exposure (Randhawa et al., 1986, 1987). If antenatal glucocorticoid therapy has a such an effect on the human, this would help to protect the premature lung from oxygen-induced damage in those individuals who require hyperoxic therapy.

8.4 Role of Endogenous Glucocorticoids in the Regulation of Fetal Lung Development

Numerous lines of evidence indicate that fetal lung maturation is influenced by endogenous plasma glucocorticoids. A temporal relationship has been identified between the fetal plasma glucocorticoid peak during gestation, the dramatic changes in lung morphology and the increased maturity of the pulmonary surfactant system in the lamb (Kitterman *et al.*, 1981), rat (Holt & Oliver, 1968; Sosenko *et al.*, 1986) and guinea pig (Sosenko & Frank, 1987a; Kelly *et al.*, 1990). In the human, an increase in glucocorticoid receptors in the fetal lung occurs prior to elevations in pulmonary surfactant (Ballard & Ballard, 1974). Furthermore, the levels of cortisol in cord blood (Smith & Shearman, 1974) and amniotic fluid (deM. Fencl & Tulchinsky, 1975) increase markedly after approximately 35 weeks of gestation. This corresponds to the time when a mature lecithin-sphingomyelin ratio is found in the

amniotic fluid (Gluck & Kulovich, 1974). A significant proportion of lecithin (PC) originates from pulmonary surfactant. An increased lecithin-sphingomyelin ratio in the amniotic fluid therefore indicates an increase in pulmonary surfactant production and release, and is routinely taken as an index of fetal lung maturity. These observations have lead to the general consensus that both animal and human fetal lung development is regulated by the level of endogenous glucocorticoids. It would appear therefore that prenatal steroid therapy, at an appropriate dose and time, accelerates the maturation of the lung through normal physiological mechanisms.

8.5 Effects of Glucocorticoids on Fetal Lung and Somatic Growth

In addition to the well-documented benefits of antenatal steroid therapy, it is important to appreciate the potential deleterious effects of this therapy on the fetus. Of major concern is the negative effect that glucocorticoids have on somatic and organ growth. The administration of dexamethasone to the pregnant rat, utilizing a dose regime equivalent to that employed clinically, leads to a significant reduction in whole body and lung weight of the newborn and rats delivered one day prematurely (Frank & Roberts, 1979; Frank *et al.*, 1985). Furthermore, the deficit in fetal pulmonary growth following the treatment of pregnant rhesus monkeys with betamethasone persists for at least one month during subsequent in utero growth and development (Johnson *et al.*, 1981). The mechanism by which exogenous steroids inhibit growth in the fetus is poorly defined. Early work indicates that the smaller lungs of steroid-treated fetal rabbits and guinea pigs is related to a reduction in DNA synthesis (Carson *et al.*, 1973; Kotas *et al.*, 1974; Sanfaçon *et al.*, 1977). There is conflicting data in the literature over whether prenatal growth inhibition is reversible or whether it will cause a permanent deficit postnatally. Some investigators believe that if an organ is inhibited during a phase of rapid growth, for example immediately before or after birth, the organ will be permanently affected (Winick, 1968; Mosier, 1971). In contrast, the inhibition of lung growth following prenatal steroid treatment in the rabbit has been shown to be reversible as a result of postnatal catch-up growth (Kotas *et al.*, 1974).

The fetal lung is a target for antenatal steroids, whether it is an inhibitory

action on growth or a more favourable induction of pulmonary maturation. While the latter has been shown to be beneficial in the clinical setting, the former action of glucocorticoids may exacerbate the problems experienced by preterm neonates. A growth retarded lung at birth, due to prenatal steroid treatment, in addition to the likelihood of poor postnatal pulmonary growth and lung damage, will complicate short-term neonatal recovery and may ultimately lead to sustained abnormalities in pulmonary development.

9. Dexamethasone Therapy for Chronic Lung Disease

Up until recently, the management of infants with CLD was primarily supportive, consisting of fluid restriction, diuretics, mechanical ventilation and supplementary oxygen. The improvements in pulmonary function in animal models of lung disease following 9α-fluoromethylprednisolone administration (Sahebjami *et al.*, 1974) provided some basis for the use of corticosteroids as a treatment for CLD. The steroid now employed is the synthetic corticosteroid, dexamethasone. Dexamethasone has a glucocorticoid activity 30 times greater than endogenous corticosteroids, while being virtually devoid of mineralocorticoid activity. As a consequence, the therapeutic use of this analogue is associated with few mineralocorticoid side-effects compared to those which occur with the naturally occurring compounds. The initial trials of dexamethasone as a treatment for neonates classified as having severe CLD were most encouraging (Mammel *et al.*, 1983,1987; Avery *et al.*, 1985). Infants exhibited improvements in pulmonary function generally within 72 hours of steroid administration, while the average time to extubation was approximately one week following the initiation of drug treatment. These studies were only controlled for 72 hours and therefore examined the short-term effect of dexamethasone on pulmonary function. More recent trials have investigated the long-term efficacy of steroid therapy (Cummings *et al.*, 1989; Harkavy *et al.*, 1989; Noble-Jamieson *et al.*, 1989). Cummings *et al.* (1989) reported that pre-term infants who were dependent upon mechanical ventilation and oxygen at two weeks of age and treated with dexamethasone were weaned from the supportive therapy significantly faster than control infants. They concluded that these neonates who were at a high risk for CLD did exhibit sustained improvements

in pulmonary function in response to steroid therapy. In contrast, for infants in whom CLD is already evident, the initiation of dexamethasone treatment appears to produce a short-term improvement (Harkavy *et al.*, 1989; Noble-Jamieson *et al.*, 1989). This is characterized by a significantly lower oxygen requirement by steroid-treated neonates compared to placebo controls during the first seven to ten days of steroid treatment, however the overall duration of oxygen therapy during hospitalization was similar in the steroid and control groups. Despite this somewhat transient response elicited by steroid therapy, in the absence of an alternative treatment for CLD, the use of dexamethasone in special care baby units has become widespread. The dexamethasone treatment regime reported in the studies cited above begins with a dose of 0.5mg/Kg body weight/day which is then gradually tapered depending upon the clinical response. It should be appreciated however that these standard regimes are not stringently followed. The severe cases of CLD often necessitate the use of larger daily doses, approaching 2mg/Kg body weight/day.

Several mechanisms have been proposed to explain the improvements in pulmonary function following steroid treatment. The induction of lung maturation elicited by corticosteroids suggests that dexamethasone may increase surfactant synthesis and/or accelerate morphological maturation of the lung (see Section 8.1). Such a mechanism is doubtful however, in that it does not explain the failure of dexamethasone to produce long-term benefits. A recent study reported that the improvements in pulmonary function in infants with CLD during dexamethasone treatment was associated with a diuresis (Gladstone *et al.*, 1989). This supports the concept that a reduction in pulmonary oedema, possibly through an alteration in membrane permeability, mediates the beneficial effects of steroid therapy. Alternatively the anti-inflammatory action of steroids may contribute towards the favourable pulmonary outcome in neonates treated with dexamethasone. Neutrophils and elastase (a proteolytic enzyme released from inflammatory cells) have been found in tracheal aspirates obtained from infants destined to develop CLD (Merritt *et al.*, 1981,1983; Ogden *et al.*, 1984). The recruitment of neutrophils to the site of pulmonary injury has the potential to exacerbate lung damage through their release of free radicals and proteolytic enzymes into the surrounding tissue. In view of the rapid and transient response of CLD sufferers to dexamethasone therapy, the

mechanism of drug action most likely involves a steroid-induced reduction in the pulmonary inflammation response. In support of this, Gerdes *et al.*, (1988) found a decrease in elastase activity in the bronchoalveolar lavage obtained from infants treated with dexamethasone for 48 to 72 hours. The failure of steroid therapy to produce a sustained improvement in pulmonary function in neonates who have already developed CLD, may be related to the severity of tissue damage at this stage which is unlikely to be altered by the use of dexamethasone.

9.1 Glucocorticoids and Growth

One of the major questions concerning the therapeutic use of dexamethasone lies in its long-term safety. The potential complications of glucocorticoid treatment are well-known and include infection (Mammel *et al.,* 1983, 1897), adrenocortical suppression (Arnold *et al.*, 1987) and neurological abnormalities (Bourchier, 1988; Noble-Jamieson, 1989). In addition, and of relevance to the subject of this thesis, are the growth suppressive qualities of glucocorticoids.

9.1.1 Effect of Glucocorticoids on Whole Body Growth

Growth retardation is a well-known feature in children suffering from Cushing's syndrome, a condition characterized by an overproduction of glucocorticoids (Strickland *et al.*, 1972). The catch-up growth observed upon removing the source of glucocorticoid overproduction is considered the most compelling evidence that steroids slow growth (Prader *et al.*, 1963). Exogenous glucocorticoids are also potent inhibitors of growth in children when employed to treat chronic conditions including asthma (Morris, 1975), nephrotic syndrome (Lam & Arneil, 1968), congenital adrenal hypoplasia (Rappaport *et al.*, 1973), juvenile rheumatoid arthritis (Ward *et al.*, 1966), inflammatory bowel syndrome (McCaffery *et al.*, 1970) and chronic active hepatitis (Clark & Fitzgerald, 1984). The frequency with which glucocorticoids are administered appears to have a significant effect on the capacity to suppress growth. A daily dose of prednisolone of 0.6mg/Kg body weight suppresses growth in children with rheumatoid arthritis, while 2mg/Kg prednisolone given on alternate days maintains normal growth (Byron *et al.*, 1983).

It must be appreciated however, that with careful employment, glucocorticoids have the capacity to promote growth in children by adequately treating the underlying disease that is adversely affecting growth.

The inhibitory effect of glucocorticoids on growth could occur by influencing the secretion and/or activity of growth hormone, thyroid hormone and the sex steroids, all of which control growth. Many investigations have focused on the relationship between steroid treatment and growth hormone, however, explanations regarding the mechanism of glucocorticoid-induced growth retardation remain unclear. Although a decreased concentration of growth hormone has been found in adults treated with steroids (Frantz & Rabkin, 1964; Hartog *et al.*, 1964), plasma growth hormone concentrations were not found to be suppressed in corticosteroid-treated children (Morris *et al.*, 1968b). Since the growth hormone measurements were made 12 to 16 hours after the last dose of prednisolone, it was concluded that prolonged suppression of growth hormone release does not occur in steroid-treated children. This study does not however provide information about the short term effects of the treatment. It is possible that corticosteroids transiently inhibit growth hormone secretion and that the level had returned to normal by the time the measurement was made. If a decrease in growth hormone secretion does occur in children with glucocorticoid-induced growth retardation, it would be expected that exogenous administered growth hormone may improve growth. Growth acceleration has been observed in children with Cushing's syndrome and children with rheumatoid arthritis receiving long-term corticosteroid therapy and who were also treated with growth hormone (Butenandt, 1979). This beneficial effect has not been observed however in growth retarded children with asthma and nephrotic syndrome (Morris *et al.*, 1968a).

More recent work has focused on the effects of corticosteroids on the somatomedin system. Somatomedins are peptide hormones with insulin-like activity that appear to mediate the growth-promoting effects of growth hormone. Somatomedins promote collagen formation by increasing the transport of amino acids; protein, RNA and DNA synthesis; and the incorporation of sulphate into proteoglycans in cartilage cells (Phillips & Vassilopoulou-Sellin, 1980). Normal somatomedin levels have been demonstrated in children with Cushing's syndrome

(Gourmelen *et al.*, 1982), however somatomedin bioactivity is decreased in similar individuals (Van der Brande & DuCaju, 1974). Similarly, an abrupt fall in serum somatomedin activity has been reported when 2mg/Kg methylprednisolone is administered to children with nephrotic syndrome (Elders *et al.*, 1975). This data together with *in vitro* studies (Binoux *et al.*, 1980) suggest that glucocorticoids influence the somatomedin system through increasing the availability of somatomedin inhibitors.

Further evidence exists that glucocorticoids may affect growth by directly inhibiting collagen metabolism. Linear growth requires the formation of new bone, which in turn requires the synthesis of type I collagen. Type I collagen is first synthesized as a precursor, procollagen. Significant decreases in this precursor have been recorded in children receiving corticosteroids for either asthma or inflammatory bowel syndrome (Hyams *et al.*, 1988). This would indicate that corticosteroids may act by inhibiting type I procollagen production and/or increasing the catabolism and excretion of this propeptide.

9.1.2 Specific Effects of Glucocorticoids on Tissue Growth

Animal studies have reinforced clinical observations, demonstrating that glucocorticoids lead to a suppression of growth in the young and a whole body atrophy in adult animals (Loeb, 1976; Tomas *et al.*, 1979; Kelly & Goldspink, 1982,1983,1984; Kelly *et al.*, 1986). More specifically, these animal studies have demonstrated that glucocorticoids have a differential effect on protein metabolism in various tissues. The anabolic effect that glucocorticoids have on the liver was reported some time ago and characterized by an increased hepatic protein content following cortisone administration (Munro, 1964). A more recent series of experiments has investigated the growth patterns of the musculature and lymphoid tissues of the rat in response to dexamethasone (2.5mg/Kg body weight/ day for 1 to 10 days). Growth was enhanced in the heart, reduced in slow-twitch skeletal muscle and abolished in fast-twitch skeletal muscle (Kelly & Goldspink, 1982; Kelly *et al.*, 1986). Of the tissues examined, the lymphoid organs showed the greatest sensitivity to dexamethasone undergoing a rapid and marked atrophy (Kelly &

Goldspink, 1983,1984). These different responses to dexamethasone treatment were explained by steroid-induced changes in tissue protein synthesis and/or protein breakdown.

9.2 Growth in Infants with Chronic Lung Disease Treated with Dexamethasone

The well-established growth-suppressive qualities of glucocorticoids have major implications in their role as a potential therapy for CLD. Infants with this severe neonatal disorder do not thrive (see Section 3.3.1). These patients should not, if possible, be exposed to an agent which has the capacity to further hinder optimal growth. More specifically, successful growth and repair of the lung in neonates with CLD is vital if they are to break free from their dependence on respiratory support and ultimately recover. Animal studies have demonstrated a high level of dexamethasone binding to the lung, which among the tissues studied, was only surpassed by the thymus (Granberg & Ballard, 1977). More recently, a relatively high level of glucocorticoid receptor mRNA has been reported in the lung (Kalinyak *et al.*, 1987). These results indicate that the lung may represent a highly sensitive target tissue for glucocorticoid activity. The adverse effect of dexamethasone on the growth of the musculature and lymphoid tissues of the of the rat, at a dose comparable to the clinical regime employed for CLD, questions the effect of steroid therapy on pulmonary growth in preterm infants.

10. Rationale and Research Objectives

Premature infants who develop acute respiratory distress and subsequently succumb to severe CLD experience a whole body growth deficit. More specifically, evidence suggests that the immature lung does not grow and develop properly in such a disease state. Pre- and postnatal treatment and support is employed clinically, in an attempt to alleviate the respiratory distress of preterm babies and to prevent the onset of secondary complications. In situations where a premature birth threatens, antenatal glucocorticoid treatment may be initiated due to the ability of these steroids to induce lung maturation. The provision of supplementary oxygen

is essential in supporting neonatal respiration, while postnatal glucocorticoid treatment is employed in the hope of improving a rapidly deteriorating respiratory status.

Previous animal studies have reported that glucocorticoids and hyperoxia can themselves inhibit growth. There are limitations in this data however when investigating pulmonary growth in the premature infant, either in the animal model employed or the specific tissues studied. This thesis, with the use of more appropriate animal models and experimental systems, has investigated the following hypotheses:

A. The provision of supplementary oxygen to premature infants has the potential to suppress lung growth and repair by inhibiting pulmonary protein synthesis.

B. The hyperoxic-induced inhibition of pulmonary protein synthesis is mediated via an increased concentration of oxidized glutathione in the lung.

C. Despite the obvious benefits of glucocorticoid treatment for acute and chronic lung disease, these steroids have the potential to inhibit pulmonary protein synthesis, and thereby hinder lung growth and repair in the premature infant.

CHAPTER 2. MATERIALS AND METHODS

2.1 Experimental Animals

All animals were obtained from colonies maintained within the School of Biochemical and Physiological Sciences, at Southampton University. The animals were housed in a room controlled for temperature (22°C) and light (12 hours, 06:00 to 18:00).

Rats

Wistar rats were allowed free access to water and fed *ad libitum* on PRD diet (Special Diet Services, Lavender Hill, Cambridgeshire) containing 19.7% protein. All experiments involved the use of male, adolescent animals which were four to five weeks of age (approximately 100 g).

Guinea Pigs

Dunkin-Hartley strain guinea pigs were allowed free access to water and fed *ad libitum* on sterilised hay and RGP diet (Special Diet Services, Lavender Hill, Cambridgeshire) containing 17.4% protein.

Neonatal animals were obtained by normal parturition at full-term and nursed by their dams for two days. Adolescent animals were four to five weeks of age (approximately 250 g). Timed pregnancies with an accuracy of ±1 day were carried out by taking daily vaginal smears from virgin guinea pigs to establish their oestrus cycles. Microscopic examination identified ovulation as the day before the influx of leucocytes into the vagina. Fertilization was then achieved by housing the females with a male three days before the next ovulation. Following a successful fertilization, gestational day zero was taken as the day of ovulation. Using this method, a normal gestation ends with birth on day 68.

2.2 Oxygen Exposure

Oxygen exposure was carried out in a 25 litre perspex chamber flushed with oxygen (BOC) at a rate of 3 litres/minute. On average four neonatal guinea pigs were housed in a chamber together with a lactating dam. Adolescent guinea pigs or rats were housed three and six to a chamber respectively. Oxygen concentration, continuously monitored with an oxygen electrode (IL 407 apparatus; Instrumentation Laboratory, Lexington, MA, USA) varied between 95 and 98%. The temperature ranged from 24 to 26°C. Control animals were maintained in identical cages which were flushed with room air at a similar rate to the oxygen supply. Animals were weighed at the beginning of each experiment, which always commenced at 09:00 h, and daily thereafter.

To ensure optimal nutritional support for the neonatal guinea pigs, several additional measures were taken. The lactating dam assigned to each chamber was rotated between the hyperoxic environment and room air every 24 hours to prevent oxygen-induced illness and consequent poor mothering. In addition, a supplementary feeding regime was initiated in the evening of the first experimental day and three times daily thereafter. Pups were removed from their hyperoxic chamber for no longer than five minutes and presented with half strength infant feed formula, SMA (Wyeth Nutrition, Maidenhead, Berks.) via a rubber teat.

2.3 Dexamethasone Administration

Adolescent Rats

Dexamethasone acetate (Sigma Chemical Company) was administered subcutaneously as a suspension in 0.9% NaCl. All experimental animals received the steroid at a dose of 2.5mg/Kg body weight/day, while the controls received an equivalent volume (0.1ml) of 0.9% NaCl.

Pregnant Guinea Pigs

Daily subcutaneous injections of dexamethasone acetate (2.0mg/Kg body

weight suspended in 0.9% NaCl) or an equivalent volume (1.0ml) of 0.9% NaCl commenced on day 55 of gestation. On gestational day 61, a Caesarian section was carried out under halothane anaesthesia (2-4%), maintained with nitrous oxide (0.4 litre/minute) and oxygen (1.5 litre/minute). The pregnant dam was placed supine on the operating table and the surgical site was prepared by shaving. A longitudinal incision was made through the skin and peritoneum to expose the uterus and its contents. The pups were then promptly removed, the oropharynx cleared and the umbilical cord double clamped and cut. All pups were delivered within four to five minutes of the initiation of anaesthesia. Following delivery, the pups were actively stimulated by rubbing gently with cotton wool. Gentle shaking encouraged the clearing of fluid from the tracheo-bronchial tree. The pups were then dried in a stream of warm air. Any pup in whom respiration was not established within the first two to three minutes by this approach was exposed to bursts of dry 100% oxygen.

All dexamethasone injections together with daily body weight recordings were carried out between 09:00 and 10:00 h.

2.4 Measurement of *in vivo* Pulmonary Protein Synthesis in the Rat

Materials

Reagents were purchased as follows:

L-[4-^{3}H]phenylalanine (specific radioactivity 28Ci/mmol), The Radiochemical Centre, Amersham.

Liquiscint scintillation fluid, National Diagnostics.

Quinine Sulphate, BDH.

Ninhydrin, leucylalanine, ß-phenylethylamine, phenylalanine decarboxylase and pyridoxal phosphate were all obtained from Sigma Chemical Company.

Solutions

L-[4-^{3}H]phenylalanine combined with 150mM phenylalanine in 0.9% NaCl to give approximately 48μCi/ml stored at -20°C

5mM ß-phenylethylamine " "

0.3M Perchloric acid	stored at room temperature	
6M Hydrochloric acid (HCl)	"	"
0.01M Sulphuric acid (H_2SO_4)	"	"
3M Sodium hydroxide (NaOH)	"	"
0.5M sodium phosphate buffer pH8.0	"	"

1.28×10^{-3}M Quinine Sulphate stored in the dark at room temperature.

The following solutions were prepared on the day of use:

0.14U/ml Phenylalanine decarboxylase and 4.05×10^{-3}M pyridoxal phosphate suspended in 0.5M sodium citrate buffer, pH6.3

2mM Leucylalanine

50mM Ninhydrin.

Experimental Procedure

The rate of protein synthesis in the rat lung was measured *in vivo* after an intravenous, bolus injection of phenylalanine to flood the precursor pool(s) (Garlick *et al.*, 1980) (see over). All experiments were carried out between 08:00 and 11:00 to minimise diurnal variation in the measured rate of protein synthesis (Garlick *et al.*, 1973). Rats were individually removed from their cages and restrained in a tea towel during the injection. The tail was immersed in warm water in order to dilate the lateral tail veins, hence making the site of injection more accessible. The injection consisted of 150μmol of phenylalanine, including 48μCi of L-[4-^{3}H]phenylalanine in 1.0ml of 0.9% NaCl/100 g body weight and was administered over 1 minute. After injection, animals were returned to their original environment for a further 9 minutes. At 10 minutes exactly, animals were decapitated. A blood sample was collected for 15 seconds, from which an aliquot of plasma was obtained by centrifugation. The lung was then rapidly removed, rinsed in ice-cold saline, blotted dry, frozen in liquid nitrogen, weighed and stored at -20°C until analysis.

Sample preparation involved homogenizing 200 mg of tissue in 25 volumes of ice-cold 0.3M perchloric acid with a ground glass homogenizer. An aliquot of the homogenate was retained for the measurement of tissue protein, while the remainder was centrifuged at 2000 rpm for 10 minutes at 4°C in a Sorvall RT6000

centrifuge. The supernatant which represented the intracellular pool of amino acids was decanted into vials and frozen. The pellet which contained the protein bound phenylalanine was washed twice with 2.5ml aliquots of ice-cold 0.3M perchloric acid and hydrolysed in 3mls 6M HCl at 110°C for 12 hours. The acid was removed by evaporation under vacuum at 110°C and the remaining amino acids were resuspended in 1.1mls of 0.5M sodium citrate buffer, pH6.3.

The determination of the specific activity of phenylalanine necessitated its enzymatic conversion to ß-phenylethylamine. This involved the incubation of 1.0ml of supernatant or 0.5ml of hydrolysate with 0.25ml or 0.5ml of a suspension of phenylalanine decarboxylase (14U/ml) and pyridoxal phosphate ($4.05x10^{-3}$M) respectively overnight at 52°C. On the following morning, ß-phenylethylamine was extracted by adding 0.5ml 3M NaOH and shaking vigorously with 10mls of chloroform/n-heptane (1:3, v/v). The organic layer was removed, added to 5mls of chloroform plus 4mls of 0.01M H_2SO_4 and shaken vigorously. The upper (aqueous) layer was removed for liquid scintillation counting and ß-phenylethylamine analysis.

For the radioactivity measurements, 1.0ml (of supernatant) or 2mls (of hydrolysate) of the aqueous extract were added to 10mls of Liquiscint scintillant. Each sample was counted in an LKB 1219 scintillation counter for 20 minutes at 20-30% efficiency.

1.0ml (of supernatant) or 0.04ml (of hydrolysate made up to 1.0ml with 0.01m H_2SO_4) of the aqueous extract were assayed for ß-phenylethylamine, using 0.5 to 20.0 nmols ß-phenylethylamine as a standard. 2.5mls of 0.5M sodium phosphate buffer, 1.0ml of 50mM ninhydrin and 0.5ml of leucylalanine were added to 1.0ml of the samples and standards in duplicate. Following incubation at 60°C for 1 hour, samples and standards were cooled on ice for 5 minutes and placed in water at room temperature for 10 minutes before the fluorescence was read at 495nm (excitation 390nm). With each experiment, enzyme blanks were routinely analyzed in parallel with the tissue samples and standards.

Selection of a Suitable Precursor Pool for the Measurement of Protein Synthesis

Protein synthesis is measured by following the incorporation of a suitable radiolabeled amino acid into protein, and requires the knowledge of the specific radioactivity of that amino acid in the precursor pool for protein synthesis. Studies in the perfused lung have demonstrated that when ^{14}C-phenylalanine is present in the perfusate at normal plasma concentrations, the specific radioactivity of tRNA-bound phenylalanine, the immediate precursor for protein, was different to that of phenylalanine in the free extracellular and intracellular pools (Watkins & Rannels, 1980). Under these conditions, rates of protein synthesis were in error if calculated from the specific radioactivity of the free (intracellular or extracellular) amino acid pools. Under these conditions an accurate determination of the rate of protein synthesis requires the direct analysis of the specific radioactivity of tRNA-bound phenylalanine. The measurement of the specific radioactivity of amino acids acylated to tRNA is however technically difficult. In the perfused lung, this problem can be circumvented if the concentration of extracellular phenylalanine is increased appreciably (Watkins & Rannels, 1980). Under such conditions, the specific radioactivity of intracellular and extracellular phenylalanine is equal to that of tRNA-bound phenylalanine and therefore the extracellular value can be used with validity to calculate rates of pulmonary protein synthesis. Conversely in isolated fibroblasts and macrophages, the specific radioactivity of aminoacyl-tRNA fails to reach that of the extracellular or intracellular pool upon increasing the extracellular concentration of the marker amino acid (Hammer & Rannels, 1981; Hildebran *et al*., 1981). Analysis of the specific radioactivity of tRNA-bound amino acid is therefore essential in these isolated cells in determining synthesis rates if substantial errors are to be avoided. In the present work, protein synthesis in the lung has been measured *in vivo* following the method of Garlick *et al*. (1980). The intravenous injection of ^{3}H-phenylalanine administered with a large dose of unlabelled phenylalanine is believed to flood the intracellular and extracellular pools of free phenylalanine and the tRNA-bound phenylalanine pool, enabling the specific radioactivity of the former to be used in the protein synthesis determination. Proof that isotopic equilibrium is reached within the whole lung is supported by previous *in vitro* (Watkins & Rannels, 1980) and *in vivo* (Laurent, 1982) work, however absolute validation would require the comparison of the specific radioactivity of phenylalanine in the extracellular, intracellular and aminoacyl-tRNA pools.

Calculation of the Rate of Protein Synthesis

The fractional rate of protein synthesis, K_s, is expressed as the percentage of tissue protein synthesized per day and was calculated from:

$$K_s = \frac{S_B \times 100}{S_A t}$$

S_A and S_B are the specific radioactivities of phenylalanine in the free tissue pool (i.e. intracellular and extracelluar) and bound into protein respectively, and t is the incorporation time in days.

Calculation of the Rate of Protein Breakdown

Since the growth of a tissue arises as a consequence of imbalances between the rates of protein synthesis and rates of protein breakdown, the fractional rate of protein breakdown, K_b, was calculated from the predetermined fractional synthetic rate, K_s and the growth rate, K_g, i.e.,

$$K_b = K_s - K_g$$

The fractional growth rate was calculated as the percentage change in protein mass which occurred over a specified number of days close to the determination of protein synthesis (see over).

2.5 Measurement of *in vivo* Pulmonary Protein Synthesis in the Guinea Pig: Validation of an Intraperitoneal Route for the Administration of ^{3}H-phenylalanine

As the guinea pig does not have a suitable intravenous injection site, an alternative route for radiolabel administration was identified. After careful consideration, an intraperitoneal route was chosen. The original methodology for measuring protein synthesis *in vivo* relies on certain assumptions (Garlick *et al.*, 1980) regarding the distribution of labelled amino acids. For this reason, preliminary

The Calculation of Rates of Protein Breakdown by Difference

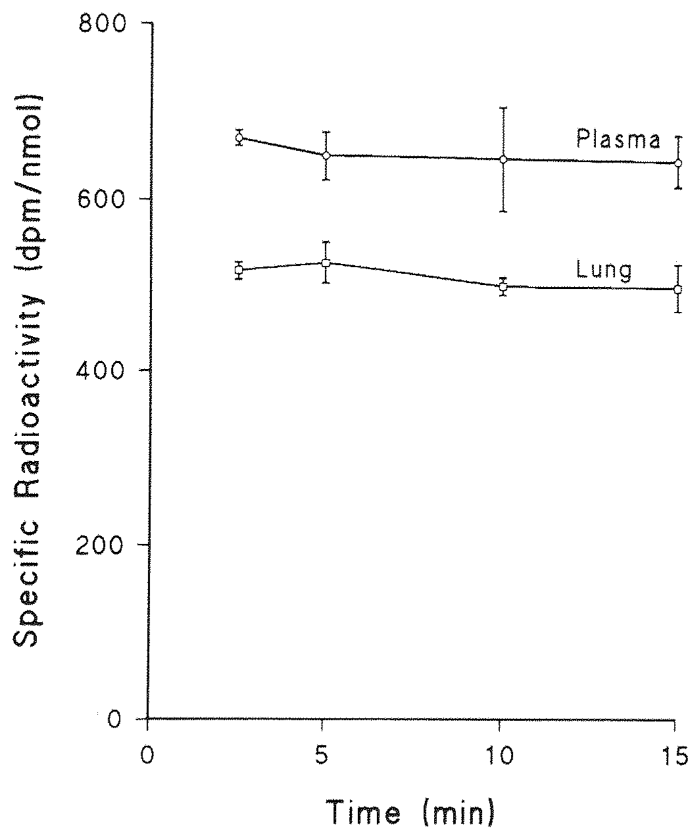
The accuracy of the derived values of protein breakdown (K_b) will depend upon the accuracy of the measured rates of tissue synthesis and growth. Of the two, the latter is most likely to be in error. Ideally, growth should be measured at the specific time at which synthesis was determined. In practice however, tissue growth is measured over a longer time period (ranging from 1 to 5 days in this thesis). When growth is linear, as the control tissues, this does not present a problem. It must be appreciated however, that the daily rate of tissue growth when measured over a number of days during an experimental procedure (for example hyperoxic exposure or dexamethasone treatment), will not necessarily represent the rate at the beginning or end of the experiment. For this reason, the growth and breakdown rates of experimental tissues are very often less accurate than those of the control tissues.

studies were necessary to establish the kinetics of radiolabel distribution in the guinea pig following the injection via an intraperitoneal route.

For the preliminary study, neonatal guinea pigs were injected intraperitoneally with radiolabelled tracer. The injection consisted of 150μmol of phenylalanine including 48μCi of L-[4-^{3}H]phenylalanine in 1.0ml of 0.9% NaCl/100 g body weight. At 2.5, 5.0, 10.0 and 15.0 minutes after the injection, animals were decapitated. A blood sample was collected in an heparinised tube over the following 15 seconds, from which an aliquot of plasma was obtained immediately by centrifugation. Tissues were rapidly dissected, rinsed in ice-cold saline, blotted dry, frozen in liquid nitrogen and stored at -20°C until analysis. The specific radioactivity of free phenylalanine in the plasma and in the tissues at different times after radiolabel injection was measured as described in section 2.4.

Figure 8 illustrates the specific radioactivity (S_A) of free phenylalanine in the plasma and lung at different times after the intraperitoneal injection of labelled amino acid. The specific radioactivity in the lungs of neonatal guinea pigs reached 76% of that in the plasma at 2.5 minutes after the injection. This suggested that following an intraperitoneal injection, a rapid equilibrium occurred between the plasma and the lung in parallel to that observed following an intravenous injection in the rat (Garlick *et al.*, 1980; Goldspink, 1987). The specific radioactivity measurements remained relatively constant in the plasma and lung following the injection. Based on these observations, a 10 minute incorporation period was undertaken in all subsequent *in vivo* protein synthesis experiments which employed the guinea pig as the animal model.

Figure 8. Changes in Specific Activity of Free Phenylalanine in the Plasma and Lung of Neonatal Guinea Pigs



Neonatal guinea pigs were killed at 2.5, 5.0, 10.0 and 15.0 minutes after an i.p. injection of ^{3}H-phenylalanine. The specific activities of free phenylalanine were determined at each time point in the plasma and lung as described by Garlick *et al.* (1980). Each point represents the mean ± S.D. derived from 4 animals

2.6 Isolation of Ribosomal Particles and Polysomes from Rat Lung on Sucrose Density Gradients

Materials

All chemicals were purchased from Sigma Chemical Company.

Solutions

Homogenizing buffer: 150mM potassium chloride (KCl), 10mM Tris-HCl, pH7.4. 10mM magnesium chloride ($MgCl_2$).

0.59 and 1.46M sucrose in homogenizing buffer.

All solutions were prepared on the day before use and stored at 4°C.

Experimental Procedure

Lungs were minced with scissors and homogenized in 3 volumes of ice-cold buffer containing 150mM KCl, 10mM Tris-HCl, pH7.4 and 10mM $MgCl_2$ using 12 strokes of a dounce homogenizer (Rannels *et al.*, 1979). Aliquots of the homogenates were retained for RNA analysis and the remainder were centrifuged at 10,000 x g for 10 minutes at 4°C in the JA-20 rotor of a Beckman J2.2 centrifuge. Triton X-100 was added to the resulting supernatants to give a final concentration of 1% (v/v). Samples (0.75 to 0.9ml) were layered onto freshly prepared 0.59 to 1.46M linear sucrose gradients made with the homogenizing buffer. Under conditions where the experimental treatment led to a reduction in pulmonary RNA content, the concentration of the extract was adjusted to ensure an equivalent amount of RNA was loaded onto both control and experimental gradients. The gradients were centrifuged at 300,000 x g in a TST41.14 rotor (Kontron Instruments) for either 220 minutes or 7 hours for the resolution of polysomes and ribosomal particles respectively. The contents of each gradient was then pumped (2ml/min) from the bottom using a peristaltic pump, through the flow cell of a UA-5 spectrophotometer (Instrumentation Specialities Co.) and the absorbance at 254nm was recorded on a chart recorder. The fractions representing 40 S, 60 S and 80 S particles were collected for the measurement of RNA content. The polysome content of each sample was quantitated by weighing a photocopied absorbance profile.

2.7 Measurement of Pulmonary RNA Content

The RNA content of the lung was determined using the method of Fleck and Munro (1962)

Basis of the Reaction

RNA and DNA are separated in tissue samples during a digestion in alkali which hydrolyses the RNA into acid soluble mononucleotides. Upon acidification of the digest, the DNA and protein are precipitated. The acid soluble fraction is retained and the absorbance at 260nm is read to determine the RNA content.

Solutions

0.08M, 0.3M, 4.0M Perchloric acid	stored at 4°C
0.3M Potassium hydroxide (KOH)	" "
1mg/ml bovine serum albumin (BSA) in 0.1M NaOH	stored at -20°C

Assay Procedure

Lung homogenates (100 mg in 5mls 0.3M perchloric acid) were centrifuged at 2000 rpm for 10 minutes at 4°C in a Sorvall RT6000 centrifuge. The supernatant was discarded while the pellet was washed twice with 4ml aliquots of ice-cold 0.08M perchloric acid before being dissolved in 3mls of KOH for 1 hour at 37°C. The alkaline digest was acidified by adding 1ml 4M perchloric acid and the precipitate was centrifuged at 2000rpm for 10 minutes at 4°C. The absorbance of the supernatant was read at 260nm against a KOH blank and the concentration of RNA in the sample was calculated from:

$$\text{mg RNA/g tissue} = \frac{A_{260} - KOH_{bl}}{31.25 \times \text{homog. wt.}}$$

31.25 = Absorbance of 1 mg hydrolysed RNA at 260nm

Measurement of RNA in the 40 S, 60 S and 80 S ribosomal particles

Bovine serum albumin (0.2ml), acting as a carrier protein, was added to

each pooled fraction. The alkaline digestion employed 0.3ml 0.3M KOH while the precipitation of the protein required 0.1ml 4M perchloric acid. The precipitate was centrifuged at 2000rpm for 10 minutes at 4°C and the absorbance of the supernatant was read at 260nm against 37% sucrose in 150mM KCl, 10mM Tris-HCl, pH7.4, 10mM $MgCl_2$. The RNA content of each fraction was calculated from:

$$1 \text{ unit of absorbance at } 260\text{mM} \equiv 40\mu g \text{ RNA/ml}$$

and expressed as μg/mg homogenate RNA.

2.8 Preparation of a Cell-free Protein Synthesizing System From Rat Lung

The protocol employed was based upon an original description by Eisenstein and Harper (1984) who characterized a protein synthesizing system from rat liver.

Materials

Reagents were purchased as follows:

L-[4-^{3}H]phenylalanine (specific radioactivity 28Ci/mmol) and NCS tissue solubilizer, The Radiochemical Centre, Amersham.

Optiphase scintillation fluid, LKB Scintillation Products.

Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (disodium salts), Boehringer Mannheim Biochemicals.

Creatine phosphate (dipotassium salt) Calbiochem Behring.

Edeine was a generous gift from Dr. Virginia Pain, Department of Biochemistry, University of Sussex.

All other chemicals were obtained from Sigma Chemical Company.

Preparation of the Reaction Mix

Small aliquots of the individual stock solutions were stored at the appropriate temperature and thawed if frozen on the day of use:

1.38M Potassium acetate (KAc)	stored at 4°C
500mM HEPES (K0H), pH7.55	" "
314mM Magnesium acetate (MgAc)	" "
393mM Dithiothreitol (DTT)	stored at -20°C
8mM Spermidine HCl pH7.5	" "
2.57mM amino acid mix (minus Leu, Lys, Tyr, Phe)	stored at -20°C.
25.7mM ATP	stored at -80°C
10mM GTP	" "
257mM creatine phosphate	" "

5.14mM leucine, lysine, tyrosine, phenylalanine were prepared on the day of use.

Storage of Inhibitors

90mM Oxidized glutathione (GSSG)

4.5mM 7-methylguanosine-5'-triphosphate (m^7GTP)

0.5mM Edeine, were all made up in distilled water and stored at -20°C.

Preparation of the Postmitochondrial Supernatant

Adolescent male Wistar rats (100 g body weight) were used as the source of tissue. Animals were killed by cervical dislocation, the lung quickly dissected out, rinsed in ice-cold buffer (final concentrations: 165mM KAc; 2mM MgAc; 50mM HEPES (KOH), pH7.55; 2.5mM DTT), blotted dry and placed on ice in the homogenization buffer. All procedures beyond this point were carried out at 2°C. 1 g of tissue was minced with scissors and homogenized in 2 volumes of ice-cold buffer using 10 strokes of a dounce homogenizer. The homogenate was centrifuged at 12,000 x g for 10 minutes at 4°C in the JA-20 rotor of a Beckman J2.2 centrifuge. The freshly prepared postmitochondrial supernatant was decanted and used immediately.

Reaction procedure

87.5μl of the postmitochondrial supernatant,

87.5μl of the reaction mix, final concentrations:

165mM KAc

2mM MgAc

50mM HEPES (K0H), pH7.55

1mM ATP

0.5mM GTP

10mM creatine phosphate

2.5mM DTT

2.25 U/ml creatine phosphokinase

0.15mM spermidine HCl pH 7.5

all amino acids at 100μM

and 25μl of water or another addition (see below) were added together in LP3 tubes and kept on ice. The reaction was initiated by adding 25μl (25μCi) L-[4-^{3}H]phenylalanine to give a final reaction volume of 225μl. Immediately after this, the tube was vortexed for 2 seconds, a 25μl zero time sample was removed and the tube was placed in a water bath at 30°C. Additional 25μl aliquots were removed at appropriate time intervals. Upon reaction completion, an aliquot of the remaining assay mix was diluted 15 fold and retained for the measurement of protein content.

The 25μl water aliquot was substituted for GSSG when assaying the influence of this compound on the system. Alternatively, specific inhibitors of initiation, edeine (10μM) or m^{7}GTP (500μM) were routinely added to determine the ability of the cell-free system to reinitiate.

Filter Paper Processing

Upon removal from the reaction, the 25μl aliquots were spotted onto numbered Whatman No.1 filter paper discs (24mm diameter) and placed into ice-cold 10% trichloroacetic acid containing 2mM phenylalanine. Ten minutes after the last disc was added, the filters were carried through a series of washes:

1. Ice-cold 5% trichloroacetic acid for 10 minutes to remove unincorporated [^{3}H]phenylalanine.

2. Boiling 5% trichloroacetic acid for 10 minutes to hydrolyse charged tRNA.
3. Fresh ice-cold 5% trichloroacetic acid for 10 minutes.
4. Absolute alcohol followed by acetone with gentle swirling for 1 minute.

The discs were dried under a heat lamp and placed into scintillation vials. To each filter, 0.5ml NCS tissue solubilizer was added. The vials were capped and incubated at 52°C for 1 hour to solubilize the protein from the filter. After allowing the vials to cool, 10mls of Optiphase scintillant was added and each sample was counted in a Raytest 4700 scintillation counter for 2 minutes at 40% efficiency.

The rate of protein synthesis in the cell-free system was calculated from the final specific radioactivity of the reaction mix (2.44×10^{12} dpm/mmol/phe) and expressed as moles of phenylalanine incorporated/mg postmitochondrial supernatant protein/minute.

2.9 Analysis of Ribosomal Particles and Polysomes in the Cell-free System on Sucrose Density Gradients

Cell-free systems were prepared from rat lung as described above, with a final reaction volume of 1.3mls and without radioactive phenylalanine. 5 minutes after the initiation of the incubation at 30°C, the samples were placed on ice to terminate the reaction and Triton X-100 was added to give a final concentration of 1% (v/v). Samples (1.2mls) were layered onto freshly prepared, linear sucrose gradients in 150mM KCl, 10mM Tris, pH7.4 and 10mM $MgCl_2$. The gradients were centrifuged at 300,000 x g in a TST41.14 rotor (Kontron Instruments) for either 220 minutes or 7 hours for the resolution of polysomes and ribosomal particles respectively. The absorbance at 254nm of the gradients was monitored using a UA-5 spectrophotometer (Instrumentation Specialities Co.) as described in section 2.6. The recorded absorbance profile was photocopied and weighed to quantitate polysomal and ribosomal material.

2.10 Protein Synthesis in the Reticulocyte Lysate

Materials

Reagents were purchased as follows:

L-[U-^{14}C]leucine (specific radioactivity 312 Ci/mmol), The Radiochemical Centre, Amersham.

Ready-solv scintillation fluid, Beckman.

ATP, GTP and Creatine phosphates (disodium salts), Boehringer Mannheim Biochemicals.

The reticulocyte lysate, haem and haem controlled inhibitor (HCI) were a generous gift from Dr. Virginia Pain, Department of Biochemistry, University of Sussex.

All other chemicals were obtained from Sigma Chemical Company.

Preparation of the Reaction Mix

Small aliquots of the individual stock solutions were stored at the appropriate temperature and thawed if frozen on the day of use:

750mM Potassium chloride (KCl)	stored at -20°C
20mM MgAc	" "
30mM Glucose	" "
10mM ATP	" "
2mM GTP	" "
100mM Tris-HCl, pH7.5	" "
1mM amino acid mix minus leucine	" "
840μM leucine	" "
L-[U-^{14}C]leucine	stored at 4°C

The following solutions were prepared on the day of use:

70mM creatine phosphate

1.3mg/ml creatine phosphokinase.

Storage of Inhibitors

20mM GSSG stored at -20°C

5mM m^7GTP " "

80 U/μl HCI in 20% glycerol, 240mM KCl, 16mM Tris-HCl, pH7.5, 0.8mM ethylenediaminetetra-acetic acid (EDTA), 5.6mM ß-mercaptoethanol, stored at -80°C. One unit of HCI is defined as the amount of protein which inhibits protein synthesis in the reticulocyte in the presence of haem by 50% after a 90 minute incubation.

Preparation of the Reticulocyte Lysate

The reticulocyte lysate was stored in aliquots under liquid nitrogen and the haem (1mM in Tris-HCl, pH7.5) was stored in aliquots at -20°C. Immediately before use, the lysate was removed from storage and thawed in the presence of haem at twice the concentration needed for optimal protein synthesis. This was previously established to be 15μM. For a haem deficient preparation, no addition was made during the thawing of the lysate.

Reaction Procedure

50μl of lysate

30μl of reaction mix, final concentrations:

75mM KCl

2mM MgAc

1mM ATP

0.2mM GTP

10mM Tris-HCl, pH7.5

all amino acids at 100μM

and 10μl of water or another addition (see below) were added together in LP3 tubes kept on ice. The reaction was initiated by adding 10μl (0.5μCi) L-[U-^{14}C]leucine to give a final reaction volume of 100μl. Immediately after this, the tube was vortexed for 2 seconds, a 10μl zero time sample was removed and the tube was incubated at 30°C. Additional 10μl aliquots were removed at appropriate time intervals.

The 10μl water aliquot was substituted for GSSG, HCl (2400U/ml) or m^7GTP (400μM) when assaying the influence of these compounds on the system.

Filter Paper Processing

Upon removal from the reaction, the 10μl aliquots were spotted onto numbered Whatman No.1 filter paper discs (25mm diameter) and placed into ice-cold 10% trichloroacetic acid containing 10mM leucine. 10 minutes after the last disc was added, the filters were carried through a series of washes:

1. Ice-cold 5% trichloroacetic acid containing 5mM leucine for 10 minutes to remove unincorporated [^{14}C]leucine.
2. Boiling 5% trichloroacetic acid containing 5mM leucine for 10 minutes to hydrolyse charged tRNA.
3. Fresh 5% trichloroacetic acid at room temperature for 10 minutes.
4. Absolute alcohol followed by acetone with gentle swirling for 1 minute.

After the filters had dried, 40μl 15% (w/v) hydrogen peroxide was added to each disc. The filters were left at room temperature for 1 hour, after which time the samples had bleached, thus preventing colour quenching during scintillation counting. The filters were sorted into scintillation vial inserts, 5mls of Ready-solv scintillant were added and each sample was counted in a Raytest 4700 scintillation counter for 5 minutes at 40% efficiency.

Rates of protein synthesis in the reticulocyte lysate were expressed as cpm of leucine incorporated/10μl reaction mix/minute.

2.11 Measurement of Total and Oxidized Glutathione in the Lung and Blood

Total and oxidized glutathione concentrations were determined in fresh samples employing the enzymatic recycling procedure described by Griffith (1980).

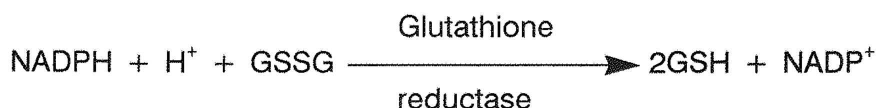
Basis of the Reaction

Reduced glutathione (GSH) reacts nonenzymatically with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to produce GSSG and the coloured ion 2-nitrobenozic

acid (TNB).

$$2GSH + DTNB \longrightarrow GSSG + TNB$$

GSSG, in the presence of NADPH and glutathione reductase, is reduced enzymatically to regenerate GSH.



$$NADPH + H^+ + GSSG \xrightarrow[\text{reductase}]{\text{Glutathione}} 2GSH + NADP^+$$

The regenerated GSH reacts with DTNB again, giving rise to enzymatic recycling.

To assay for total glutathione, NADPH, DTNB and the sample were equilibrated, during which time GSH is oxidized to GSSG. The reaction was initiated by adding glutathione reductase and the formation of TNB was monitored spectrophotometrically. The assay was made specific for GSSG by adding 2-vinyl pyridine, which derivitises reduced glutathione, allowing GSSG alone to be detected in the assay.

Materials

NADPH was purchased from Park Scientific. All other chemicals were purchased from Sigma Chemical Company.

Solutions

97% 2-vinyl pyridine	stored at -20°C
6% (v/v) Acetic acid	stored at 4°C
10% (w/v) Sulphosalicyclic acid	" "
0.3M Perchloric acid	" "

The following solutions were made up on the day of use:

0.3mM NADPH and 6mM DTNB in 125mM phosphate, 6.3mM EDTA buffer pH7.5.

179 U/ml Glutathione reductase in 3.6M $(NH_4)_2SO_4$.

20mM Glutathione.

Assay Procedure

Lungs (100 to 150 mg) were homogenized in 15 volumes of ice-cold 0.3M perchloric acid with an Ultra Turrax tissue homogenizer. Aliquots of the homogenate were retained for the measurement of protein and the remainder were centrifuged at 2000 rpm for 15 minutes at 4°C in a Sorvall RT6000 centrifuge. Alteratively, 500μl of whole blood was mixed with 100μl 6% (v/v) acetic acid and 400μl 10% (w/v) sulphosalicyclic acid and centrifuged in a microfuge at 10,000 x g for 5 minutes. The supernatants were retained and kept on ice. The glutathione stock solution was diluted over a range of 0.1 to 5.0 nmoles/ml. 200μl of the samples or standards in duplicate were equilibrated with 100μl of DTNB and 700μl of NADPH at 30°C for 5 minutes. 5μl glutathione reductase was added to each sample and immediately after this, the tube was vortexed for 2 seconds. The formation of TNB was monitored as an increase in absorbance at 412nm over 1 minute at 30°C in an LKB Biochrom Ultraspec II spectrophotometer. For the measurement of GSSG alone, the supernatants were incubated with 10μl of 2-vinyl pyridine at room temperature for at least 1 hour. Subsequently the samples were assayed as described for total glutathione. The glutathione content of the samples was calculated by reading the change in absorbance against the standard curve, and the results were expressed as nmoles/mg tissue protein or nmoles/mg haemoglobin.

2.12 Measurement of Antioxidant Enzyme Activities in the Lung

Tissue Preparation

Lungs (250 mg) were homogenized in 10 volumes of 10mM phosphate, 30mM KCl buffer pH7.4 with an Ultra-Turrax tissue homogenizer, and sonicated on ice (MSE Soniprep) for 60 seconds (6 x 10 second bursts with 5 second rests). Aliquots were removed for the measurement of DNA and the remainder was incubated on ice for 30 minutes with 1% (v/v) absolute alcohol. Samples were then vortexed with 1% (v/v) Triton X-100 and centrifuged in a microfuge for 5 minutes at 10,000 x g. The supernatants were decanted and frozen until analysis.

Measurement of Superoxide Dismutase Activity

Basis of the Reaction

Superoxide dismutase (SOD) was determined by the method of Marklund (1985). The assay is based on the autoxidation of pyrogallol, a reaction dependent upon the superoxide ion and as a consequence, inhibited in the presence of SOD. This reaction has a coloured product and can be monitored as an increase in absorption at 420nm. CAT is included in the reaction to remove any hydrogen peroxide which would accelerate the reaction as pyrogallol is also a good peroxidase substrate. SOD exists in two forms in eukaryotic cells, the mitochondrial enzyme, Mn-SOD and the cytosolic enzyme, CuZn-SOD. CuZn-SOD is inhibited by cyanide ions, hence Mn-SOD can be assayed specifically by the addition of cyanide to the reaction mixture.

Materials

All reagents were purchased from Sigma Chemical Company.

Solutions

The following solutions were prepared on the day of use:

50mM Tris-HCl, 1mM diethylenetriamine pentaacetic acid (DTPA), 0.1μM CAT buffer, pH8.2 for the analysis of total SOD.

50mM Tris-HCl, 1mM (DTPA), 0.1μM potassium cyanide buffer pH 8.2 for the analysis of Mn-SOD.

2.4mM pyrogallol in 0.01M HCl.

Assay Procedure

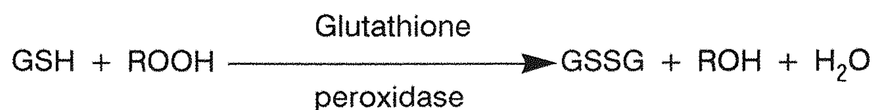
Bovine liver Cu/Zn-SOD was used as the standard and diluted over a range of 10 to 50U/ml. 50μl of standard or sample in duplicate, were added to 1ml of the appropriate assay buffer contained in the reaction cuvette. The reaction was initiated by adding 50μl of 2.4mM pyrogallol. The autoxidation was monitored as an increase in absorbance at 420nm in an LKB Bromma Ultralab 2086 MK II Kinetic Analyzer at 25°C over 2 minutes. At regular intervals throughout the assay, blanks were run which contained the assay buffer alone, plus pyrogallol. Total SOD and

Mn-SOD activity in the sample were calculated by measuring the magnitude of the reaction rate inhibition against that of the standard solutions and the reaction rate of the corresponding blanks. The results were expressed as units of SOD/mg tissue DNA.

Measurement of Glutathione Peroxidase Activity

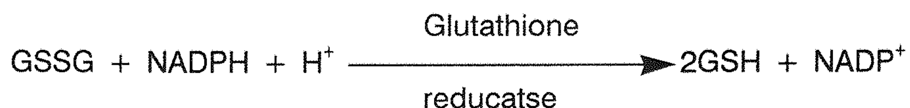
Basis of the Reaction

Glutathione peroxidase (GSH-Px) was determined using the method of Beutler (1979). The assay is based on the recycling of glutathione by glutathione reductase and GSH-Px present in the sample. In the presence of a peroxide, GSH-Px catalyses the oxidation of glutathione.



$$GSH + ROOH \xrightarrow[\text{peroxidase}]{\text{Glutathione}} GSSG + ROH + H_2O$$

The reduction of glutathione is catalysed by glutathione reductase, a reaction which utilizes NADPH as a cofactor.



$$GSSG + NADPH + H^+ \xrightarrow[\text{reducatse}]{\text{Glutathione}} 2GSH + NADP^+$$

This assay employs t-butyl hydroperoxide as the peroxide. The rate of GSSG production hence GSH-Px activity is measured by means of the glutathione reductase reaction, by following the oxidation of NADPH at 360nm.

Materials

NADPH was purchased from Park Scientific. All other chemicals were purchased from Sigma Chemical Company.

Solutions

The substrate solution was prepared on the day of use:

100mM Tris-HCl, 5mM EDTA, 0.22mM NADPH, 2.0mM GSH, 1U/ml glutathione reductase, pH8.0

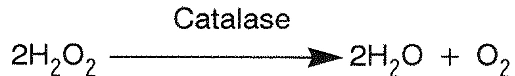
Assay Procedure

The reaction was initiated by adding 100µl 7mM t-butyl hydroperoxide to the reaction cuvette containing 10µl of sample and 1ml of the substrate solution. The oxidation of NADPH was measured as a decrease in absorbance at 360nm (using $\epsilon=6.22$) in an LKB Bromma Ultralab 2086 MK II Kinetic Analyzer at 37°C over 2 minutes. The activity of GSH-Px in the sample was calculated from:
1 unit of GSH-Px will catalyze the oxidation of 1µmole NADPH per min at 37°C and the results were expressed as units of GSH-Px/mg tissue DNA.

Measurement of Catalase Activity

Basis of the Reaction

Catalase (CAT) activity was measured by the method of Aebi (1984). The assay follows the reaction:



$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The activity of CAT is measured by following the decomposition of hydrogen peroxide spectrophotometrically.

The kinetic properties of CAT are such that it is not possible to saturate the enzyme with substrate since there is a rapid inactivation of catalase by hydrogen peroxide at concentrations above 100mM. The enzymatic decomposition of hydrogen peroxide is therefore a first order reaction, and was recorded as the initial rate (occurring over 0 to 30 seconds) in the presence of 10mM peroxide.

Materials

All reagents were purchased from Sigma Chemical Company.

Solutions

50mM phosphate buffer, pH7.0 stored at 4°C.

30mM hydrogen peroxide, prepared on the day of use.

Assay Procedure

Bovine liver CAT was used as a standard and diluted over a range from 25 to 300U/ml in the assay buffer. 50μl of standard or sample in duplicate were added to 950μl of the assay buffer contained in the reaction cuvette. The reaction was initiated by adding 500μl of 30mM hydrogen peroxide. The decomposition of the peroxide was monitored as a decrease in absorbance at 240nm in an LKB Biochrom Ultraspec II spectrophotometer at 25°C. The activity of CAT in the sample was calculated by reading the change in absorbance against the standard curve. The results were expressed as units of CAT/mg tissue DNA.

2.13 Measurement of Phosphatidylcholine and its Individual Molecular Species in the Lung

Total lipids were extracted from lung tissue using chloroform and methanol (Bligh & Dyer, 1959). An internal standard of dimyristoyl phosphatidylcholine (50nmol in trifluorethanol) was added to the extraction mixture to assess recovery. Phosphatidylcholine (PC) was isolated from the total lipid extract using disposable aminopropyl Bondelut columns (Jones Chromatography, Hengoed, Mid. Glamorgan, U.K.) (Caesar *et al.*, 1988). Individual molecular species of PC, dipalmitoyl phosphatidylcholine (DPPC) and palmitoyl oleoyl phosphatidylcholine (POPC) were analyzed using reverse phase high performance liquid chromatography (HPLC) on a C-18 column (Postle, 1987). The post-column fluorescence derivative formation technique using diphenyl hexatriene enabled direct quantitation of peaks eluting from HPLC regardless of their degree of acyl unsaturation. Results were expressed as μg total PC/mg tissue DNA and the percentage of which consitiutes DPPC and POPC.

2.14 Measurement of Pulmonary Protein Content

The protein content of the lung was determined by the method of Smith *et al.* (1985). This method enables the use of a small final reaction volume (210μl) and consequently allows a large number of samples to be analyzed rapidly with the use of microtitre plates.

Basis of the Reaction

Bicinchoninic acid, sodium salt, is utilized to monitor the cuprous ion (CU^{1+}) produced during the incubation of protein with the cupric ion (Cu^{2+}) in an alkaline environment (biuret reaction). The water soluble salt of bicinchoninic acid is a stable and highly sensitive reagent for Cu^{1+}, with which it forms an intense purple complex. The coloured complex is stable, increases in intensity over a broad range of protein concentrations and has an absorption maximum at 562nm.

Materials

Reagents were purchased as follows:

Crystalline BSA, Boehringer Mannheim Biochemicals.

Bicinchoninic acid, Sigma Chemical Company.

Solutions

1mg/ml BSA in 0.1M NaOH	stored at -20°C
0.3M Perchloric acid	stored at 4°C
50mM sodium phosphate buffer, pH7.5	" "
4% Cupric sulphate ($CuSO_4 . 5H_2O$)	" "

A working reagent containing 50 volumes of bicinchoninic acid and 1 volume of 4% $CuSO_4 . 5H_2O$ was prepared on the day of use.

Assay Procedure

Lung homogenates (200 mg in 5mls of 0.3M perchloric acid) were diluted approximately 10 fold with the assay buffer and sonicated on ice (MSE Soniprep) for 20 seconds (4 X 5 second bursts with 5 second rests) to solubilise any particles. BSA was used as a standard over a range of 0.2 to 1.0mg/ml in the

assay buffer. 10μl aliquots of standards and samples were pipetted in triplicate into microtitre plates to which was added 200μl of the working reagent. Following an incubation at 37°C for 30 minutes, the absorbance of the plate was read at 570nm (Dynatech Microelisa Autoreader MR580). The protein content of the samples was calculated by reading the absorbance against the standard curve and was expressed as mg/g tissue.

2.15 Measurement of Pulmonary DNA Content

The DNA content of the lung was determined using the method of Sterzel *et al.* (1985) which is an automated version of the method of Cesarone *et al.* (1979).

Basis of the Reaction

This technique utilizes the fluorochrome Hoechst 33258 which binds with high affinity to the base pairs in the large grooves of the DNA double helix. The binding is thought to be localized near the methyl group of thymidine. Upon interacting with the double helix, electrons in the double bonds of the fluorochrome become delocalized. This enhances the fluorescence of Hoechst 33258 to a degree which is proportional to the concentration of DNA in the sample.

Materials

All reagents were purchased from Sigma Chemical Company.

Solutions

Calf thymus DNA, 1mg/ml in 50mM phosphate buffer, pH7.5	stored at -20°C.
$3x10^{-4}$ Hoechst 33258	" "
9% NaCl	stored at 4°C
400mg/ml Brij	" "
10mM phosphate, 30mM KCl buffer, pH7.4	" "

The following solutions were prepared on the day of use:

$3x10^{-5}$ Hoechst 33258, protected from the light

Working diluent containing 0.9% NaCl, 40mg/ml Brij.

Assay Procedure

Lung homogenates (250 mg in 2.5mls of assay buffer) were diluted approximately 10 fold with the assay buffer. The homogenates were sonicated on ice (MSE Soniprep) for 20 seconds (4 X 5 second bursts with 5 second rests) to solubilize any particles. The DNA standard was diluted over a range of 1 to 10μg/ml and was routinely standardized by recording the absorbance at 260nm on the day of use. 1ml of samples and standards in duplicate were transferred to autoanalyzer cups, placed on the carousel of an adapted Technicon AAII based autoanalyzer system. The contents of each cup was mixed in a coil with working diluent, pumped into a blackened box where the fluorochrome was introduced and mixed again before being fed into the flow cell of a Perkin-Elmer LS-3B fluorescence spectrophotometer. The flow cell was excited and the fluorescence was read at 450nm and recorded on a chart recorder. The DNA content of the samples was calculated by reading the fluorescence against the standard curve, and expressed as mg/g tissue.

2.16 Measurement of Haemoglobin Concentration

Basis of the Reaction

Haemoglobin, in the presence of alkaline potassium ferricyanide is oxidized to methaemoglobin. Methaemoglobin reacts with potassium cyanide to form cyanmethaemoglobin which has an absorption maximum at 540nm.

Materials

All reagents were purchased from Sigma Chemical Company.

Solutions

Drabkins solution: Sodium bicarbonate, potassium ferricyanide, potassium cyanide, 0.05% Brij 35, stored at room temperature.

Cyanmethaemoglobin in Drabkins solution ≡ 18g/100ml whole blood, stored at 4°C.

Reaction Procedure

5mls of Drabkins solution was added to 20μl of whole blood or standard solution. Following a 20 minute incubation at room temperature the absorbance of the samples were read at 240nm in an LKB Biochrom Ultraspec II spectrophotometer. The haemoglobin content of the samples was calculated by reading the absorbance against the standard curve and was expressed as mg/100mls of whole blood.

2.17 Statistical Analysis of Results

The results presented represent the mean ± S.D. Differences between means were assessed for statistical significance using an unpaired Student's t-test. Means were regarded as significantly different from each other when a value of $P<0.05$ was obtained.

CHAPTER 3: THE EFFECT OF HYPEROXIA ON THE RATE OF PROTEIN SYNTHESIS AND THE GLUTATHIONE STATUS OF THE LUNG

3.1 INTRODUCTION

Premature birth before approximately 32 weeks of gestation is often associated with a complicated neonatal course due to the immaturity of the lung. As a consequence of an insufficient pulmonary surfactant system and a lack of alveolar development, such premature infants are unable to breathe independently, and experience acute respiratory distress (Farrell, 1982). Treatment for this condition involves both mechanical ventilation to open up the airways of the immature lung and the provision of supplementary oxygen to ensure oxygenation of the body. Although vital for survival, this intensive neonatal therapy often exacerbates the precarious state of the baby by damaging the delicate lung tissue. Infants with severe lung injury ultimately contract CLD, a fibrolytic lung disease which ensures that the baby continues to be dependent upon respiratory support (O'Brodovich & Mellins, 1985). A successful recovery from CLD is only possible if the infant can break free from intensive neonatal care, which in turn depends upon optimal pulmonary growth and repair. Unfortunately, in addition to the well documented delay in whole body growth (Markestad & Fitzhardinge, 1981; Vohr *et al.*, 1982; Kurzner *et al.*, 1988), lung function tests and morphological analysis suggest an abnormal pattern of lung growth and development in babies with CLD (Bryan *et al.*, 1973; Wheeler *et al.*, 1984; Sobonya *et al.*, 1982; Tepper *et al.*, 1986).

The high concentrations of inspired oxygen administered to premature infants is an obvious candidate for contributing towards their growth deficit. Hyperoxic exposure (≥95% oxygen, 24 h) inhibits the rate of protein synthesis in the lung of the adolescent rat (Kelly, 1988). Since the growth of a tissue requires the net accumulation of protein, any factor which decreases the rate at which protein is synthesized, will compromise tissue growth.

The mechanism by which hyperoxia leads to a decreased rate of protein synthesis has not, up until now, been investigated. Two lines of evidence suggest

however that the inhibition may be mediated as a result of a perturbation in the glutathione status of the lung. Glutathione acts as a cofactor for glutathione peroxidase in the reduction of hydrogen peroxide and lipid hydroperoxides. The oxidized form of glutathione (GSSG) is reduced by the NADPH-dependent enzyme, glutathione reductase. Under conditions of oxidative stress however, the recycling of glutathione is disturbed, leading to significant increases in the concentration of GSSG in the lung (White *et al.*, 1986, 1987; Jenkinson *et al.*, 1987, 1988a). Second, GSSG has a potent inhibitory action on protein synthesis in the reticulocyte lysate (Kosower *et al.*, 1971, 1972).

Previous studies investigating the effect of hyperoxia on the rate of protein synthesis and the glutathione status of the lung were derived from adult and adolescent animals. As a result, adequate explanations cannot be drawn from this work concerning events occurring in the lung of the human infant. Recently the neonatal guinea pig has been cited as a more appropriate model for the use in animal research, which has relevance to the human situation. The guinea pig has a relatively long gestational period compared to other small laboratory animals. In association with this, the pattern of pulmonary development in this species follows a similar time course to that seen in the human lung (Lechner & Banchero, 1982).

In this chapter I report my findings on the effects of both 24 and 72 hour hyperoxic exposures (≥95% oxygen) on the rate of protein synthesis in the lung of the neonatal guinea pig. The objective of this study was to gain a greater insight into the potential that hyperoxic therapy has in suppressing lung growth in the newborn. To parallel, with respect to age, the work initially carried out using the adolescent rat, this study was extended to the adolescent guinea pig. Together with the protein synthetic measurements, the glutathione status of the lung was also determined in these animals following oxygen exposure. These measurements were taken as an initial step in testing the hypothesis that following hyperoxia, an increased concentration of GSSG in the lung mediates the decreased rate of pulmonary protein synthesis.

3.2 EXPERIMENTAL PROTOCOL

Neonatal guinea pigs were obtained by normal parturition at term and nursed by their dams for two days. On the morning of the third day of life, animals were randomly divided into five groups. Group 1 were killed immediately and their lungs were analyzed as part of the growth rate determinations. For the acute experiment, animals in groups 2 and 3 were maintained in room air or hyperoxia (≥95% oxygen) for 24 hours. For the chronic experiment, animals in groups 4 and 5 were kept in room air or hyperoxia for 72 hours. The adolescent guinea pigs were divided into two groups which were maintained in either room air or hyperoxia (≥95% oxygen) for 24 hours. For full details of the oxygen exposure, refer to Chapter 2, section 2.2.

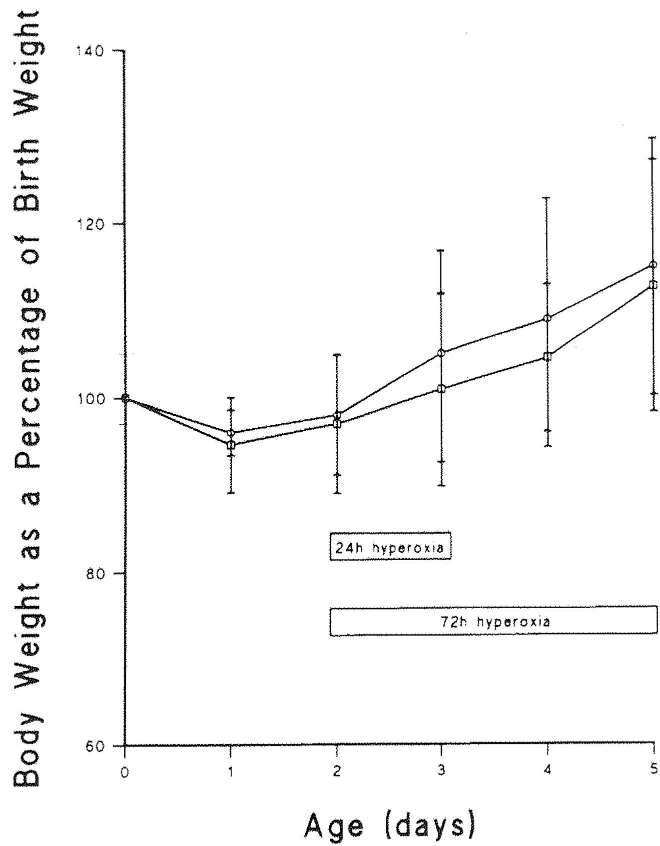
Following the appropriate experimental exposure, animals were injected intraperitoneally with a bolus dose of ^{3}H-phenylalanine and killed by decapitation. The lungs were then taken for the measurement of the rate of protein synthesis, glutathione status (including the blood) and tissue protein. Refer to Chapter 2, sections 2.5, 2.11 and 2.14 for an explanation of the methodology employed.

3.3 RESULTS

3.3.1 The Effect of Hyperoxia on Whole Body Weight of Neonatal Guinea Pigs

Figure 9 illustrates the body weight profile of newborn guinea pigs maintained in room air or exposed to hyperoxia. Following birth, guinea pigs lost 5 to 6% of their body weight over the first 24 hours. This weight loss was transient however, so that by the third day of life, i.e. at the beginning of the experiment, all animals were gaining weight. During the experimental period the body weight profile of the oxygen-exposed pups and the air controls followed a similar course. The average weight gain for both groups was 4% per day when measured over 4 days.

Figure 9. Body Weight Profile of Neonatal Guinea Pigs Exposed to Air or Hyperoxia



Full-term pups were obtained by normal parturition and nursed by their dams for 2 days. On the morning of the third day of life, pups were either maintained in room air (○) or exposed to hyperoxia (□) for the following 24 or 72 hours. Each point represents the mean ±S.D. n=7 and 9 for the 21% oxygen and ≥95% oxygen groups repectively.

3.3.2 Protein Turnover in the Lung of Neonatal Guinea Pigs Following Hyperoxia

The influence of an acute hyperoxic exposure (24 hours) on lung growth of neonatal guinea pigs was assessed by measuring the rate of protein deposition between the third and fourth day of life. Table 1 shows that the rate of lung growth was appreciable in both the normoxic and hyperoxic groups, averaging 7.6 to 8.1% per day. When the individual components of tissue growth (rates of pulmonary protein synthesis and breakdown) were examined however, it became apparent that hyperoxia was exhibiting a significant effect on the protein turnover of the lung (Table 2). After 24 hours exposure to ≥95% oxygen, there was a small (12%) but significant decrease in the fractional rate of protein synthesis. This reduction was reflected in the total amount of pulmonary protein synthesized, which was 13% lower than that of the control group. The ability of the lung to grow in a hyperoxic environment, despite a compromised rate of protein synthesis, was explained by the lower rate of pulmonary protein breakdown, which fell by 15% compared to the air controls (Table 2).

The inhibition of pulmonary protein synthesis in the adolescent rat was twice as great as that presently seen in the newborn guinea pig following 24 hours hyperoxia (Kelly, 1988). In addition, the rate of pulmonary protein breakdown increases in the rat under such conditions, resulting in a hyperoxic-induced atrophy of the lung. To determine whether the relatively small response of the neonatal guinea pig to 24 hours hyperoxia was the beginning of a larger response, resembling that of the adolescent rat, the effect of prolonged oxygen exposure on pulmonary protein synthesis was investigated. Following 72 hours in ≥95% oxygen, the lungs of neonatal guinea pigs were significantly heavier (22%) than the air controls (Table 3). This was due in part to the leakage of protein-rich fluid from the plasma into the lung, as a consequence of oxygen-induced tissue damage. The difference between the mean lung weights in the control and hyperoxic exposed group (Table 3) would suggest that the presence of pulmonary oedema could account for up to 0.3 g of tissue weight and would therefore produce an erroneously high measurement of total lung protein (calculated as the product of lung weight and protein per 100 mg of tissue). For this reason, the effect of prolonged hyperoxia on pulmonary protein and hence growth of the lung has not

Table 1. Lung weight, Protein and the Rate of Pulmonary Growth in Neonatal Guinea Pigs Following a 24 Hour Hyperoxic Exposure

Treatment	Age (days)	Tissue wt (g)	Protein content (mg)	Daily growth rate (K_g) (%/day)
Zero Control	2	0.98±0.17	146.2±25.6	
24 h 21% Oxygen	3	1.04±0.18	158.6±25.1	8.1
24 h ≥95% Oxygen	3	1.10±0.21	157.8±30.1	7.6

Total protein was measured by the method of Smith *et al*. (1985). Growth rates were derived from the changes in the pulmonary protein mass between 2 day old (zero controls) and 3 day old animals. The values presented are the means ± S.D. n=4, 10 and 16 for the zero controls, 24 h 21% oxygen and 24 h ≥95% oxygen groups respectively.

Table 2. Protein Turnover in the Lung of Neonatal Guinea Pigs Following a 24 Hour Hyperoxic Exposure

Treatment	Fractional rate of synthesis (K_s) (%/day)	Fractional rate of breakdown (K_b) (%/day)	Total Protein synthesized (mg/day)
24 h 21% oxygen	25.2±3.0	17.1	40.0±7.5
24 h ≥95% oxygen	22.1±2.8* (-12%)	14.5 (-15%)	35.0±8.6 (-13%)

The fractional rate of synthesis was determined as described by Garlick *et al.* (1980). The total protein synthesized per day represents the product of the fractional rate of synthesis and the total protein mass. The fractional rate of breakdown was calculated from the predetermined fractional rate of synthesis and the growth rate ($K_b=K_s-K_g$). The values presented are the means ± S.D. n=10 and 16 for the 21% oxygen and the ≥95% oxygen groups respectively. Values in parentheses show the percentage change from the 21% oxygen group. *$P<0.05$, statistically significant from the 21% oxygen group.

been tabulated. Despite the evidence of tissue damage upon extending the hyperoxic period to 72 hours, the inhibition of pulmonary protein synthesis was no greater than that elicited following the 24 hour oxygen exposure (Table 3). The inability to obtain a meaningful measurement of lung growth in neonatal guinea pigs after 72 hours in ≥95% oxygen, meant that it was not possible to calculate the rate of pulmonary protein breakdown in this group of animals.

Table 3. Lung Weight and Pulmonary Protein Synthesis in Neonatal Guinea Pigs Following a 72 Hour Hyperoxic Exposure

Treatment	Tissue wt (g)	Fractional rate of synthesis (%/day)
72 h 21% oxygen	1.18±0.06	25.2±3.0
72 h ≥95% oxygen	$1.51 \pm 0.13^{**}$ (+22%)	$21.5 \pm 1.7^{*}$ (-15%)

The fractional rate of protein synthesis was determined as described by Garlick *et al.* (1980). The values presented are the means ± S.D. n=6 and 9 for the 21% oxygen and ≥95% oxygen groups respectively. $^{*}P<0.01$, $^{**}P<0.001$, statistically significant from the 21% oxygen group.

3.3.3 The Effect of Hyperoxia on the Glutathione Status of the Lung and Blood of Neonatal Guinea Pigs

To investigate the hypothesis that an increased concentration of GSSG in the lung following hyperoxia mediates the inhibition of pulmonary protein synthesis, the glutathione status of the lung of neonatal guinea pigs exposed to ≥95% oxygen was measured and compared to the air controls (Table 4). Small (13%) increases in the concentration of both reduced and oxidized glutathione in the lung were evident after 24 hours exposure to hyperoxia. An extended period of oxygen

Table 4. The Glutathione Status of the Lung and Blood of Neonatal Guinea Pigs Following Hyperoxia

Treatment	LUNG (nmoles/mgPr)		BLOOD (nmoles/mgHb)	
	Reduced Glutathione	Oxidized Glutathione	Reduced Glutathione	Oxidized Glutathione
24 h 21% oxygen	14.92±3.32	0.55±0.11	2.46±0.65	0.04±0.02
24 h ≥95% oxygen	16.91±4.26	0.62±0.19	2.52±0.64	0.09±0.06
72 h ≥95% oxygen	14.95±2.60	0.41±0.11 (-25%)	4.17±1.63* (+70%)	0.14±0.06* (+250%)

Total and oxidized glutathione was measured by the method of Griffith (1980). Values presented are the means ± S.D. derived from at least 6 animals. Values in parentheses show the percentage change from the 21% oxygen group. *P<0.01, statistically significant from the 21% oxygen and 24 h ≥95% oxygen group.

exposure did not however result in further increases in pulmonary glutathione. Following 72 hours in ≥95% oxygen, the concentration of reduced glutathione was almost identical to the air controls. Furthermore, pulmonary GSSG was 25 to 35% lower than that of the control group and the animals maintained in hyperoxia for just 24 hours (Table 4).

The failure to detect an increased concentration of pulmonary GSSG following either 24 or 72 hours hyperoxia may reflect an initial accumulation, and as a consequence, and increased release of GSSG from the tissue into the circulation. To test this possibility, the glutathione status of the blood was measured. Table 4 shows that after 24 hours hyperoxia, there was an appreciable (125%) but non-significant increase in the concentration of GSSG in the blood. When animals were maintained in hyperoxia for a continuous 72 hours, the concentration of GSSG in the blood was further increased and was significantly higher (by 250%) than the blood of animals maintained in room air. In association with this, the concentration of reduced glutathione in the blood rose significantly following the prolonged hyperoxic exposure (Table 4).

3.3.4 Lung Protein Content and the Rate of Pulmonary Protein Synthesis in Adolescent Guinea Pigs Following Hyperoxia

The greater resistance of the neonatal guinea pig compared to the adolescent rat to oxygen-induced alterations in pulmonary protein metabolism could reflect an age and/or a species difference. To investigate such a phenomena, the present study was extended to the adolescent guinea pig.

Unlike the response of the adolescent rat to 24 hours hyperoxia (Kelly, 1988), lung wet weight and pulmonary protein were not reduced in the adolescent guinea pig (Table 5). The small (11%) increase in total lung protein of oxygen-exposed guinea pigs did not reflect a greater concentration of pulmonary protein, but a slightly higher (8%) lung mass in this group of animals. In contrast to the neonatal guinea pigs, the adolescent animals were resistant to a hyperoxic-induced inhibition of lung protein synthesis. The fractional rate of pulmonary protein synthesis was in fact 14% greater following hyperoxia (Table 5). This higher mean

Table 5. Lung Weight, Lung Protein and the Rate of Pulmonary Protein Synthesis in Adolescent Guinea Pigs Following a 24 Hour Hyperoxic Exposure

Treatment	Tissue wt (g)	Protein conc (mg/g)	Protein content (mg)	Fractional rate of synthesis (%/day)
24 h 21% oxygen	2.08±0.17	137.0±9.0	285.3±26.0	26.7±2.7
24 h ≥95% oxygen	2.24±0.21	141.0±4.0	316.2±30.8	30.4±5.6

Total protein was measured by the method of Smith *et al*. (1985). The fractional rate of protein synthesis was determined as described by Garlick *et al*. (1980). The values presented are the means ± S.D. derived from 6 animals.

value is not representative of the group as a whole. Three out of the six animals exhibited a pulmonary fractional synthetic rate of over 30%, while the values of the remaining animals were very similar to those of the controls (see Appendix 1.).

3.3.5 The Effect of Hyperoxia on the Glutathione Status of the Lung and Blood of Adolescent Guinea Pigs

Hyperoxia did not significantly affect the glutathione status of either the lung or blood of adolescent guinea pigs (Table 6). Such a response is essentially similar to that observed in neonatal guinea pigs after a 24 hour exposure to ≥95% oxygen (Table 4). The trend towards an increased concentration of GSSG in the blood of the newborn pups was not, however, evident in the older animals.

Table 6. The Glutathione Status of the Lung and Blood of Adolescent Guinea Pigs Following a 24 Hour Hyperoxic Exposure

Treatment	LUNG (nmoles/mgPr)		BLOOD (nmoles/mgHb)	
	Reduced Glutathione	Oxidized Glutathione	Reduced Glutathione	Oxidized Glutathione
24 h 21% oxygen	19.04±3.18	0.57±0.24	10.64±0.97	0.06±0.03
24 h ≥95% oxygen	18.12±1.89	0.51±0.07	9.07±0.94	0.06±0.02

Total and oxidized glutathione was measured by the method of Griffith (1980). Values presented are the means ± S.D. derived from 6 animals.

3.4 DISCUSSION

The deleterious effect of high concentrations of oxygen on cellular growth and, more specifically, on protein synthesis in mammalian systems of non-pulmonary origin has been appreciated for some time (Reuckert & Mueller, 1960; Hollenberg, 1971; Goetz, 1975; Almis-Kanigür *et al.*, 1982, 1983). More recently, an inhibition of protein synthesis in the lung of the rat was documented following a 24 hour hyperoxic exposure (Kelly, 1988). The provision of elevated inspired oxygen concentrations is a vital component of intensive neonatal care, employed to maintain the lives of very premature babies who develop acute and chronic lung disease. It is feasible that this hyperoxic support is wholly or partly responsible for the deficit in whole body and pulmonary growth cited in infants with severe CLD (Bryan *et al.*, 1973; Markestad & Fitzhardinge, 1981; Sobonya *et al.*, 1982; Vohr *et al.*, 1982; Wheeler *et al.*, 1984; Tepper *et al.*, 1986; Kurzner *et al.*, 1988). The studies outlined in this chapter have investigated the effect of hyperoxia on pulmonary protein synthesis in the neonatal guinea pig. It is hoped that this animal model is more applicable to the human infant, and has been employed here to assess the potential that supplementary oxygen has in suppressing growth and repair of the newborn lung.

Neonatal guinea pigs exposed to hyperoxia for 24 hours exhibited a small (12%) but significant decrease in the rate of pulmonary protein synthesis. Accompanying this fall, was a (15%) reduction of similar magnitude in the rate of protein breakdown in the lung. As a result, the lungs of the oxygen exposed animals grew at a rate comparable to the air controls over the 24 hour experimental period (K_g = 8.1 and 7.6%/day for the control and hyperoxic groups respectively). In contrast, in the adolescent rat, both a 25% decrease in lung protein synthesis and a 18% increase in pulmonary protein breakdown in response to a 24 hour hyperoxic exposure has been observed (Kelly, 1988). As a result, the rat lung underwent an atrophy following oxygen exposure (K_g = -17%/day), while pulmonary growth in the air controls is approximately 5%/day (Goldspink, 1987; Fussell & Kelly, 1990). An accelerated rate of protein breakdown in the rat lung following hyperoxia is an expected phenomenon. Oxygen free radicals are very reactive

towards proteins, modifying their structure and ultimately increasing their susceptibility to degradation by proteolytic enzymes (Davies & Goldberg, 1987a,b; Dean, 1987; Prinsze *et al.*, 1990).

When rats are exposed to hyperoxia for longer than 24 hours, their food intake is reduced (Kelly, 1988). Care was needed therefore when the effect of prolonged hyperoxia on lung growth of neonatal guinea pigs was investigated, to prevent a contribution from nutritional factors. The influence of food restriction on the rate of pulmonary protein synthesis has been investigated, however the results are conflicting (Gacad *et al.*, 1972; Rannels *et al.*, 1979; Preedy *et al.*, 1988). Gacad *et al.* (1972) observed that 48 hours starvation decreases *in vitro* protein synthesis in the lung of male rabbits. In support of this, a reduction in protein synthesis, estimated in perfused lungs in situ, has been observed after starving adult, male rats for 72 hours (Rannels *et al.*, 1979). In contrast, more recent work did not detect a decrease in pulmonary protein synthesis when measured *in vivo* in 200 g female rats following a 24 or 48 hour fast (Preedy *et al.*, 1988).

To ensure adequate nutritional support for the neonatal guinea pigs maintained in a hyperoxic environment for 72 hours, a supplementary feeding regime was adopted. During the 3 days in oxygen or air, pups accepted 2 to 3mls of the supplemental feed 3 times daily, suckled from their dam and fed from the solid food provided in the cages. These observations, together with the similar body weight profile of the oxygen- and air-exposed animals, strongly suggest that food intake was not adversely affected by a 72 hour hyperoxic exposure.

The heavier lungs of guinea pigs maintained in hyperoxia for 72 hours was indicative of pulmonary oedema. Oxygen-induced damage of the capillary endothelial cells allows the passage of serum proteins and fluid from the capillary lumen into the interstitial spaces of the alveolar wall. Pulmonary oedema ensues if the flow of fluid out of the capillaries exceeds the ability of the pulmonary lymphatics to pump the fluid out of the lung. Despite this evidence of tissue damage upon increasing the period of hyperoxic exposure from 24 to 72 hours, a greater inhibition of pulmonary protein synthesis did not occur. It is possible that in response to tissue injury, repair mechanisms become operational, which have the

capacity to overcome the hyperoxic-induced inhibition of protein synthesis in the lung. Unfortunately, only a limited amount of information regarding pulmonary protein metabolism could be drawn from this experiment. The presence of pulmonary oedema made it impossible to obtain a meaningful measurement of lung growth over the 72 hour hyperoxic period. As a consequence, the rate of protein breakdown in the lung could not be calculated. It is possible that the small reduction in protein synthesis in the lung may have been accompanied by an increase in the rate of protein degradation and hence a retardation in pulmonary growth.

The failure of hyperoxia to influence pulmonary protein metabolism in the neonatal guinea pig to the extent observed in the adolescent rat suggests the presence of an age and/or species difference. The protein synthetic activity of the lung of adolescent guinea pigs was not however reduced following hyperoxia. This finding would suggest that species variation between the rat and the guinea pig explains the sensitivity of the lung to a hyperoxic-induced inhibition of protein synthesis. Furthermore, the relatively small hyperoxic response of the neonatal guinea pig declined as a function of age, such that it was no longer evident in animals at four to five weeks of age. This is surprising in that the neonates of rats, mice and rabbits have an increased tolerance to hyperoxic-induced lung injury compared to adults (Frank *et al.*, 1978; Yam *et al.*, 1978).

To summarize, although hyperoxia inhibits protein synthesis in the lung of the neonatal guinea pig, a compensatory fall in the rate of protein breakdown prevents a reduction in the rate of pulmonary growth. It should be appreciated however, that this response to acute hyperoxia (24 hours, ≥95% oxygen) can change upon increasing the duration of oxygen exposure. This study, in demonstrating that the immature lung is sensitive to hyperoxia, suggests that supplementary oxygen, administered to premature babies with CLD, has the potential to disrupt pulmonary protein metabolism. The presently cited species difference, with respect to the sensitivity of the lung to hyperoxia, makes it difficult however, to speculate whether neonatal oxygen therapy actually inhibits protein deposition in the lung. In addition, the degree of prematurity of the individual, the duration and concentration of oxygen exposure and an additional complication of

undernutrition may all affect the degree to which pulmonary growth and repair in the preterm infant is influenced by the provision of supplementary oxygen.

The mechanism by which hyperoxia inhibits protein synthesis in the lung is not, at present, understood. The hypothesis under investigation in this thesis suggests that GSSG accumulates in the lung during oxidative stress, and that this oxidized species mediates the inhibition of pulmonary protein synthesis. This chapter began to test this hypothesis by assessing the glutathione status of the lung during hyperoxia.

It was surprising that the hyperoxic insult failed to induce an increase in the concentration of reduced glutathione in the lung of the neonatal and adolescent guinea pigs. Glutathione provides reducing equivalents, required for the removal of reactive oxygen species through the action of glutathione peroxidase. Increases in glutathione in the lung of both neonatal and adult rats have been reported within 24 hours of hyperoxia (Kimball *et al.*, 1976; Yam *et al.*, 1978; Deneke *et al.*, 1983). Such a response is believed to represent an important protective mechanism against oxidant injury. It is possible that the time course at which the various components of the pulmonary antioxidant defense are induced, differs in the guinea pig. An induction of SOD and CAT may occur initially in response to hyperoxia, followed by a later stimulation of the glutathione system. A small but non-significant increase in lung tissue concentrations of GSSG accompanied the decrease in pulmonary protein synthesis in the neonatal guinea pigs after a 24 hour period in hyperoxia. Upon increasing the oxygen exposure to 72 hours, pulmonary GSSG content did not continue to rise, but actually fell. Previous investigations into the effect of an *in vivo* hyperoxic exposure on the concentration of GSSG alone, rather than total glutathione are scarce. Significant increases have been observed in the lung of the rat following a 52 hour exposure to 100% oxygen respectively (White *et al.*, 1986, 1987). It is difficult to extrapolate the results from these studies to the present investigation, in that a different species, age of animal and duration of oxygen exposure were employed.

The changes observed in the concentration of lung tissue GSSG in the neonatal guinea pig may suggest that this oxidized species initially (before 24 hour)

accumulated in the lung following hyperoxia. Several mechanisms may then have become operational, so that after 24 hours of hyperoxia, much of the excess GSSG had been removed, and that by 72 hours of oxygen exposure, the glutathione redox status of the lung had been restored. One such mechanism to maintain glutathione in the reduced state under conditions of oxidative stress has been reported in the rat, and involves a concerted increase in the concentration and/or activities of glutathione reductase and glucose-6-phosphate dehydrogenase (Kimball *et al.*, 1976; Yam et al., 1978; Warshaw *et al.*, 1985; Crouch *et al.*, 1988). These enzymes, together with NADPH, reduce GSSG and thereby replenish the glutathione consumed during the removal of hydrogen peroxide and lipid peroxides. An alternative mechanism by which a tissue maintains a high GSH/GSSG ratio may involve the transport of GSSG out of the cell and into the circulation. Oxidant stress in the isolated perfused rat lung, achieved by hydroperoxide infusion or by reoxygenation of the lung following a period of hypoxia, results in the release of GSSG into the perfusate, as the tissue concentration of GSSG rises (Jenkinson *et al.*, 1987; Jenkinson *et al.*, 1988b). Studies examining the kinetics of GSSG release versus the intracellular concentration, suggests that during oxidative stress the release of this oxidized species from the lung involves the operation of a carrier mediated transport mechanism (Jenkinson *et al.*, 1987).

In support of a transport mechanism removing GSSG from the lung, a progressive increase in the concentration of GSSG was observed in the blood of neonatal guinea pigs in response to hyperoxia. The significantly lower concentration of GSSG in the lung following a 72 hour oxygen exposure may therefore be explained by the significantly higher concentration of this oxidized species in the blood at this time point. The source of GSSG detected in the blood of the guinea pig is unlikely to be purely of pulmonary origin however, but probably represents a number of tissues. The liver, for example, is an organ in which the efflux of GSSG has been extensively characterized (Eklöw *et al.*, 1981; Nicotera *et al.*, 1985).

In summary, the hyperoxic-induced inhibition of pulmonary protein synthesis in the neonatal guinea pig was not associated with a significant accumulation of GSSG in the lung. Despite this, the changes in the concentration of GSSG in the lung and the blood following the acute (24 hour) and chronic (72 hour) oxygen

exposure, can be explained by an initial accumulation of GSSG in the lung followed by its release into the blood. An early increase in the concentration of this oxidized species may have mediated the decreased rate of pulmonary protein synthesis which was evident at 24 hours. The contribution played by GSSG towards the sustained fall in protein synthesis in the lung at 72 hours is however questionable.

CHAPTER 4. FURTHER INVESTIGATION INTO THE MECHANISM INVOLVED IN THE HYPEROXIC-INDUCED INHIBITION OF PULMONARY PROTEIN SYNTHESIS

4.1 INTRODUCTION

The supposition that the hyperoxic-induced inhibition of pulmonary protein synthesis is mediated via GSSG was introduced in Chapter 3 and has been investigated to a greater degree in the present section. Briefly, the hypothesis originates from two lines of evidence. First, oxidative stress is associated with an elevated concentration of GSSG in the lung (White *et al.*, 1986, 1987; Jenkinson *et al.*, 1987, 1988b). Second, a disturbance in the glutathione redox status could influence pulmonary protein synthesis, since increased concentrations of GSSG has an inhibitory effect on the protein synthetic activity of the reticulocyte lysate (Kosower *et al.*, 1972).

The influence of GSSG on protein synthesis in the reticulocyte lysate has been extensively characterized and is described in detail in Chapter 1, section 7.3. Briefly, when haem supplemented lysates are incubated with GSSG (0.05 to 2.0mM), the rate of protein synthesis rapidly declines after a lag period of 6 to 9 minutes. Thereafter, protein synthesis ceases completely. These changes are accompanied by the disaggregation of polysomes and an accumulation of 80 S monomers, indicating that the translational block is occurring at peptide chain initiation (Kosower *et al.*, 1972). The inhibition by GSSG involves the activation of a translational inhibitor, I(GSSG), which is associated with an increased cAMP-independant kinase that specifically phosphorylates the α subunit of the initiation factor eIF-2 (Ernst *et al.*, 1978a). Evidence suggests that I(GSSG) and $hpOI_2$, the inhibitor of protein synthesis which is activated in the lysate exposed to high concentrations of oxygen, is the same protein (Almis-Kanigür *et al.*, 1982, 1983). This suggests that under hyperoxic conditions, an increased concentration of GSSG in the reticulocyte activates an inhibitor, which in turn mediates the inhibition of protein synthesis in the system. These studies lend considerable support to the hypothesis presently under investigation.

The present work has employed both *in vivo* and *in vitro* systems to investigate the contribution played by GSSG in inhibiting protein synthesis in the lung following hyperoxia. In Chapter 3, I reported that pulmonary protein synthesis in the guinea pig was not as sensitive to hyperoxia as that previously reported in the adolescent rat (Kelly, 1988). In addition, high concentrations of oxygen did not significantly alter the glutathione status of the lung in this species. For this reason, the adolescent rat which exhibits a relatively high sensitivity to hyperoxia was employed as the model in these further investigations of the regulatory role of GSSG.

A significant (25%) inhibition in pulmonary protein synthesis has been demonstrated in the adolescent rat following a 24 hour exposure to ≥95% oxygen. This fall in protein synthesis was accompanied by a 36% reduction in the translational efficiency of the lung, while ribosomal capacity was unaffected (Kelly, 1988). The initial approach undertaken in the present study was to extend this previous work. The compromised efficiency of the rat lung to synthesize protein was investigated by defining the step in translation impaired by hyperoxia. The overall rate of protein synthesis in eukaryotic cells is, in the majority of cases, regulated by changes at peptide chain initiation (Rannels *et al.*, 1978a,b; Flaim *et al.*, 1982; Kelly & Jefferson, 1985). Furthermore, this is the translational step which is blocked in the reticulocyte lysate when treated with GSSG (Kosower *et al.*, 1972). Ribosomal aggregation in the lung of oxygen exposed rats was therefore studied to determine whether peptide chain initiation was impaired in this system. In addition to further defining the inhibition of protein synthesis, the glutathione status of the rat lung was assessed following a 24 hour hyperoxic exposure. This was necessary to investigate whether the decreased rate of pulmonary protein synthesis induced by hyperoxia is in fact accompanied by an accumulation of GSSG in the lung.

The second approach taken was to investigate the ability of GSSG to inhibit protein synthesis in the lung. This involved the development of a cell-free system from rat lung, to assess the effect of GSSG on pulmonary protein synthesis.

Cell-free systems permit the identification of the mechanisms involved in

protein synthesis. Moreover, the ability to change conditions *in vitro*, allows manipulations to be made, and the effect of such manipulations on specific steps of protein synthesis to be examined. With the exception of the reticulocyte lysate, the preparation of mammalian cell-free systems has been hindered by their low rate of protein synthetic activity (Manchester, 1970), and limited ability to reinitiate on endogenous mRNA (Falvey & Staehelin, 1970). Recently however, *in vitro* systems derived from the mammalian liver have emerged with greatly improved protein synthetic characteristics (Eisenstein & Harper, 1984; Morley & Jackson, 1985; Kimball *et al.*, 1989). The rate of protein synthesis achieved in these systems range from 10 to 20% of the *in vivo* rate, while 40 to 80% of amino acid incorporation is due to the initiation of new polypeptide chains.

Before studying the characteristics of protein synthesis in a cell-free system derived from the lung, a preliminary study was carried out in the reticulocyte lysate. This work was undertaken in order to reproduce the inhibitory effects of various components, notably GSSG, before they were employed in the lung extract.

My initial objectives in the development of a lung cell-free system were to establish a system in which protein synthesis proceeded at a rate comparable to that obtained in the hepatic systems described above. Second, it was necessary to demonstrate that a significant proportion of synthesis was due to re-initiation, to allow the examination of the effects of added components which may act at initiation. When these criteria were met, the potential of GSSG to regulate pulmonary protein synthesis was examined.

4.2 EXPERIMENTAL PROTOCOL

Adolescent, male Wistar rats (initial body weight 100 g) were randomly divided into two groups, and were maintained in either room air or hyperoxia (≥95% oxygen) for 24 hours. Following the appropriate experimental exposure, animals were injected intravenously with a bolus dose of ^{3}H-phenylalanine and killed by decapitation. The lungs were then taken for the measurement of protein synthesis, ribosomal and polysomal aggregation, tissue RNA, glutathione status

(including the blood) and tissue protein. Refer to Chapter 2, sections 2.4, 2.6, 2.7, 2.11 and 2.14 respectively for an explanation of the methodology employed.

Adolescent, male Wistar rats were also used as the source of tissue for the preparation of the lung cell-free protein synthesizing system. The protocol followed for the measurement of protein synthesis and the analysis of polysomal aggregation in the system are detailed in Chapter 2, sections 2.8 and 2.9 respectively. Protein synthesis was monitored in the reticulocyte lysate as described in Chapter 2, section 2.10.

4.3 RESULTS

To confirm the earlier work, that hyperoxia inhibits pulmonary protein synthesis (Kelly, 1988), which forms the basis of the present study, protein synthesis was measured in the lung of male, Wistar rats following 24 hours exposure to ≥95% oxygen. In addition, the ribosomal cycle of the lung and the glutathione status of the lung and blood were examined after this period of hyperoxia. These measurements were undertaken to investigate the mechanism involved in the reduced rate of pulmonary protein synthesis following hyperoxia, and more specifically, to deduce whether GSSG was a contributing factor.

4.3.1 The Effect of Hyperoxia on Protein Synthesis in the Rat Lung

The fractional rate of protein synthesis was significantly reduced in the lungs of animals exposed to ≥95% oxygen for 24 hours (Table 7). As a result, the total amount of pulmonary protein synthesized per day was 35% lower than that of the air controls. This decrease will have contributed to the smaller quantity of protein present in the lungs of the hyperoxic-exposed rats (Table 7).

4.3.2 The Effect of Hyperoxia on the Ribosomal Cycle of the Rat Lung

Having confirmed that pulmonary protein synthesis in the adolescent rat is sensitive to hyperoxia, subsequent steps were carried out to further define the

Table 7. Protein Content and Pulmonary Protein Synthesis in Adolescent Rats Following a 24 Hour Hyperoxic Exposure

Treatment	Protein content (mg)	Fractional rate of synthesis (%/day)	Total protein synthesized (mg/day)
24 h 21% oxygen	107.7±23.6	40.2±4.0	43.2±8.9
24 h ≥95% oxygen	97.0± 5.7	31.8±4.8*	28.8±7.9*
	(-10%)	(-20%)	(-35%)

Total protein was measured by the method of Smith *et al*. (1985). The fractional rate of protein synthesis in the lung was determined as described by Garlick *et al*. (1980). Total protein synthesized per day represents the product of the fractional rate of synthesis and the protein mass. The values presented are the means ± S.D. n=4 and 6 for the control and ≥95% oxygen groups respectively. Values in parentheses show the percentage change from the control group. *$P<0.05$, statistically significant from the control group.

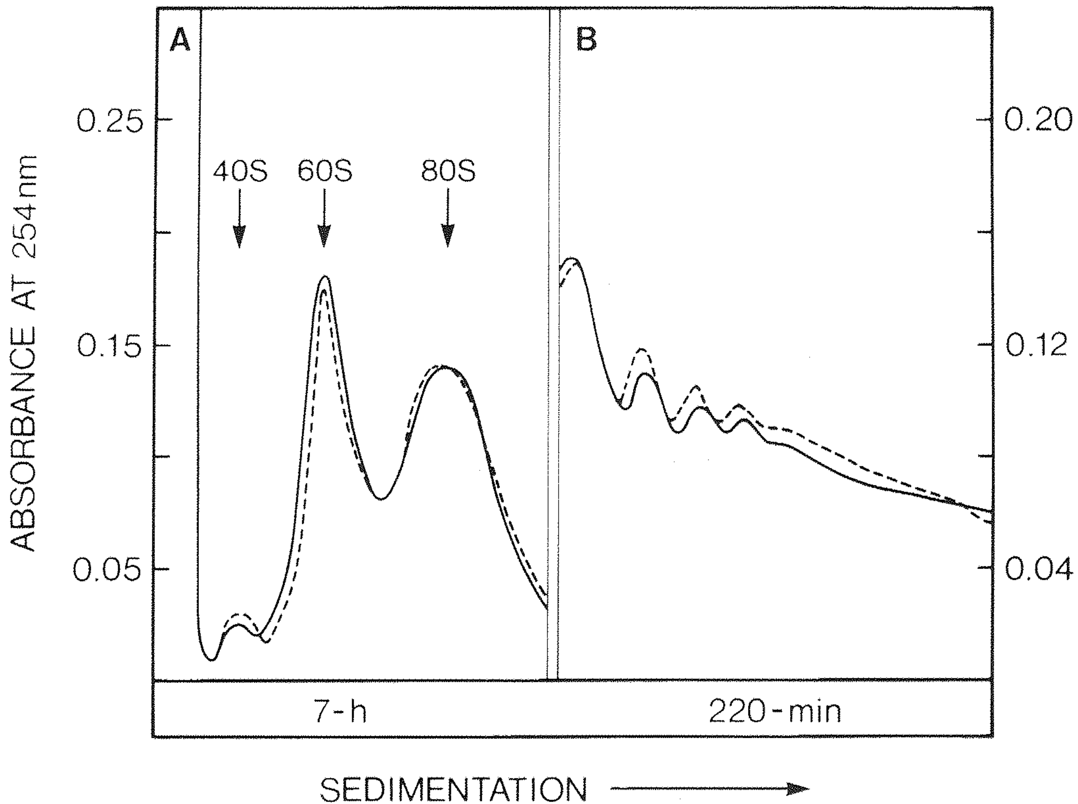
nature of this inhibition. The decreased rate in protein synthetic activity in the lung following hyperoxia was accompanied by a significant reduction in the translational activity of ribosomes, while the ribosomal content of the lung was unchanged (Kelly, 1988). In an attempt to detect whether the compromised translational efficiency was due to an impaired rate of peptide chain initiation, polysomal and ribosomal aggregation in the lung was analyzed after a 24 hour hyperoxic exposure.

Figure. 10A shows that the relative proportions of ribosomal subunits and monomers were very similar in the lungs of animals maintained in either room air or under high concentrations of oxygen. This observation was confirmed when the RNA content of the peaks representing these ribosomal particles were analyzed (Table 8). Concomitant with the unchanged profile of pulmonary ribosomal aggregation in the hyperoxic-exposed rats, a loss of polysomal material was not evident in the lungs of these animals (Fig. 10B). These results indicate the overall reduction in pulmonary protein synthesis following hyperoxia, cannot be explained by a specific block at peptide chain initiation.

4.3.3 The Effects of Hyperoxia on the Glutathione Status of the Lung and Blood in the Rat

The hypothesis that an accumulation of GSSG in the lung mediates the reduced rate of pulmonary protein synthesis under hyperoxic conditions, was investigated by measuring the glutathione status of the lungs of animals exposed to $\geq$95% oxygen and those maintained in room air (Table 9). A significant increase (54%) in the concentration of reduced glutathione in the lung was evident after 24 hours exposure to hyperoxia. This perturbation was not however accompanied by a similar elevation in the oxidized form of this tripeptide (Table 9). The inability to detect an accumulation of pulmonary GSSG in the oxygen exposed rats, may reflect an increased release of this oxidized species into the circulation, in response to an increased production within the lung. The glutathione status of the blood did not however reflect the occurrence of such a phenomenon. While the concentration of reduced glutathione in the blood was not altered following hyperoxia, the blood GSSG concentration was significantly lower in animals exposed to $\geq$95% oxygen

Figure 10. Sucrose Density Gradient Analysis of Polysomes and Ribosomal Particles from Lungs of Rats Exposed to Hyperoxia



Lungs of rats maintained in room air (------) or exposed to hyperoxia (24 h, ≥94% oxygen) (——) were homogenized as described in Chapter 2, section 2.6. The postmitochondrial supernatant (0.75ml for ribosomal particles, 0.9ml for polysomes) was layered onto 0.59 to 1.46M linear sucrose gradients. Ribosomal subunits and monomers (A) and polysomes (B) were resolved by centrifugation at 300,000 x g in a TST41.14 rotor (Krontron Instruments) for 7 h and 220 min respectively at 2°C. The gradients were monitored for absorbance at 254nm.

Table 8. The Effect of Hyperoxia on RNA Content and Ribosomal Aggregation in the Lung

Treatment	Tissue RNA (mg/g)	RNA content of ribosomal peaks (μg/mg tissue RNA)		
		40 S	60 S	80 S
24 h 21% oxygen	2.64±0.27	27±4	62±13	87±12
24 h ≥95% oxygen	2.63±0.31	26±3	56± 2	85±10

Lung homogenates were prepared as described in Chapter 2, section 2.6 and an aliquot was retained for the measurement of tissue RNA as described by Fleck and Munro (1962). Following centrifugation of the postmitochondrial supernatant (0.75ml) on sucrose density gradients, RNA content of the 40, 60 and 80 S peaks was determined and expressed relative to total RNA content of the homogenate. The values presented are the means ± S.D. derived from 3 determinations.

Table 9. The Glutathione Status of the Lung and Blood of Adolescent Rats Following a 24 Hour Hyperoxic Exposure

Treatment	LUNG (nmoles/mgPr)		BLOOD (nmoles/mgHb)	
	Reduced Glutathione	Oxidized Glutathione	Reduced Glutathione	Oxidized Glutathione
24 h 21% oxygen	11.35±2.04	0.48±0.22	7.95±1.41	0.09±0.02
24 h ≥95% oxygen	17.44±2.19** (+54%)	0.46±0.21	7.60±1.17	0.06±0.02* (-33%)

Total and oxidized glutathione was measured by the method of Griffith (1980). Values presented are the means ± S.D. n=4 and 6 for the control and ≥95% oxygen groups respectively. Values in parentheses show the percentage change from the control group. *$P<0.05$, **$P<0.01$, statistically significant from the control group.

compared to that of the air controls.

4.3.4 Protein Synthesis in the Reticulocyte Lysate in Response to a Variety of Inhibitory Factors

Another approach taken to determine if GSSG plays a role in reducing pulmonary protein synthesis under hyperoxic conditions involved the development of a cell-free system derived from the mammalian lung. Such a system was required to determine whether protein synthesis in the lung is sensitive to GSSG at concentrations which are likely to exist under conditions of oxidative stress. Before commencing this work, preliminary studies were conducted with the reticulocyte lysate, the most widely used cell-free system. Extensive work with the reticulocyte has greatly enhanced our understanding of both the mechanisms and the regulation of eukaryotic peptide chain initiation. More importantly, with respect to the present investigation, a detailed examination of the inhibitory activity of GSSG on protein synthesis was carried out in this *in vitro* system.

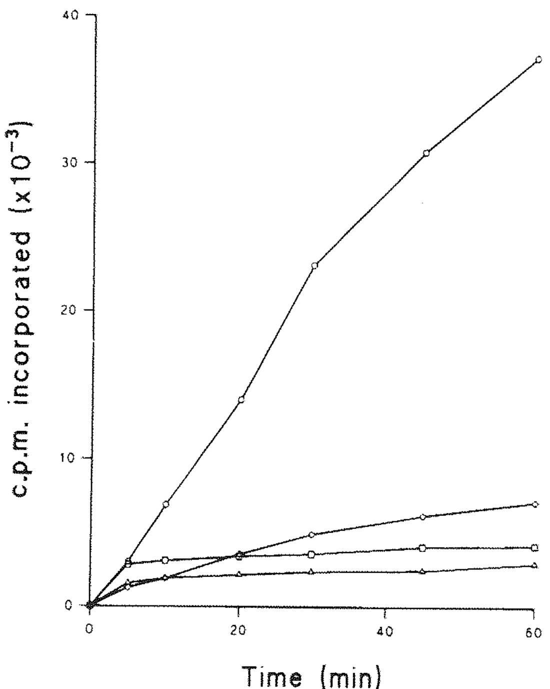
The Effect of Haem Deficiency and the Haem Controlled Inhibitor

The ability of the reticulocyte lysate, under optimal conditions, to exhibit a linear rate of protein synthesis for prolonged periods is illustrated in Fig. 11. In the absence of haem however, protein synthesis proceeded at the initial control rate for only 5 minutes and then declined abruptly, yielding biphasic kinetics. The inhibition of protein synthesis in the haem deprived lysate is due to the activation of a translational inhibitor, HCl. Upon adding purified HCl to haem-supplemented lysates, protein synthesis was immediately reduced and had completely ceased within ten minutes of incubation (Fig. 11).

The Effect of 7-methylguanosine-5'-triphosphate

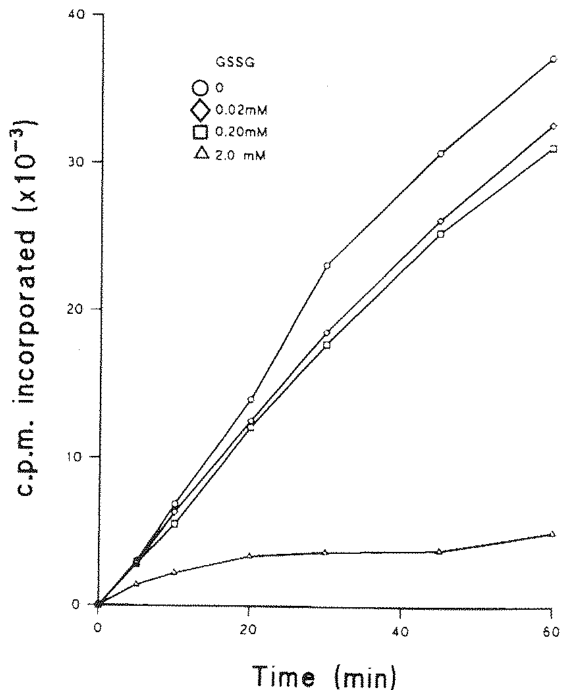
The methylated sequence $m^7G^{5'}$ppp....... ("cap" structure) is a general feature at the 5' end of many eukaryotic mRNA's, where it is believed to function as a recognition signal (Rottman *et al.*, 1974; Hickey *et al.*, 1976). Peptide chain initiation in systems in which the message possess this 5' terminal sequence is inhibited by

Figure 11. The Effect of Haem Deficiency, HCl and m^7GTP on Protein Synthesis in the Reticulocyte Lysate



Protein synthesis reaction mixtures were incubated under four conditions: plus haem (○); minus haem (□); plus haem plus 2,400U/ml HCl (△); plus haem plus 400μM m^7GTP (◇). Incubation was at 30 °C for 60 minutes and protein synthesis was determined by the incorporation of [^{14}C]leucine (final specific radioactivity 0.56Ci/mmol) into protein in 10μl aliquots of the reaction mixture. Each point represents the mean value from duplicate samples.

Figure 12. The Effect of GSSG on Protein Synthesis in the Reticulocyte Lysate



Protein synthesis reaction mixtures were incubated with GSSG to produce final concentrations of 0.02 to 2.0mM GSSG as indicated. All additions were made at 0 added minutes. Incubation was at 30°C for 60 minutes and protein synthesis was determined by the incorporation of [^{14}C]leucine (final specific radioactivity 0.56Ci/mmol) into protein in 10μl aliquots of the reaction mixture. Each point represents the mean from duplicate samples.

the cap analogue, 7-methylguanosine-5'-triphosphate (m^7GTP). m^7GTP is believed to interact with the active site on the initiation factor(s) or ribosomes which are involved in the recognition and binding of the 5' end moiety of mRNA (Canaani *et al.*, 1976). The inhibitory effect of this cap analogue on protein synthesis in haem-supplemented lysates is clearly shown in Fig. 11. Amino acid incorporation proceeded at a considerably slower rate in the presence of the m^7GTP, bringing about an 80% inhibition of protein synthetic activity after a 60 minute incubation period.

The Effect of Oxidized Glutathione

Figure 12 illustrates the influence of increasing concentrations of GSSG (0.02 to 2.0mM) on protein synthesis in haem-supplemented lysates. The addition of GSSG at 0.02 and 0.2mM had negligible inhibitory activity upon the system. The incorporation of radiolabelled amino acid proceeded at the control rate for approximately 20 minutes and then slowed slightly, to produce a 15% inhibition of protein synthesis at 60 minutes. The profound inhibitory characteristics of this oxidized species were clearly evident however, when the final concentration was increased to 2.0mM (Fig. 12).

4.3.5 Characteristics of Protein Synthesis in the Cell-free System Derived from the Mammalian Lung

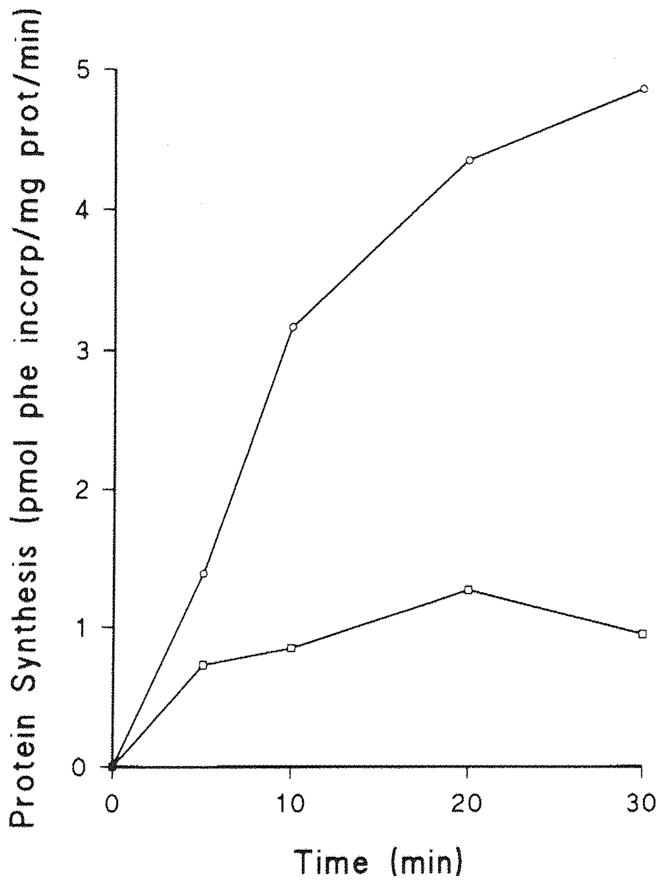
The conditions employed in the development of an *in vitro* protein synthesizing system from rat lung were based upon those reported to be optimal for a rat liver system (Eisenstein & Harper, 1984). This particular hepatic system represented a significant improvement on previous mammalian cell-free systems, as it exhibited a relatively high rate of re-initiation on both endogenous and exogenous mRNA. A characteristic feature of the protocol was a 10-fold dilution of the liver extract prior to assay, the main effect of which was to maintain an initial rate of protein synthesis for at least 90 minutes. The difference from the method described by Eisenstein and Harper (1984), that was initially employed in the preparation of the lung extract, involved the use of the sodium salt of creatine phosphate in place of the potassium salt. A typical time course of incorporation under these conditions

is illustrated in Fig. 13. The rate of protein synthesis was linear for only 5 minutes and was equivalent to just 1% of the *in vivo* rate. In an attempt to improve the performance of the system, a number of conditions were re-examined. Particular attention was paid to eliminate the exogenous addition of sodium and chloride ions, in the light of the inhibitory effect that these ions have at non-physiological concentrations (Cooper *et al.*, 1968; Weber *et al.*, 1977). The sodium salt of creatine phosphate was substituted for the potassium salt, sodium hydroxide and hydrochloric acid, previously utilized for pH neutralization, were replaced with potassium hydroxide and glacial acetic acid and finally the postmitochondrial supernatant was not diluted prior to assay. While reducing the amount of sodium and chloride was only found to be slightly beneficial, the use of a more concentrated extract markedly stimulated the protein synthetic performance of the system. Under these revised conditions, protein synthesis was constantly maintained at an initial rate for 10 minutes (Fig. 13). For this reason, the protein synthetic rate was calculated over the first 10 minutes and expressed as an initial rate. Over a series of 10 experiments, the initial rate of protein synthesis in the *in vitro* system derived from the mammalian lung was 0.31 $\pm$ 0.07 pmol phenylalanine incorporated per mg postmitochondrial supernatant per minute. This rate corresponds to 5% of the rate at which protein is synthesized in the lung of the adolescent rat *in vivo* (see appendix 2.), comparing favourably with other such mammalian systems (Eisenstein & Harper, 1984; Morley & Jackson, 1985; Kimball *et al.*, 1989).

Further measures taken to achieve a greater protein synthetic activity involved an attempt to eliminate both exogenous and endogenous nucleases. To avoid exogenous nuclease contamination, the system was prepared utilizing sterile procedures. All plasticware, glassware and dissection instruments were autoclaved. The water used for the preparation of solutions was treated with diethylpyrocarbonate and autoclaved and disposable gloves were worn at all times. To reduce the activity of ribonucleases endogenous to the lung, the human placental ribonuclease inhibitor (200U/ml extract) was incorporated into the system. None of these procedures were effective however, in further stimulating amino acid incorporation (data not shown).

Ribosomal and polysomal aggregation in the system were analyzed on

Figure 13. Protein Synthesis in the Cell-free System Prepared from Rat Lung.



Protein synthesis in the system before (□) and after (○) a variety of refinements were made is illustrated (see text). The incorporation of [^{3}H]phenylalanine into protein in 25μl aliquots of the incubation mixture was determined at 30°C at the times indicated. Each point represents the mean value from duplicate samples.

sucrose density gradients after a 5 minute incubation, a time point at which protein synthesis was proceeding at the optimal rate (Fig. 14). The profile of ribosomal particles was typically characterized by a relatively large 60 S peak and small 40 S and 80 S peaks. In addition, this strange ribosomal profile was unaltered when derived from a system in which protein synthesis was markedly inhibited (data not shown). On the other hand, polysomal aggregation responded to agents which inhibited the protein synthetic activity of the system (see below), and therefore was considered a more reliable means of gaining further information upon changes in the overall rate of protein synthesis.

Since the lung cell-free system was developed to investigate its sensitivity to GSSG, a species which acts at peptide chain initiation in the reticulocyte, it was essential that the system was able to actively initiate the synthesis of new polypeptides. The ability of the system to reinitiate was examined by the addition of specific blockers of initiation, edeine or m^7GTP. The mode of action of m^7GTP was outlined in section 4.3.4. The antibiotic complex edeine, upon binding to the small ribosomal subunit, inhibits initiation, in an analogous fashion to m^7GTP, by preventing 43 S initiation complex binding to mRNA (Vázquez, 1979). Both of these agents markedly inhibited protein synthesis in the lung cell-free system (Table 10), indicating that 40 to 60% of amino acid incorporation into protein was due to reinitiation. The 25% loss of polysomal material in the presence of edeine (Fig. 15) verified that this agent was in fact acting as an inhibitor of initiation.

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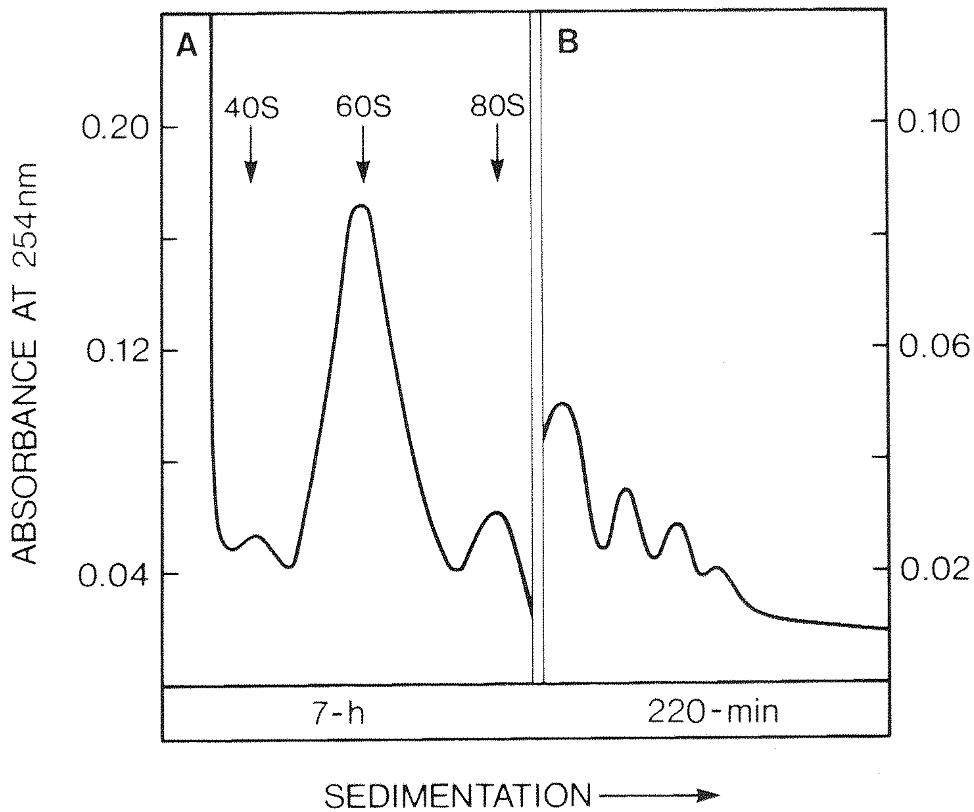
Figure 14. Sucrose Density Gradient Analysis of Polysomes and Ribosomal Particles from the Lung Cell-free System



The lung cell-free system was incubated under optimal conditions for protein synthesis for 5 min in the absence of labelled amino acid. Aliquots (1.2ml) were then layered onto 0.59 to 1.46M linear sucrose gradients. Ribosomal subunits and monomers (A) and polysomes (B) were resolved by centrifugation at 300,000 x g in a TST41.14 rotor (Krontron Instruments) for 7 hours and 220 min respectively at 2°C. The gradients were monitored for absorbance at 254nm.

Table 10. Initial Rates of Protein Synthesis in the Cell-free System Prepared from Rat Lung Incubated with Specific Inhibitors of Initiation

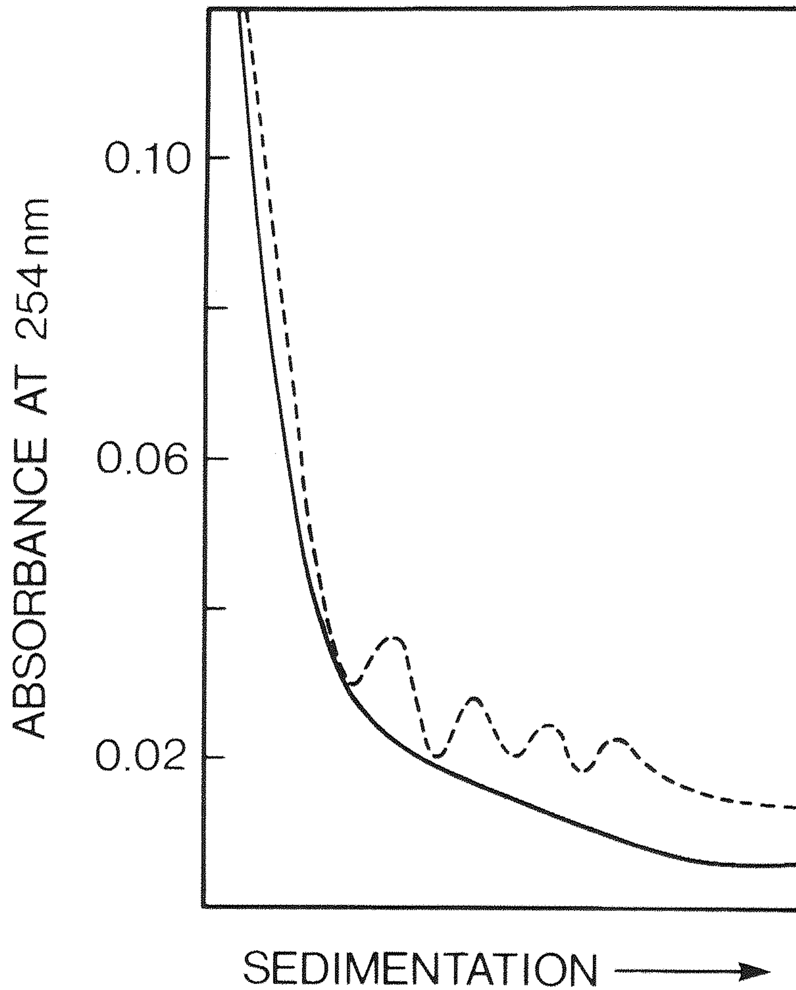
Experiment	Additions	Initial rate of synthesis (pmol phe incorp/ mg prot/min)	Inhibition (%)
I	None	0.25	-
	Edeine (10μM)	0.10	60
II	None	0.24	-
	m^7GTP (500μM)	0.15	38

Protein synthesis reaction mixtures (225μl) were incubated at 30°C for 30 min and protein synthesis was determined by the incorporation of [^{3}H]phenylalanine (final specific radioactivity 0.5Ci/mol) into protein in 25μl aliquots of the incubation mixture. In experiments I and II, which were carried out on different days, edeine and m^7GTP respectively, were added at 0 min. Each value represents the mean from duplicate samples.

4.3.6 The Effect of Oxidized Glutathione on Protein Synthesis in the Lung Cell-free System

The homogenizing buffer and the reaction mix employed in the preparation of the lung *in vitro* system routinely contained dithiotheritol to produce a final concentration of 2.5mM. Initially, the effect of GSSG was investigated under these conditions. An appreciable decrease in the initial rate of protein synthesis was not achieved until the concentration of GSSG was increased to 5mM (Table 11). When GSSG (5mM) was added to a lung extract in which dithiotheritol had been omitted,

Figure 15. The Effect of Edeine on A_{254} Profiles of Polysomes Isolated from the Lung Cell-free System on Sucrose Density Gradients



The lung cell-free system was incubated under optimal conditions for protein synthesis for 5 min in the absence of labelled amino acid and in the absence (------) or presence (——) of 10μM edeine. Aliquots (1.2ml) were then layered onto 0.59 to 1.46M linear sucrose gradients and centrifuged at 300,000 x g in a TST41.14 rotor (Krontron Instruments) for 220 min at 2°C. The gradients were monitored for absorbance at 254nm.

the inhibition of protein synthesis was 2-fold higher than that in the dithiotheritol-supplemented system (Table 11). This result suggested that what originally appeared to be a relative insensitivity of pulmonary protein synthesis to GSSG, was in fact due to the efficient reduction of this species by dithiotheritol. Upon repeating the dose response in the absence of dithiotheritol, pulmonary protein synthesis was markedly sensitive to GSSG between concentrations of 0.2 and 0.5mM (Table 11).

4.3.7 Polysomal Aggregation in the Lung Cell-free System in the Presence of Oxidized Glutathione

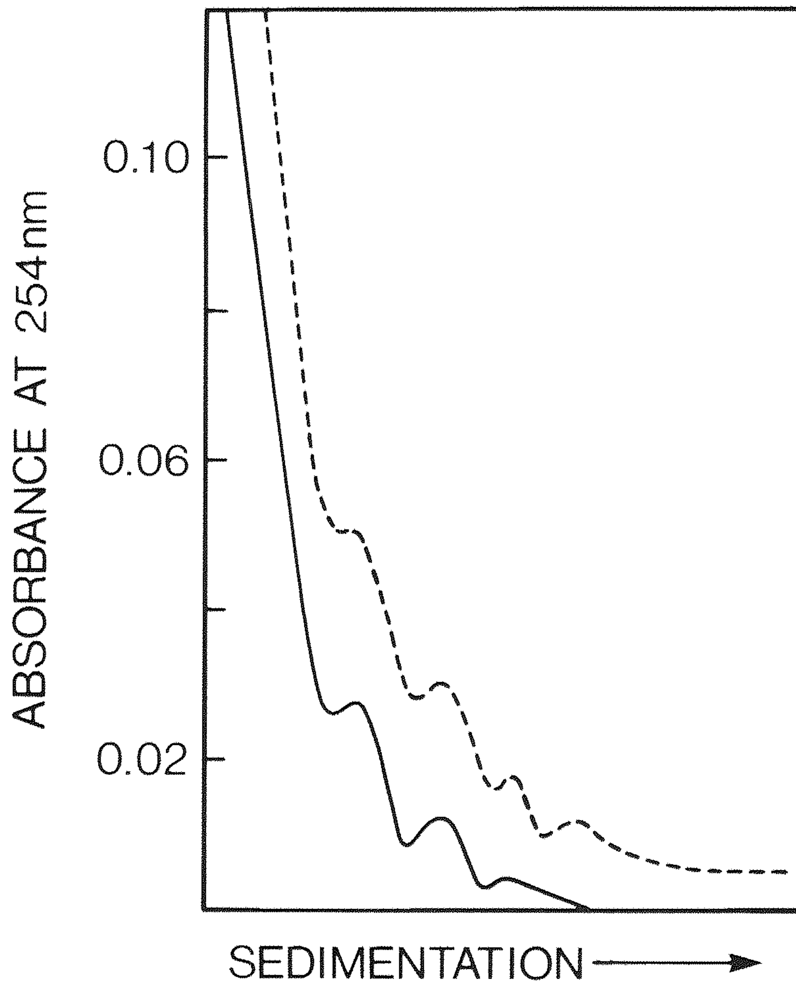
Polyribosomal analysis in the GSSG-treated lung cell-free system was used to further define the overall fall in protein synthesis. In the presence of GSSG, a 40% loss of polysomal material (Fig. 16) accompanied the appreciable reduction in amino acid incorporation. These changes are analogous to those seen in the reticulocyte lysate upon treatment with GSSG, and are indicative of a block at peptide chain initiation.

Table 11. Initial Rates of Protein Synthesis in the Cell-free System Prepared from Rat Lung Incubated with GSSG, in the Presence or Absence of Dithiotheritol

Experiment		Additions	Initial rate of synthesis (pmol phe incorp/ mg prot/min)	Inhibition (%)
I	+DTT	None	0.32	-
		GSSG 1.0mM	0.29	9
		2.0mM	0.26	19
		5.0mM	0.15	53
		10.0mM	0.05	84
II	+DTT	None	0.28	-
		GSSG 5.0mM	0.17	39
	-DTT	None	0.19	-
		GSSG 5.0mM	0.03	84
III	-DTT	None	0.22	-
		GSSG 0.2mM	0.20	9
		0.5mM	0.14	36
		1.0mM	0.08	64
		2.0mM	0.03	86

Protein synthesis reaction mixtures (225μl) were incubated at 30°C for 30 min and protein synthesis was determined by the incorporation of [^{3}H]phenylalanine (final specific radioactivity 0.5Ci/mol) into protein in 25μl aliquots of the incubation mixture. In experiment I, GSSG was added at 0 min at increasing concentrations as indicated to a system containing 2.5mM dithiotheritol (DTT). In experiment II, 5.0mM GSSG was added at 0 min to systems which contained either 2.5mM or no DTT. In experiment III, GSSG was added at 0 min at increasing concentrations as indicated to a system containing no DTT. Experiments I, II and III were carried out on different days. Each value represents the mean ± S.D. from duplicate samples.

Figure 16. The Effect of GSSG on A_{254} Profiles of Polysomes Isolated from the Lung Cell-free System on Sucrose Density Gradients



The lung cell-free system was incubated under optimal conditions for protein synthesis for 5 min in the absence of labelled amino acid and in the absence (------) or presence (——) of 1mM GSSG. Aliquots (1.2ml) were then layered onto 0.59 to 1.46M linear sucrose gradients and centrifuged at 300,000 x g in a TST41.14 rotor (Krontron Instruments) for 220 min at 2°C. The gradients were monitored for absorbance at 254nm.

4.4. DISCUSSION

The goal of this study was to determine whether GSSG was involved in the mechanism by which hyperoxia inhibits protein synthesis in the lung. This investigation utilized two systems which examined the effects of hyperoxia and GSSG on *in vivo* and *in vitro* pulmonary protein synthesis respectively.

When adolescent rats are exposed to hyperoxia (≥95% oxygen, 24 hours), the significant (25%) inhibition of protein synthesis in the lung is related to a reduction (36%) in translational efficiency (Kelly, 1988). To extend this previous investigation, two approaches were conducted *in vivo*. First it was determined whether the compromised translational efficiency following hyperoxia was due to an impairment at peptide chain initiation in the lung. This is the translational step which GSSG blocks in the reticulocyte lysate. Second, the glutathione redox status of the lung following hyperoxia was measured, to investigate whether the concentration of GSSG increases in parallel with a compromised rate of pulmonary protein synthesis.

Peptide chain initiation in the lung following hyperoxia was assessed by examining ribosomal aggregation on sucrose density gradients. Polysomal disaggregation and 80 S monomer accumulation were not, however, observed in the lungs of rats exposed to high concentrations of oxygen, suggesting that the defect in translational efficiency was not due to a specific block at initiation. Information regarding the changes in translational events in response to hyperoxia is scarce. One study, in association with protein synthesis measurements, examined peptide chain initiation and elongation in primary cultures of porcine aortic endothelial cells maintained in hyperoxia (Jornot *et al.*, 1987). Protein synthesis in the cells was reduced almost linearly in relation to the duration of exposure to 95% oxygen. After two days in hyperoxia, the inhibition of incorporation of ^{3}H-phenylalanine into total protein was significant, and had reached 71% after a five day exposure. In agreement with the present work, a specific lesion at peptide chain initiation was not observed in the hyperoxic-exposed cells. When elongation was assessed, by measuring the ribosomal transit time, it became apparent that

following five days of hyperoxia, the synthesis and release of completed proteins in the endothelial cells was decreased by approximately 50%. If hyperoxia was specifically inhibiting *in vivo* pulmonary protein synthesis at elongation, we would have expected to have observed an increased polysome content upon sucrose density gradient analysis. It is feasible, however, that high concentrations of oxygen induce lesions at both initiation and elongation in the lung, explaining the identical proportions of 80 S monomers and polysomes in the control and hyperoxic-exposed animals. Protein synthesis in mammalian tissues has been investigated widely with respect to nutritional and hormonal control. In many situations, the overall rate of translation is controlled at peptide chain initiation (Rannels *et al.*, 1978 a,b; Flaim *et al.*, 1982; Kelly & Jefferson, 1985). The inability to detect a specific block at initiation in the present study and in previous work with cultured endothelial cells (Jornot *et al.*, 1987) is interesting, in suggesting a role for elongation in the regulation of protein synthesis under hyperoxic conditions.

The glutathione status of the lungs of animals maintained in ≥95% oxygen exhibited a significant (54%) increase in the concentration of reduced glutathione. Elevations of a similar magnitude in pulmonary glutathione have previously been reported in both adult and neonatal rats following a 24 hour period in hyperoxia (90% to 98% oxygen) (Kimball *et al.*, 1976; Yam *et al.*, 1978; Deneke *et al.*, 1983). Glutathione provides reducing equivalents for the elimination of hydrogen peroxide and other toxic hydroperoxides. An increased concentration of reduced glutathione in the lung, under conditions in which the production of oxygen free radicals will rise, therefore constitutes an important event in decreasing the susceptibility of the lung to oxygen-induced tissue injury. Of greater relevance to the hypothesis under investigation, was the failure to detect an increased concentration of GSSG in the lungs of the oxygen-exposed rats, despite the decreased rate of pulmonary protein synthesis in these animals. White *et al.* (1986, 1987) reported a 5.6 to 7.2 fold increases in lung GSSG concentration in rats after 52 hours in >99% oxygen. This discrepancy may be related to the different durations of hyperoxic exposure (24 versus 52 hours) and/or the age of the animals. The rats used by White *et al.* (1986, 1987) were between 300 and 400 g, i.e. approximately 12 weeks old, in contrast to the 3 to 4 week old adolescent animals presently employed. Younger animals have a greater resistance to oxygen-induced lung injury and this is most

likely related to a rapid adaptation of pulmonary antioxidant defence capacity compared to adults (Yam *et al.*, 1978). For example, increases in the concentrations and/or activities of glutathione reductase and glucose-6-phosphate dehydrogenase have been reported in the lungs of young rats within 24 hours of hyperoxia (Yam *et al.*, 1978; Warshaw *et al.*, 1985; Crouch *et al.*, 1988). These enzymes, along with NADPH, reduce GSSG and thereby regenerate glutathione required for the continued removal of toxic hydroperoxides. The failure of the activity of these enzymes to increase in adult rats within 24 hours of hyperoxia (Kimball *et al.*, 1976; Yam *et al.*, 1978) may contribute to the reported accumulation of GSSG in the lungs of older rats (White *et al.*, 1986, 1987). Alternatively, GSSG may be transported from the lung of the adolescent rat during hyperoxia, thereby maintaining a high GSH/GSSG ratio. In support of this, conditions of oxidative stress result in an elevated concentration of GSSG in the isolated perfused lung in associated with an increased release of this oxidized species into the perfusate (Jenkinson *et al.*, 1987, 1988b). The significantly lower concentration of GSSG in the blood of animals following exposure to ≥95% oxygen disputes, however, that such a mechanism operates during *in vivo* hyperoxic stress.

The second approach undertaken in the present study to test the hypothesis under investigation, was to examine the potential of an increased concentration of GSSG to inhibit pulmonary protein synthesis. This necessitated the development of a cell-free protein synthesizing system from the mammalian lung, in which the effects of increasing concentrations of GSSG could be examined.

In the past, the development of cell-free systems from non-erythroid sources have suffered from low rates of protein synthesis (Manchester, 1970) and the inability to initiate translation of endogenous mRNA (Falvey & Staehelin, 1970). Recently, three independent groups have developed *in vitro* systems from the mammalian liver which exhibit relatively high protein synthetic activity, of which a considerable proportion was due to reinitiation (Eisenstein & Harper, 1984; Morley & Jackson, 1985; Kimball *et al.*, 1989). In addition, the system described by Kimball *et al.* (1989) maintained the characteristics of protein synthesis inhibition present in the intact hepatocyte following amino acid deprivation. The successful preparation of a mammalian cell-free system from a new source, namely the lung,

was based very closely upon the conditions required for optimal protein synthesis in an hepatic extract (Eisenstein & Harper, 1984). The lung cell-free system synthesized protein at a linear rate for up to ten minutes at approximately 5% of the *in vivo* rate (see Appendix 2). Furthermore, the use of specific inhibitors of initiation demonstrated that initiation of new polypeptides accounted for 40 to 60% of the observed incorporation of ^{3}H-phenylalanine into protein. These characteristics compare favourably with those reported in the improved cell-free systems derived from the liver (Eisenstein & Harper, 1984; Morley & Jackson, 1985; Kimball *et al.*, 1989).

The present study demonstrated that pulmonary protein synthesis is sensitive to GSSG. Furthermore, the inhibitory effect on protein synthetic activity was accompanied by polysome disaggregation, indicating a specific block at peptide chain initiation. In the absence of a reducing agent, protein synthesis in the lung cell-free system was inhibited in the presence of 0.2mM GSSG, and this inhibition reached 40% when the concentration was increased to 0.5mM. These concentrations had no inhibitory effect when dithiothreitol was incorporated into the system. Similarly, the addition of dithiothreitol (1.0mM) at zero time to reticulocyte lysates treated with 0.2 to 0.25mM GSSG, effectively blocks the inhibition of protein synthesis and the activation of the GSSG-induced inhibitor I(GSSG) (Ernst *et al.*, 1978a,b). This reducing agent is ineffective however in restoring protein synthesis in GSSG-treated lysates after a 10 minute incubation period, that is to say, once I(GSSG) has been formed. These observations indicate the addition of dithiothreitol to GSSG-treated cell-free systems reduces this oxidized species thereby preventing the activation of I(GSSG) and consequently, the inhibition of protein synthesis.

Protein synthesis in the reticulocyte lysate is inhibited by as little as 0.05mM GSSG (Kosower *et al.*, 1972). Although the endogenous concentration of GSSG in rabbit reticulocytes is not known, total glutathione has been reported to be 2 to 3mM (Kosower *et al.*, 1972; Freedman *et al.*, 1974). In other mammalian systems, notably the human and rat erythrocyte (Srivastava & Beutler, 1968) and rat liver and kidney (Tietze, 1969), GSSG represents 0.2 to 5.0% of total glutathione. The extrapolation of this figure to the concentration of GSSG required to inhibit protein synthesis in the rabbit reticulocyte implies that GSSG may be a physiological

regulator of protein synthesis in the system. Furthermore, the inhibition of protein synthesis in GSSG-treated lysates is more rapid at a high initial rate of protein synthesis and is delayed when the rate of synthesis is low (Kosower *et al.*, 1972). This relationship gives further support to the premise that GSSG is involved in the control systems which regulate protein synthesis.

The potential of GSSG to influence pulmonary protein synthesis following hyperoxia will depend upon the relationship between the concentration present in the tissue under oxidative stress and that amount (0.2 to 0.5mM) required to inhibit protein synthetic activity. The concentration of GSSG in the lung of young rats under basal conditions is approximately 0.05mM (Jenkinson *et al.*, 1988b; Table 9). Following hyperoxia (>99% oxygen, 52 hours), White *et al.* (1986, 1987) reported a 560 to 721% increase in the concentration of GSSG in the lung of the rat. The extrapolation of this increase upon the basal concentration quoted above gives rise to 0.28 to 0.36mM GSSG. Pulmonary protein synthesis is therefore sensitive to GSSG at concentrations which have been reported in the lung following an *in vivo* hyperoxic exposure.

In summary, the results presented in this chapter are conflicting in their support for the hypothesis that GSSG is involved in the mechanism by which hyperoxia inhibits pulmonary protein synthesis. The overall reduction in translational efficiency in the lung following hyperoxia was not defined to a single step, but may be explained by an impaired rate of peptide chain initiation and elongation. This may imply that hyperoxia inhibits pulmonary protein synthesis via more than one mechanism. A role for GSSG is supported by the inhibitory effect of this species on the initiation of pulmonary protein synthesis, while alternative mechanism(s) may operate at elongation. The main weakness of this hypothesis lies in the dispute as to whether pulmonary GSSG increases under hyperoxic conditions to a concentration required to influence protein synthesis. While a sufficient accumulation of GSSG has been reported in the lung of the adult rat after a 52 hour hyperoxic exposure (White *et al.*, 1986, 1987), this was not detected in the present investigation. The possibility remains however that the concentration of GSSG in the lung of the adolescent rat rose to a "critical" level, mediated an inhibition of pulmonary protein synthesis, before being efficiently eliminated from the tissue when

the measurement was made at 24 hours. To test this proposal, would require the analysis of the lung glutathione redox status at additional time points between 0 and 24 hours after hyperoxic exposure.

CHAPTER 5. THE EFFECTS OF MATERNAL DEXAMETHASONE ADMINISTRATION ON GROWTH AND MATURATION OF THE FETAL LUNG

5.1 INTRODUCTION

For pregnancy to produce a healthy individual at birth, ideally requires its continuum to full-term. The alveoli, which constitute the machinery for gaseous exchange, begin to develop in the human lung at approximately 30 weeks of gestation (Langston *et al.*, 1984; Hislop *et al.*, 1986), and continue to divide into early childhood (Thurlbeck, 1975; Thurlbeck, 1982). In association with this, the lung develops biochemically during the latter part of gestation in number of species including man. The biochemical components of lung development which are important for the successful adaptation of the newborn lung to an ex utero environment are the surfactant and antioxidant systems. Pulmonary surfactant, in reducing the surface tension at the alveolar air-liquid interface, prevents lung collapse at the end of expiration, while the antioxidant system provides defence against reactive, hence potentially damaging oxygen free radicals. The developmental profiles of surfactant associated PC and the antioxidant enzymes, SOD, GSH-Px and CAT in the lung of the fetal rabbit (Khosla *et al.*, 1983; Frank & Groseclose, 1984), rat (Tanswell & Freeman, 1984; Sosenko *et al.*, 1986) and guinea pig (Kelly *et al.*, 1990; Rickett & Kelly, 1990) are characterized by significant increases during the final period of gestation. An essentially similar pattern exists in the human fetal lung, where PC increases gradually from approximately week 20 of gestation (Adams *et al.*, 1970) and catalase activity increases three fold from 15 weeks of gestation to term (McElroy *et al.*, 1990).

As a consequence of the time course of pulmonary development, an infant born prematurely possesses a lung which is both morphologically and biochemically immature. A premature birth after 32 weeks of gestation is not generally associated with a complicated neonatal course, since the lung has developed sufficiently to support respiration in the outside world. The immaturity of the lung at 24 to 32 weeks is such, however, to manifest in severe respiratory problems for infants born at this gestational age. The insufficiency of the surfactant system is believed to be the major factor responsible for the characteristic development of RDS. Infants who

contract this acute respiratory condition are unable to breathe independently, and as a result, require the provision of mechanical ventilation and supplementary oxygen. As a consequence of the technological advances in intensive neonatal care, the mortality from RDS has dramatically decreased. Unfortunately, this favourable outcome has occurred at the expense of an increased incidence of complications relating to the aggressive nature of respiratory support (Papageorgiou & Stern, 1986). These debilitating secondary conditions of RDS, detailed in Chapter 1, section 3., may manifest in fatalities, or at least result in an incomplete or very slow recovery.

Animals studies in the late 1960's and early 1970's demonstrated precocious lung maturation in the fetal lamb and rabbit (Liggins, 1969; Kotas & Avery, 1971; Wang *et al.*, 1971) upon administration of glucocorticoids to mothers at an appropriate time prior to delivery. These findings promoted the use of antenatal steroid therapy in the clinical setting, to prevent the associated complications of lung immaturity in situations which threatened a premature birth. The efficacy of prenatal glucocorticoids in lowering the incidence of RDS was first demonstrated by Liggins & Howie (1972). Since this initial report, numerous studies have confirmed the usefulness of antenatal steroids to accelerate fetal lung maturation (Block *et al.*, 1977; Papageorgiou *et al.*, 1979; Collaborative Group, 1981; Doyle *et al.*, 1986). To summarize, the prenatal administration of either betamethasone or dexamethasone (10 to 12mg/day) 24 to 48 hours before delivery, has the following benefits upon infants born prematurely between 26 to 34 weeks of gestation: a reduced incidence of RDS, a decreased severity of the condition when it does occur and an increased survival.

The initiation of antenatal glucocorticoid therapy for the prevention of RDS promoted a number of animal studies to investigate the potential risks associated with the treatment. The negative effect of prenatal steroid on somatic and lung growth was first demonstrated in the rabbit fetus exposed to hydrocortisone (Kotas & Avery, 1971; Carson *et al.*, 1973; Kotas *et al.*, 1974). Subsequent work reported a marked inhibitory effect of glucocorticoids on lung and body weight in the premature rat utilizing a regime comparable to that used in human pregnancies (dexamethasone 0.2mg/Kg body weight/day for 48 hours prior to delivery) (Frank

& Roberts, 1979; Frank *et al.*, 1985). Of further concern, regarding the long-term effects of glucocorticoids on fetal development, the pulmonary growth deficit observed in the steroid-treated rhesus monkey fetus was found to persist for at least 4 weeks after discontinuation of treatment (Johnson *et al.*, 1981). A favourable outcome for a preterm infant will only be compromised by a growth retarded lung at birth, especially if this is superimposed on poor postnatal pulmonary growth and lung damage, frequently associated with a premature delivery. These animal studies, in demonstrating the growth suppressive effects of steroid exposure on the fetus, cautioned against the indiscriminate and prolonged use of potent glucocorticoids during pregnancy.

Previous animal work investigating the favourable and detrimental effects of antenatal glucocorticoid exposure on fetal lung development have been conducted in separate species, utilizing variable steroid regimes. The present chapter has utilized the guinea pig to assess both fetal lung growth and maturation in response to prenatal glucocorticoid treatment. This enabled a parallel examination of the benefits and potential risks associated with antenatal steroids. Lung weight, lung protein and the rate of pulmonary protein synthesis were determined as indices of pulmonary growth, while maturation was investigated by assessing the surfactant status, glutathione content and antioxidant enzyme activities in the lung. At birth the guinea pig possesses a high degree of physical and functional maturity compared to other small laboratory animals. In addition, advanced morphological lung development has been described in the newborn (Lechner & Banchero, 1982). The similarity in lung structure between the guinea pig and human at birth, suggests that this animal model is appropriate when investigating the problems which accompany clinical prematurity. Furthermore, the maturity associated with the guinea pig enables the pups to be delivered by Caesarian section up to 7 days before full gestation which is 68 days, and survive for several hours. This permitted the measurement of *in vivo* pulmonary protein synthesis in the preterm animal. In the present study, dexamethasone was administered daily to the pregnant dam from gestational day 55 to day 60 before the premature pups were delivered by Caesarian section on day 61. The natural increases in pulmonary PC, glutathione and the activities of SOD, GSH-Px and CAT begin at approximately day 60 of gestation in this species (Kelly *et al.*, 1990; Rickett & Kelly, 1990). By commencing

dexamethasone treatment on gestational day 55, any induction of these parameters should have been evident on day 61, when under control conditions, these indices of pulmonary maturation are still relatively low.

5.2 EXPERIMENTAL PROTOCOL

On day 55 of gestation, Dunkin-Hartley guinea pigs were randomly divided into 2 groups and housed in individual cages. Group 1 received a daily subcutaneous injection of 0.9% NaCl, while group 2 was administered dexamethasone acetate (2.0mg/Kg body weight/day) for 6 days. On gestational day 61, pups were delivered by Caesarian section, the details of which are described in Chapter 2, section 2.3. Following the intraperitoneal injection of a bolus dose of ^{3}H-phenylalanine, the pups were killed by decapitation. The lungs were then taken for the measurement of protein synthesis, glutathione status, antioxidant enzyme activity, surfactant status, tissue protein and tissue DNA. Refer to Chapter 2, sections 2.5, 2.11, 2.12, 2.13, 2.14 and 2.15 for an explanation of the methodology employed.

5.3 RESULTS

5.3.1 The Effects of Prenatal Dexamethasone on Whole Body and Lung Weight of the Fetal Guinea Pig

Table 12 compares the body and lung weights of guinea pigs on day 61 of gestation following prenatal saline or dexamethasone treatment. The greater mean body weight and lung weight in the experimental group does not appear to be an effect of the steroid, but reflects the disparity between litter sizes. The guinea pigs which made up both the control and steroid treated groups were taken from 3 separate litters. The individual litter numbers were 7,3 and 4 for the control and 2,4 and 4 for the dexamethasone group. The litter of 7 control pups possessed low body weights while the litter of 2 dexamethasone treated animals had both large body and lung weights (see Appendix 3.).

Table 12. Fetal Body and Lung Weight Following Prenatal Dexamethasone Treatment

Maternal treatment	Body wt. (g)	Lung wt. (g)
Control	51.4±11.5	1.25±0.15
Dexamethasone	65.3± 8.8	1.39±0.36
	(+27%)	(+11%)

Pregnant guinea pigs were injected subcutaneously with 0.9% NaCl or dexamethasone acetate (2.0mg/Kg body weight) from day 55 to day 60 of gestation, prior to fetal delivery on gestational day 61. The values presented represent the means ± S.D. n=14 and 9 (body wt) and n=14 and 6 (lung wt). Values in parentheses show the percentage change from the control group.

5.3.2 The Effects of Prenatal Dexamethasone on Fetal Lung DNA, Protein and Pulmonary Protein Synthesis

Previous animal studies have reported that prenatal glucocorticoid administration slows fetal lung cell mitosis in favour of differentiation (Carson *et al.*, 1973; Kotas *et al.*, 1974). In the present study the indices of pulmonary maturation (see section 5.3.3) were expressed per unit DNA. It was important therefore to examine DNA content in addition to protein metabolism in the fetal lung when investigating the potential growth suppressive qualities of dexamethasone.

The inability of maternal steroid treatment to influence fetal lung protein or DNA was indicated in the almost identical concentrations of these parameters in the control and experimental groups (Table 13). The slightly higher total protein and DNA content in the lung following dexamethasone treatment is not significant, and reflects the greater lung mass in the small litter (n=2) which was incorporated into

Table 13. Fetal Lung Protein, DNA and Protein Synthesis Following Prenatal Dexamethasone Treatment

Maternal treatment	DNA conc. (mg/g)	DNA content (mg)	Protein conc. (mg/g)	Protein content (mg)	Fractional rate of synthesis (%/day)
Control	5.2±0.7	6.4±1.2	66.5±11.1	80.1±14.6	12.0±4.6
Dexamethasone	5.2±0.7	7.6±1.6	67.8±9.4	93.9±13.8	12.5±5.7
		(+19%)		(+17%)	

Tissue DNA was measured by the method of Sterzel *et al*. (1985). Tissue protein was measured by the method of Smith *et al*. (1985). The fractional rate of protein synthesis was determined as described by Garlick *et al*. (1980). The values presented represent the means ± S.D. n=13 and 10 for the control and dexamethasone treated groups respectively. Values in parentheses show the percentage change from the control group.

the steroid-treated group. Finally, with respect to growth, the rate at which protein was synthesized in the lung of the fetal guinea pig upon delivery was unaffected by the 6 day prenatal dexamethasone regime (Table 13).

5.3.3 The Effects of Prenatal Dexamethasone on the Pulmonary Antioxidant and Surfactant Systems in the Fetal Guinea Pig

Having established that fetal lung growth was not affected by prenatal dexamethasone administration, at the dose and timing employed, the ability of this regime to stimulate the biochemical indices of lung maturation was investigated.

The components of the pulmonary antioxidant system presently studied included the enzymatic defence provided by SOD, GSH-Px and CAT, and the non-enzymatic molecule, glutathione. The latter provides protection against oxygen free radicals by acting as a reductant in the presence of GSH-Px. Table 14 shows that maternal dexamethasone treatment did not significantly induce these components in the lung of the fetal guinea pig. The activities of pulmonary SOD and CAT were almost identical in the control and experimental groups. The concentration of total glutathione, i.e. both oxidized and reduced forms, was 30% greater in the lungs of pups whose mothers received dexamethasone. Likewise, pulmonary GSH-Px activity was 42% higher in the steroid group. The failure of these fairly substantial increments to gain statistical significance was due to the large spread of individual data points in both the control and steroid groups.

The second biochemical index of lung maturation investigated in the present study was pulmonary surfactant status. Surfactant consists largely of lipid (>80%) of which PC accounts for 80 to 90%. The concentration of total PC was measured in the fetal lung following prenatal steroid treatment together with the individual molecular species, DPPC, the major surface active component of PC, and palmitoyl oleoyl phosphatidylcholine (POPC). The relative proportions of these species change as gestation proceeds, i.e. with increasing lung development, such that DPPC rises and POPC falls (Kelly *et al.*, 1990). The DPPC/POPC ratio can therefore be taken as a marker of pulmonary maturation. Table 15 shows that the concentration of total PC and the percentage of which constitutes DPPC and POPC

was very similar in the control and dexamethasone treated groups. The unchanged antioxidant profile in response to maternal dexamethasone administration was therefore paralleled by the failure of this regime to stimulate the surfactant system of the fetal lung.

Table 14. Glutathione Content and Antioxidant Enzyme Activities of the Fetal Lung Following Prenatal Dexamethasone Treatment

Maternal treatment	Total glutathione (μmol/mgDNA)	Mn-SOD (U/mgDNA)	Cu/Zn-SOD (U/mgDNA)	CAT (KU/mgDNA)	GSH-Px (U/mgDNA)
Control	0.19±0.09	7.15±4.52	22.34±6.86	1.68±0.45	0.12±0.06
Dexamethasone	0.25±0.05 (+30%)	6.05±2.78	25.61±5.21	1.56±0.29	0.17±0.05 (+42%)

Total glutathione was measured by the method of Griffith (1980). The activity of SOD (cyanide-sensitive, Mn-SOD, and cyanide-insensitive, Cu/Zn-SOD) was measured by the method of Marklund (1985). CAT activity was measured by the method of Aebi (1984). GSH-Px activity was measured by the method of Beutler (1979). The values presented represent the means ± S.D. n=13 and 10 for the control and dexamethasone treated groups respectively. Values in parentheses show the percentage change from the control group.

Table 15. Effect of Prenatal Dexamethasone Treatment on Phosphatidylcholine Molecular Species Distribution in the Fetal Lung

Maternal treatment	Total PC	Molecular species		DPPC/POPC
		DPPC	POPC	
	(μmols/mgDNA)	(% total PC)		
Control	1.13±0.30	33.2±6.0	18.9±5.5	2.03
Dexamethasone	1.21±0.28	31.9±6.0	21.6±7.5	1.97

Total phosphatidylcholine (PC) and the individual molecular species, dipalmitoyl phosphatidylcholine (DPPC) and palmitoyl oleoyl phosphatidylcholine (POPC) were determined as described by Postle (1987). The values presented represent the means ± S.D. n=13 and 10 for the control and dexamethasone groups respectively.

5.4 DISCUSSION

Observations from animal studies that glucocorticoids accelerate fetal lung maturation (Liggins, 1969; Kotas & Avery, 1971; Wang *et al.*, 1971) led to numerous clinical trials which investigated the efficacy of antenatal steroids in lowering the incidence of RDS (Liggins & Howie, 1972; Block *et al.*, 1977; Papageorgiou *et al.*, 1979; Doyle *et al.*, 1986). The successful outcome of these trials has since led to steroids becoming standard practice in the management of prematurity in many parts of the world, notably in the United States. A far less desirable quality of glucocorticoids is their ability to suppress whole body and organ growth, an effect which has been demonstrated following prenatal treatment in the fetus of a variety of species (Carson *et al.*, 1973; Sanfaçon *et al.*, 1977; Frank & Roberts, 1979; Johnson *et al.*, 1981).

The present work was designed to evaluate the effects of maternal dexamethasone administration on both fetal pulmonary growth and maturation within one species, namely the guinea pig. The prenatal steroid regime presently employed had no effect on lung protein in pups delivered on day 61 of gestation when compared to the saline control group. Assuming that pulmonary protein was similar in the two groups at the beginning of the experiment, this would suggest that lung growth i.e. protein accumulation, was unaffected by maternal dexamethasone administration. Furthermore, the rate of protein synthesis in the lung of the control and steroid group was almost identical. This strongly supports the view that pulmonary protein metabolism in the fetal guinea pig is resistant to dexamethasone at the dose and timing employed. Previous studies have demonstrated that prenatal steroid treatment interferes with fetal lung growth as a result of inhibiting cellular mitosis (Carson *et al.*, 1973; Kotas *et al.*, 1974). The present study did not however detect a deficit in lung cell number, reflected by DNA content per lung, following prenatal steroid treatment. These results are in agreement with a study of similar design in which dexamethasone (2mg/Kg body weight/day) was administered to pregnant guinea pigs from day 55 of gestation to term (Lechner, 1987). Analogous to the present findings, the steroid had no effect on lung weight, protein or DNA in the offspring.

The capacity of antenatal glucocorticoids to suppress somatic and organ growth was first suggested by Carson *et al.* (1973). This study reported lower body weights, lung weights and a decrease in lung cell number in fetal rabbits 48 hours after a single injection of 2mg hydrocortisone on day 24 of gestation (term is 31 days). The use of hydrocortisone, rather than dexamethasone, administered directly to the fetus and not via the pregnant dam, prevents a direct comparison of this result with the present investigation. When dexamethasone (0.2mg/Kg body weight) is administered to pregnant rats, 48 and 24 hours prior to fetal delivery on gestational day 21 (1 day premature) or at term, the offspring exhibit significantly lower body and lung weights (Frank & Roberts, 1979; Frank *et al.*, 1985). Other workers have observed a reduced DNA content in the fetal rat lung which accompanies the fall in body and lung weight following prenatal dexamethasone administration (Schellenberg *et al.*, 1987). The ability of dexamethasone to elicit a response, at a lower dose and over a shorter duration than that presently employed, would suggest that pulmonary growth in the fetal rat, compared to the guinea pig, is more sensitive to the growth suppressive qualities of glucocorticoids.

The different effects of glucocorticoids on the fetal lung in different species makes it difficult to make a definite comment regarding the influence of dexamethasone on pulmonary growth, when employed clinically. Up until recently, concerns regarding the potential risks of antenatal steroid therapy on the human fetus have been resolved by carefully monitoring whole body growth. In the short-term, premature infants who experience the benefits of antenatal glucocorticoids are not associated with a lower birth weight when compared to placebo-treated subjects (Liggins & Howie, 1972; Collaborative Group, 1981; Doyle *et al.*, 1986; Morales *et al.*, 1986). Concomitant with this, long-term follow-up studies have not detected a difference between placebo- and dexamethasone-treated groups with respect to height, weight and head circumference at 2 to 6 years of age (MacArthur *et al.*, 1982; Collaborative Group, 1984; Doyle *et al.*, 1986). Further reassurance regarding the safety of antenatal steroids was recently provided by a study which evaluated the effects of the therapy on lung growth (Wiebicke *et al.*, 1988). Pulmonary function tests performed on children over 6 years old who were part of a former collaborative study showed no differences between individuals whose

mothers received antenatal dexamethasone and those who received a placebo. Although an initial inhibitory effect of dexamethasone on lung growth cannot be ruled out by this long-term follow-up study, the results suggest that such an effect would have been accompanied by postnatal catch-up growth. Such a phenomena would be analogous to the compensatory growth reported in animal studies after a steroid-induced inhibition of lung growth (Kotas *et al.*, 1974).

Having established that fetal lung growth in the guinea pig was unaffected by the prenatal steroid regime employed, the influence of this treatment on pulmonary maturation was investigated. An unexpected finding was the failure of maternal dexamethasone to activate the fetal lung surfactant system, as determined by pulmonary PC and the distribution of DPPC and POPC. The ability of glucocorticoids to stimulate surfactant biosynthesis in the fetal lung is widely recognised and has been demonstrated in the rabbit (Rooney *et al.*, 1979), rat (Frank *et al.*, 1985) and guinea pig (Lechner, 1987) following prenatal treatment. Administration of betamethasone or dexamethasone (0.2mg/Kg body weight) to pregnant rabbits and rats significantly increases fetal lung PC content (Rooney *et al.*, 1979; Frank *et al.*, 1985). The manifestation of such a response, at a dose of steroid 10 times less than that employed in the guinea pig studies, clearly demonstrates the sensitivity of the rabbit and rat to glucocorticoid induced lung maturation. The previous work with the guinea pig reported a marked increase in fetal lung phospholipids following dexamethasone administration to the pregnant dam (Lechner, 1987). The steroid regime (2mg/Kg) commenced on day 55 of gestation and continued until full-term (68 days). The additional 7 days of dexamethasone treatment employed by Lechner (1987) may explain the apparent stimulation of pulmonary surfactant in the fetus which I was unable to demonstrate when the treatment ended on gestational day 60. A strong consensus exists that endogenous glucocorticoids influence fetal lung maturation. This opinion stems from the temporal relationship which has been identified in the rabbit, rat and guinea pig between the fetal plasma glucocorticoid peak during gestation, changes in lung morphology and surfactant system maturation (Holt & Oliver, 1968; Kitterman *et al.*, 1981; Sosenko *et al.*, 1986; Sosenko & Frank, 1987a). The predominant peak of serum cortisol in the fetal guinea pig on approximately day 59 of gestation is followed by the natural increases in pulmonary PC (Sosenko & Frank, 1987a; Kelly

et al., 1990). If the pulmonary surfactant system is unable to respond to endogenous or exogenous glucocorticoids before day 59 to 60 of gestation, this would provide an explanation for the induction of lung surfactant by dexamethasone at term (Lechner, 1987) and the inability to detect such a phenomena on gestational day 61.

The similar time course in the developmental profile of the pulmonary antioxidant and surfactant systems in the rabbit (Khosla *et al.*, 1983; Frank & Groseclose, 1984), rat (Tanswell & Freeman, 1984; Sosenko *et al.*, 1986) and guinea pig (Kelly *et al.*, 1990; Rickett & Kelly, 1990) suggest that these biochemical markers of fetal lung maturation may be under similar hormonal control. An investigation carried out in the rat supports this concept in that the antioxidant enzyme system of the fetal lung, in parallel with pulmonary surfactant, responded to maternal glucocorticoid treatment by accelerated maturation (Frank *et al.*, 1985). Antenatal steroid therapy, employed in the management of human prematurity, may therefore have dual benefits in promoting lung maturation. The benefits provided by prenatal glucocorticoids, as a result of stimulating the surfactant system of the human fetal lung, are clearly evident from the decreased incidence of RDS in premature infants following this treatment. An increased capacity of pulmonary antioxidant defence has the potential to provide a preterm infant with a greater measure of protection against the toxic complications of supplementary oxygen which may be required following a premature delivery. When such a phenomena was investigated in the guinea pig, it became apparent that in parallel with the pulmonary surfactant status of this species, the developmental pattern of the antioxidant defence system in the lung was not responsive to maternal dexamethasone.

To summarize, the adminstration of dexamethasone (2mg/Kg body weight/day) to pregnant guinea pigs from gestational day 55 to 60, failed to affect either fetal lung growth or the biochemical indices of pulmonary maturation studied. This is in contrast to previous work conducted in the rabbit and rat, which have reported a suppression of fetal lung growth and an induction of fetal lung maturation following prenatal glucocorticoid exposure late in gestation (Carson *et al.*, 1973; Rooney *et al.*, 1979; Frank *et al.*, 1985). Two explanations for this marked

species difference are discussed below, and relate to glucocorticoid receptor number in the lung and placental permeability to steroids.

One of the earliest events in the action of glucocorticoids on the fetal lung is the interaction of the steroid with specific glucocorticoid receptors in the cell cytoplasm (Ballard, 1977). The hormonal response of the lung will therefore be dictated by the number of receptors present in the tissue. During the last 3 days of gestation, measured concentrations of cytoplasmic binding sites for dexamethasone in the fetal lung vary from 0.43 to 0.52 pmoles/mg protein in the rabbit and 0.21 to 0.35 pmoles/mg protein in the rat (Giannopoulos, 1974; Granberg & Ballard, 1977). It is at this time point of gestation that prenatal glucocorticoid treatment has been reported to elicit a response in the lung of these animals (Rooney *et al.*, 1979; Frank *et al.*, 1985). In addition, the ability of antenatal steroid therapy to reduce the incidence of RDS in the premature infant is associated with the presence of cytoplasmic receptors for glucocorticoids in the lung of the human fetus from 12 weeks of gestation with a mean concentration of 0.24 pmoles/mg protein (Ballard & Ballard, 1974). In contrast, binding of dexamethasone in the cytoplasm of the guinea pig lung between day 55 and day 65 of gestation is virtually undetectable (0.01 pmoles/mg protein) (Giannopoulos, 1974). The absence of glucocorticoid receptors in the lung of this species at a time when dexamethasone was administered is probably the most likely explanation for the failure of steroid treatment to influence the investigated parameters of fetal lung development. The report by Lechner (1987), that dexamethasone administration to pregnant guinea pigs from day 55 of gestation to term stimulates pulmonary surfactant in the fetus, does not imply that the fetal lung is devoid of glucocorticoid receptors. If this observation was mediated via a direct effect of the steroid, one may speculate that the concentration of cytoplasmic binding sites for dexamethasone increases over the final 3 days of gestation.

An alternative interpretation for the presently cited species difference may relate to transplacental transfer of dexamethasone. Although the results of Lechner (1987) do not suggest that the placenta of the guinea pig presents a barrier to dexamethasone, such a characteristic may be considered in the context of the present findings. Indirect evidence does suggest that placental permeability to

steroids varies between species. For example, while Liggins (1969) was able to induce premature parturition and lung maturation in lambs when dexamethasone was directly infused into the fetus, such a response was not produced when the steroid was injected into the pregnant ewe. In contrast, the benefits experienced by the premature infant and the response elicited in the fetal rat following maternal dexamethasone administration, strongly imply that the placenta of these species is permeable to this synthetic glucocorticoid.

Speculation that the placenta of the guinea pig has a high resistance to dexamethasone has arisen from the fact that premature parturition does not occur when the hormone is given to the pregnant dam at a dose of 8mg/day for at least 7 days (Ash *et al.*, 1973). Premature delivery cannot, however, be used as an index of placental permeability in this species since the administration of dexamethasone (100μg/day for at least 7 days) also fails to induce labour when injected directly into the fetus (Ash *et al.*, 1973). The limited evidence available that dexamethasone does cross the placenta of the guinea pig is rather indirect, and describes fetal adrenal weight and thyroid hormone concentration changes after the administration of the steroid (10mg/day for 6 days) to the pregnant dam (Alwan, 1987). During fetal development, the growth and functional activity of the adrenal cortex depends upon the release of adrenocorticotrophic hormone (ACTH) from the fetal pituitary gland (Jost, 1966). The reduction in adrenal weight relative to body weight in the pups following steroid treatment suggests that dexamethasone was present in the fetal circulation and involved in a negative feedback mechanism on the pituitary, decreasing the secretion hence stimulatory activities of ACTH. In addition, the 3,5,3'-triiodothyronine/thyroxine (T_3/T_4) ratio was significantly higher in the plasma of the fetal guinea pig after maternal dexamethasone treatment. This may imply that the steroid crossed the placenta and stimulated the conversion of T_4 to T_3 in the fetus as described in the fetal sheep following the infusion of cortisol (Thomas *et al.*, 1978). The conclusions which can be drawn from this work are limited, however, since the mother of the pups which acted as controls did not receive an injection over the experimental period. A saline control group should always be included in such studies, to evaluate any significant effects of excess endogenous glucocorticoid secretion by the pregnant dam during the stress of handling and injecting. The possibility exists that the reported observations in the fetus, arose as

a consequence of an indirect effect of dexamethasone in the maternal circulation, and did not reflect the presence of the steroid in the fetus.

The dose, duration and timing of the maternal dexamethasone regime presently employed did not influence pulmonary growth or biochemical maturation of the fetal guinea pig. I believe that the most likely explanation for this "negative" response reflects the insensitivity of the fetal lung to dexamethasone owing to the virtual absence of specific glucocorticoid receptors in the tissue. As a consequence of this particular characteristic of the guinea pig, unrecognised when the experiment was instigated, this study cannot provide further information regarding the potential risks and benefits of antenatal steroids when employed in the clinical setting. The information which is continually accumulating from clinical studies would suggest however that the usefulness of prenatal steroid treatment does not occur at the expense of a growth deficit. The presence of glucocorticoid receptors in the human lung and the efficacy of antenatal steroids in reducing the incidence of RDS, illustrates the responsiveness of the human fetal lung to the "favourable" qualities of glucocorticoids. At the same time, long-term follow-up studies have alleviated concerns regarding both somatic and lung growth in infants exposed in utero to steroids.

CHAPTER 6. THE EFFECTS OF DEXAMETHASONE ON PULMONARY PROTEIN TURNOVER

6.1 INTRODUCTION

The growth suppressive qualities of corticosteroids in children have been recognized in the clinical setting for many years. Growth retardation is a well-known feature of Cushing's syndrome, a condition characterized by an overproduction of glucocorticoids (Strickland *et al.*, 1972). In addition, corticosteroids administered to individuals for prolonged periods, for various chronic conditions, have a significant growth suppressing capability (Hyams & Carey, 1988). These clinical observations are supported by animal studies, demonstrating that the net effect of excess glucocorticoids is a whole body deficit in the young and an atrophy in adults (Loeb, 1976; Tomas *et al.*, 1979; Kelly & Goldspink, 1982, 1983, 1984; Kelly *et al.*, 1986).

A greater understanding into the overall response of the body to glucocorticoids has been gained from further animal work, investigating the effects of glucocorticoids on protein metabolism in individual tissues of the body. In 1964, Munro observed that the growth of the liver, far from being suppressed, was enhanced in rats treated with cortisone. More recently, the effects of glucocorticoids on the musculature and lymphoid tissues in the rat was studied in a large investigation utilizing a standard steroid regime of 2.5mg dexamethasone per Kg body weight per day for 1 to 10 days (Kelly & Goldspink, 1982, 1983, 1984; Kelly *et al.*, 1986). Analogous to the liver, growth of the heart was shown to be enhanced in response to dexamethasone administration (Kelly & Goldspink, 1982; Kelly *et al.*, 1986). The differential effect of glucocorticoids on protein metabolism in various tissues was illustrated clearly, however, by the fact that growth was reduced in slow-twitch skeletal muscle and abolished in fast-twitch skeletal muscle (Kelly & Goldspink, 1982; Kelly *et al.*, 1986). Of the organs examined, the lymphoid tissues exhibited the greatest sensitivity to dexamethasone, with both the thymus and spleen undergoing a rapid and marked atrophy (Kelly & Goldspink, 1983, 1984).

In the 1980's, preliminary studies were initiated to investigate the response of CLD of premature infants, to dexamethasone (Mammel *et al.*, 1983,1987; Avery *et al.*, 1985). The relatively rapid (generally within 72 hours) improvement in pulmonary function following steroid treatment has since led to the widespread application of dexamethasone as a potential therapy for CLD. More recent clinical trials have given further evidence for the benefits, although somewhat transient, provided by glucocorticoids in the management of infants with chronic respiratory problems (Harkavy *et al.*, 1989; Noble-Jamieson *et al.*, 1989).

The lungs of infants with CLD are not only immature as a consequence of a premature birth, but are also damaged due to the invasive nature of supplementary oxygen and mechanical ventilation. The ultimate recovery from CLD will therefore depend upon the ability of the baby to break free from respiratory support, which in turn requires optimal pulmonary growth and repair. Unfortunately, evidence suggests that lung growth may be compromised during CLD (Bryan *et al.*, 1973; Sobonya *et al.*, 1982; Wheeler *et al.*, 1984; Tepper *et al.*, 1986). In addition, the animal studies quoted above suggest that the initiation of steroid treatment may further disrupt pulmonary protein metabolism. In severe cases of CLD, dexamethasone may be administered at a dose approaching 2mg/Kg body weight/day, comparable to the dose required to produce a negative effect on growth of the musculature and lymphoid tissues in the rat (Kelly & Goldspink, 1982, 1983, 1984; Kelly *et al.*, 1986). The high levels of both dexamethasone binding and glucocorticoid receptor mRNA in the postnatal lung (Granberg & Ballard, 1977; Kalinyak *et al.*, 1987), suggest that this tissue may be particulary sensitive to dexamethasone.

The potential of postnatal glucocorticoid treatment in influencing pulmonary growth and repair has been examined in the present chapter by assessing the sensitivity of the lung to dexamethasone. The influence of an acute (24 hour) and chronic (5 day) administration of dexamethasone (2.5mg/Kg body weight/day) on growth and protein turnover in the lung of the adolescent rat was investigated. Subsequently the changes in pulmonary protein metabolism found to be induced by dexamethasone were characterized by analyzing ribosomal and polysomal aggregation in the lung on sucrose density gradients. As a consequence of the

immaturity of the rat at birth, considerable lung growth and development occurs during the postnatal period in this species (Burri, 1974; Burri *et al.*, 1974; Thurlbeck, 1975). For example, the lungs of the adolescent rats employed in this study were still growing at a rate of 5% per day. This animal model, although not ideally representative of preterm infants, was therefore able to provide important information upon the effects of corticosteroid therapy on lung growth. In addition, this protocol enabled a meaningful comparison to be made between the influence of dexamethasone on the lung, with the earlier work conducted on the musculature and lymphoid tissues of the rat (Kelly & Goldspink, 1982, 1983, 1984; Kelly *et al.*, 1986).

6.2 EXPERIMENTAL PROTOCOL

Adolescent, male Wistar rats (initial body weight 100 g) were randomly divided into 6 groups. Group 1 were killed immediately and their lungs were analyzed as part of the growth rate determinations. For the acute experiment, animals in groups 2 and 3 received a single subcutaneous injection of 0.9% NaCl or dexamethasone acetate (2.5mg/Kg body weight) suspended in 0.9% NaCl respectively and were killed 24 hours later. In the chronic experiment, animals in groups 4 and 5 received a daily subcutaneous injection of 0.9% NaCl for 5 days, while group 6 received a daily subcutaneous injection of dexamethasone acetate (2.5mg/Kg body weight/day) for 5 days. The food intake of groups 4 and 6 was recorded daily and the average food intake of group 6 was given to group 5 animals (pair-fed controls) on the following day.

Following the appropriate experimental treatment, animals were injected with a bolus dose of ^{3}H-phenylalanine and killed by decapitation. The lungs were then taken for the measurement of protein synthesis, ribosomal and polysomal aggregation, tissue RNA and tissue protein. Refer to Chapter 2, sections 2.4, 2.6, 2.7 and 2.14 for full details of the methodology employed.

6.3 RESULTS

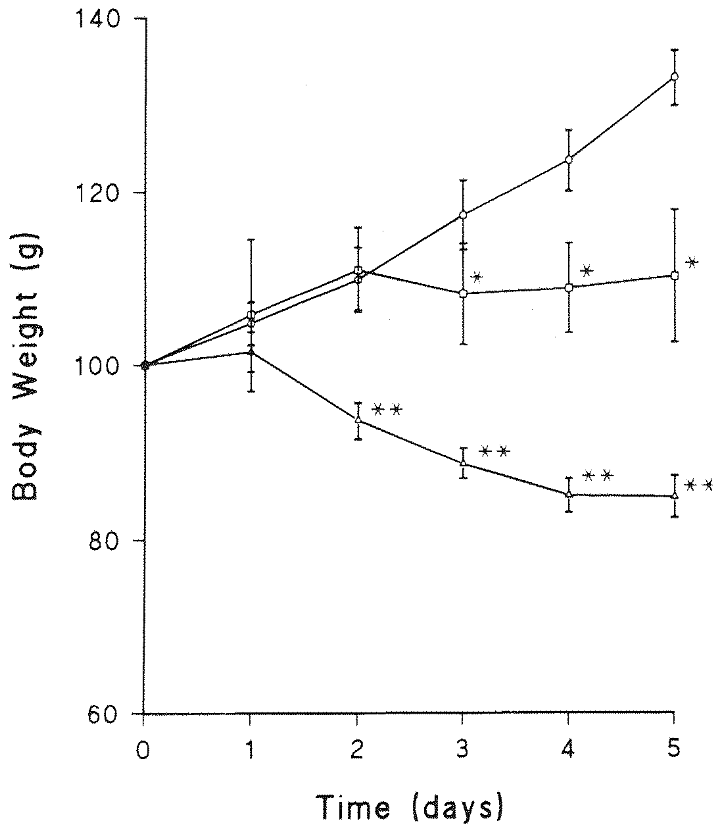
6.3.1 The Effects of Dexamethasone on Food Intake, Body Weight and Lung Growth

Previous studies have shown that dexamethasone administration at a dose of 2.5mg/Kg body weight/day has an immediate effect on both whole body and tissue growth in the rapidly growing rat (Kelly & Goldspink, 1982, 1983, 1984). These investigations did not establish, however, whether the observed changes were due in part to a reduced food intake in the steroid treated animals. In the present work, the food consumption of rats injected with dexamethasone was measured to incorporate a group of saline injected, pair-fed controls into the study. In this way, the relative contributions of dexamethasone treatment per se, and a steroid-induced change in food intake on pulmonary protein metabolism were examined

Rats injected daily with saline and fed *ad libitum* (average food intake, 17g/day) gained weight at an average rate of 6.0% per day (Fig. 17). In contrast, within 48 hours of dexamethasone treatment, animals were losing weight at an average rate of 3.0% per day. In parallel with this, by day 2, the daily food consumption of these rats had fallen by 45% and remained at this level over the following 3 days (data not shown). Whole body weight gain was optimal (7.0%/day) in the pair-fed, saline injected controls over the first 2 days, and thereafter remained constant (Fig. 17).

Having re-established the response of the whole body to dexamethasone, the sensitivity of the lung to steroid treatment was specifically examined. The influence of dexamethasone on pulmonary growth was assessed by measuring the net accumulation, or loss, of protein over the experimental period (Table 16). Changes in tissue protein content is a more reliable index of growth than wet weight measurements, since the mineralocorticoid activity of glucocorticoids (although low in the case of dexamethasone) will mediate electrolyte changes and consequently water retention. The *ad libitum* control groups exhibited an appreciable rate of lung growth which averaged 8.3 and 5.2%/day when measured

Figure 17. Changes in Whole Body Weight in Response to Dexamethasone or Pair-feeding with Animals Receiving Dexamethasone



Rats initially weighing 100 g were given daily subcutaneous injections of 0.9% NaCl (○) or 2.5mg of dexamethasone/Kg body weight (△). A third group of animals (□) received saline and were pair-fed with those rats receiving dexamethasone. Body weights were measured between 09:00 and 10:00 h each morning over the experimental period. Each point represents the mean ± S.D. derived from six animals. $^{*}P<0.01$, statistically significant from *ad libitum* controls, $^{**}P<0.001$, statistically significant from *ad libitum* and pair-fed controls.

over 1 and 5 days respectively. Within just 24 hours of dexamethasone administration, pulmonary protein accumulation was abolished, and remained suppressed over 5 days of steroid treatment. The identical rate of lung growth in the 5 day *ad libitum* and pair-fed controls indicated that in contrast to whole body weight gain, lung growth was unaffected by the degree of food restriction that the animals were subjected to (Table 16).

Table 16. The Effect of Dexamethasone on the Rate of Lung Growth

Duration of treatment (days)	Treatment	Protein Content (mg)	Daily growth rate (K_g) (%/day)
0		86.8±14.1	
1	Control	94.3±11.8	8.3
	Dexamethasone	85.2± 8.8	-0.7
5	Control	113.2± 4.6	5.2
	Pair-fed control	114.4± 4.9	5.3
	Dexamethasone	85.3± 4.9* (-25%)	-0.3

Total protein was measured by the method of Smith *et al.* (1985). Growth rates (K_g) were derived from changes in the protein mass between days 0-1 and 0-5. The values presented are the means ± S.D. derived from six animals. Values in parentheses show the percentage change from the day 5 *ad libitum* control group. *P<0.01, statistically significant from the 5 day *ad libitum* and pair-fed controls.

6.3.2 The Effects of Dexamethasone on Pulmonary Protein Turnover

The degree of tissue growth is dependent upon the balance between the rates of protein synthesis and breakdown. The effect of dexamethasone on these components was therefore studied to investigate the inability of rats to accumulate pulmonary protein following steroid administration. The rapid cessation of lung growth in rats treated acutely with dexamethasone was due to a large (38%) fall in the rate of pulmonary protein synthesis (Table 17). Following this marked, initial reduction, the rate of lung protein synthesis decreased even further during the chronic phase of steroid treatment. Pulmonary protein synthesis measured following 5 days of pair-feeding averaged 42.1±3.4%/day, thus the optimal rate of protein synthesis, together with the unaffected growth of the lung of these animals, further verified that the reduced food intake following dexamethasone did not contribute to the steroid-induced changes in pulmonary protein turnover. In subsequent measurements which further elucidated the mechanism of action of dexamethasone on protein metabolism in the lung, the 5 day saline injected, *ad libitum* controls were used as the reference, to which the 5 day steroid-treated group were compared.

The decrease in the rate of protein synthesis in the lung 24 hours after dexamethasone exposure was partially compensated for, by a small (17%) fall in the calculated rate of pulmonary protein breakdown. Furthermore, following 5 days of steroid administration, protein breakdown in the lung was depressed by 46%, in parallel with the greater fall in protein synthesis at this time point (Table 17).

The overall rate of protein synthesis in the lung is determined by the number of ribosomes in the tissue, and the translational activity of the ribosomes i.e. the rate of protein synthesis per ribosome. The mode of action of dexamethasone on inhibiting pulmonary protein synthesis at the two time points studied, was characterized by calculating the RNA/protein ratio and synthesis per unit RNA in the lung. These represent indices of ribosomal capacity and efficiency respectively (Henshaw *et al.*, 1971; Millward *et al.*, 1973). During the acute phase of steroid administration, the reduced rate of protein synthesis in the lung was due to a significant (44%) fall in translational efficiency, while synthetic capacity was

Table 17. The Effects of Dexamethasone on Protein Turnover in the Lung

Duration of treatment (days)	Treatment	Fractional rate of synthesis (%/day)	Fractional rate of breakdown (%/day)	Ribosomal efficiency (mgPr/d/mgRNA)	Ribosomal capacity (mgRNA/gPr)
1	Control	37.4±4.2	29.1	20.9±4.8	20.2±2.3
	Dexamethasone	23.4±4.4* (-38%)	24.1 (-17%)	11.7±3.0* (-44%)	19.9±3.0
5	Control	41.6±7.5	36.4	17.8±3.6	23.5±2.0
	Dexamethasone	19.6±2.4** (-53%)	19.7 (-46%)	11.7±1.1** (-35%)	16.9±2.0*** (-28%)

The fractional rate of protein synthesis was determined as described by Garlick *et al*. (1980). The fractional rate of breakdown was calculated from the fractional rate of synthesis and the growth rate ($K_b = K_s - K_b$). The values presented are the means ± S.D. derived from six animals. Values in parentheses show the percentage change from the respective control group. *$P<0.05$, **$P<0.01$, ***$P<0.001$, statistically significant from the respective control group.

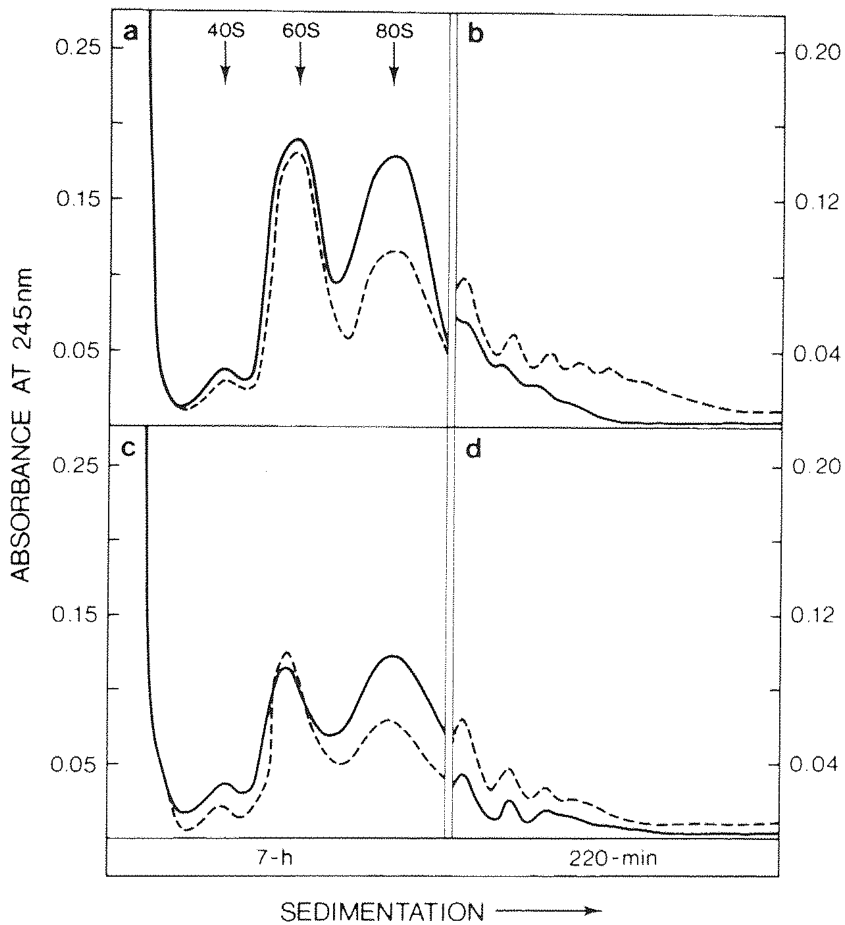
unaltered (Table 17). Upon extending dexamethasone treatment to 5 days, the greater reduction in protein synthetic activity was associated with significant losses in both ribosomal capacity (35%) and efficiency (28%).

6.3.3 The Effects of Dexamethasone on Ribosomal Aggregation in the Lung

The compromised efficiency of the lung to synthesize protein following both the acute and chronic steroid regimes was further investigated, by defining the step in translation which is impaired by dexamethasone. Hormonal control of protein synthesis in skeletal muscle has been demonstrated at peptide chain initiation (Rannels *et al.*, 1978b; Rannels & Jefferson, 1980; Kelly & Jefferson, 1985). To determine if this translational step is specifically blocked in the lungs of steroid-exposed animals, polysomal and ribosomal aggregation in the tissue were analyzed. Figure 18 (A and C) illustrates that while dexamethasone administration did not change the relative proportions of 40 S and 60 S subunits compared to controls, an increased content of 80 S monomers was evident in the lung following both the acute (Fig. 18A) and chronic (Fig. 18C) steroid treatment. These observations were confirmed by the significant (25 to 29%) increase in the RNA content of the peak representing the 80 S monomer following both durations of dexamethasone exposure (Table 18). The 40 S, 60 S and 80 S peaks in the lung are not isolated as discrete peaks (Fig. 18A and 18C), hence the selection of the gradient fractions for RNA analysis is difficult. As a consequence the actual values are likely to be rather arbitrary and may be the subject of interassay variation, The greater RNA content of the 60 S subunits and 80 S monomers in the chronic compared to the acute experiment can only be explained by the fact the ribosomal isolation and analysis were carried out on different days.

In association with the elevated 80 S monomer population, the lungs of the dexamethasone treated rats exhibited a 20 to 36% loss of polysomal material (Fig. 18B and 18D). Changes in the relative proportions of these ribosomal cycle intermediates are clearly indicative that a block at peptide chain initiation is responsible for the steroid-induced inhibition of pulmonary protein synthesis.

Figure 18. Sucrose Density Gradient Analysis of Polysomes and Ribosomal Particles from Lungs of Rats Treated with Dexamethasone



Lungs from rats treated for 24 hours (A,B) or 5 days (C,D) with either saline (------) or dexamethasone (——) were homogenised as described in Chapter 2, section 2.6. The postmitochondrial supernatant (0.75ml for ribosomal particles, 0.9ml for polysomes) was layered onto 0.59 to 1.46M linear sucrose gradients. Ribosomal subunits and monomers (A and C) and polysomes (B and D) were resolved by centrifugation at 300,000 x g in a TST41.14 rotor (Krontron Instruments) for 7h and 220min respectively at 2°C. The gradients were monitored for absorbance at 254nm.

Table 18. The Effect of Dexamethasone on RNA Content and Ribosomal Aggregation in the Lung

Duration of treatment (days)	Treatment	Tissue RNA (mg/g)	RNA content of ribosomal peaks (μg/mg tissue RNA)		
			40 S	60 S	80 S
1	Control	2.67±0.34	27±4	62±13	89± 6
	Dexamethasone	2.17±0.21* (-19%)	28±7	72±14	115±12** (+29%)
5	Control	2.58±0.24	25±6	96±28	164±17
	Dexamethasone	1.68±0.08** (-35%)	28±4	121± 5	204±11* (+25%)

Lung homogenates were prepared as described in Chapter 2, section 2.6 and an aliquot was retained for the measurement of tissue RNA as described by Fleck and Munro (1962). Following centrifugation of the postmitochondrial supernatant (0.75ml) on sucrose density gradients, RNA content of the 40, 60 and 80 S peaks was determined and expressed relative to total RNA content of the homogenate. The values presented are the means ± S.D. derived from 3 determinations. Values in parentheses show the percentage change from the respective control group. *$P<0.05$, **$P<0.01$, statistically significant from the respective control group.

6.4 DISCUSSION

The use of glucocorticoids as a potential treatment for CLD in premature babies is becoming routine practice in neonatal medicine. In the short-term at least, dexamethasone produces a rapid improvement in pulmonary function and as a consequence, accelerates weaning from oxygen supplementation and mechanical ventilation (Mammel *et al*., 1983, 1987; Cummings *et al*., 1989; Harkavy *et al*., 1989). Steroid therapy therefore has the potential to interrupt the vicious cycle produced by oxygen and mechanical ventilation, which although vital for survival, exacerbates the condition of the sick infant by damaging the delicate, immature lung.

Despite the obvious benefits provided by glucocorticoids, questions remain regarding long-term safety. Complications associated with steroid therapy in CLD of preterm infants include infection (Mammel *et al*., 1983,1987), adrenocortical suppression (Arnold *et al*., 1987) and neurological abnormalities (Bourchier, 1988; Noble-Jamieson *et al*., 1989). An added concern, which has been investigated in the present chapter, is the effect of glucocorticoids on pulmonary growth and repair. This is an important issue, since the successful continuum of the latter is essential for the ultimate recovery of the child.

The present study has shown that lung growth in the adolescent rat, was highly sensitive to circulating glucocorticoid concentrations. The rapid and complete cessation of lung growth induced by dexamethasone reflected an effect of the steroid on protein synthesis. The rate of pulmonary protein synthesis decreased markedly (38%) within 24 hours of steroid treatment and had fallen by 53% following dexamethasone administration for 5 days. The rapidity of this response to dexamethasone is comparable to that observed in the thymus of the rapidly growing rat (Kelly & Goldspink, 1984). In addition, the magnitude of protein synthesis inhibition is similar to that reported in the thymus, 39% (Kelly & Goldspink, 1984), tibialis anterior, 37% (Kelly & Goldspink, 1982), extensor digitorum longus, 31% and plantaris, 25% (Kelly *et al*., 1986). In contrast to the lung, all these tissues undergo a marked atrophy in response to dexamethasone. The ability of the lung to resist a steroid-induced catabolism was due to the compensatory fall

in the rate of protein breakdown, which increased in magnitude in association with the decreasing rates of protein synthesis at the two time points studied.

A reduction in the rate of protein synthesis may result from either a decrease in the amount of tissue RNA, hence a compromised capacity to synthesize protein, or by inefficient use of the synthetic capacity available or by a combination of these two mechanisms (Millward *et al.*, 1973). By investigating the effects of acute (24 hours) and chronic (5 days) dexamethasone administration, the primary and secondary actions of steroid treatment on pulmonary protein metabolism were elucidated. The initial fall in the rate of lung protein synthesis induced by dexamethasone, was due solely to a reduction in translational efficiency. The additional fall in protein synthesis occurring over 5 days of steroid treatment was due to combined reductions in ribosomal capacity and efficiency. Sequential changes in ribosomal efficiency followed by capacity have previously been reported in skeletal muscle in response to insulin deficiency (Pain *et al.*, 1983) and in the liver following nutrient deprivation (Millward *et al.*, 1973; Garlick *et al.*, 1975). With respect to glucocorticoid exposure, reductions in both ribosomal capacity and efficiency have been observed in smooth and skeletal muscle following 5 days of glucocorticoid exposure (Rannels *et al.*, 1978b; Rannels & Jefferson, 1980; Kelly & Goldspink, 1982; Kelly *et al.*, 1986).

The compromised efficiency to synthesize protein in the lung was further investigated by defining the step in translation impaired by dexamethasone. Elevated populations of 80 S monomers were found in the lungs of both acute and chronic-steroid treated rats. Since the dissociation of monomeric ribosomes is a prerequisite for initiation of protein synthesis (Sabol & Ochoa, 1971), this indicated that protein synthesis was limited by the reactions of peptide chain initiation. This conclusion was further confirmed by the disaggregation of polysomes which accompanied the increased 80 S monomer content in the lung following glucocorticoid treatment. An impairment at peptide chain initiation following glucocorticoid treatment has previously been reported in skeletal muscle four hours after dexamethasone administration and five days after cortisone treatment (Rannels *et al.*, 1978b; Rannels & Jefferson, 1980).

Peptide chain initiation involves a complex series of events (Chapter 1, section 5.2). Although the exact point of initiation which is regulated by dexamethasone has not been clarified in the present work, it may be considered in the context of what has been observed in other tissues in response to hormonal and nutritional control. For example, a reduced binding of Met-tRNA$_f^{met}$ to the 40 S subunit has been demonstrated in the liver in response to acute amino acid deficiency (Flaim *et al.*, 1982; Everson *et al.*, 1989) and in skeletal muscle in response to insulin deficiency (Kelly & Jefferson, 1985). Thus under these conditions, peptide chain initiation appears to be limited by a decreased rate of 43 S initiation complex formation.

A direct mode of action of glucocorticoid hormones in regulating protein turnover has clearly been shown in the isolated rat diaphragm and soleus (Kostyo & Redmond, 1966; McGrath & Goldspink, 1982). This direct response by the musculature is in keeping with the presence of glucocorticoid receptors in the sarcoplasm which display selective and high affinity binding for these hormones (Mayer *et al.*, 1974; Shoji & Pennington, 1977). The sequence of events and mechanism of action of dexamethasone on the lung has not been investigated. The complete cessation of pulmonary growth could result from a direct interaction of the hormone with the cells of the lung or could be secondary to its actions on other tissues, or possibly both. The lung cytosol of adult rats exhibits a capacity for dexamethasone binding (0.69 pmol/mg protein), which amongst the tissues studied, was only surpassed by the thymus at 0.89 pmol/mg protein (Granberg & Ballard, 1977). In agreement with this data, the greatest levels of glucocorticoid receptor mRNA has been found in the lung, with the relative abundance in other tissues being: spleen, 70%; brain, 55%; liver, 50%; kidney, 40%; heart, 35%; adrenal gland, 13%; and testis, 8% (Kalinyak *et al.*, 1987). This data would suggest that the interaction of dexamethasone with the lung is of a direct type, in which the hormone directly interacts with the tissue, and subsequently triggers the observed biological response.

The lung of the adolescent rat, when compared with the musculature and lymphoid organs, represents a tissue with a high sensitivity to the growth suppressive qualities of glucocorticoids. The dose of dexamethasone employed

(2.5mg/Kg body weight/day) is comparable to that used clinically in severe cases of CLD. It is appreciated however that several differences exist between the subject material used in the present study, namely the adolescent rat, and the premature human infant. The significant delay in weight gain observed in premature infants both during and following dexamethasone treatment (Harkavy *et al.*, 1989; Noble-Jamieson *et al.*, 1989; Yeh *et al.*, 1990) suggests that the effect of steroid administration on the whole body is similar to that seen in the rat. To draw parallels between the response of individual tissues to glucocorticoids presents more of a problem. The lung of an infant with CLD is both immature and damaged. In addition, the excessive deposition of pulmonary fibrin is characteristic of inefficient tissue repair (Taghizadeh & Reynolds, 1976), while lung function tests and morphometric analysis indicate abnormal patterns of pulmonary growth (Bryan *et al.*, 1973; Sobonya *et al.*, 1982; Tepper *et al.*, 1986) during CLD. In view of these additional variables, it is feasible that the sensitivity of pulmonary growth in the human infant may be different (diminished or accentuated) to that observed in the heathy, rapidly growing lung of the rat. Despite the obvious limitations which are always associated with the use of animal models for human research, the present work has clearly shown that glucocorticoid therapy has the potential to suppress growth and repair of the immature lung. Evidence presented in Chapters 3 and 4 indicates that high concentrations of oxygen, frequently an essential component of intensive neonatal care, also compromises lung growth. It may be concluded that to prevent a significant deficit in pulmonary growth and repair, thereby preventing a complicated clinical course, caution should be taken in the application of dexamethasone therapy to treat premature babies who succumb to CLD.

SUMMARY

Premature birth is frequently associated with severe respiratory problems. As a consequence, the preterm infant may succumb to debilitating, secondary complications which may be fatal, or at least, manifest in a slow and perhaps incomplete recovery. The provision of supplementary oxygen and administration of the synthetic glucocorticoid, dexamethasone, are two vital components of intensive neonatal care. This thesis has employed a number of animal models to investigate the possibility that hyperoxic and steroid therapy may negatively influence lung growth and repair of the human infant. An important point that repeatedly appeared throughout my studies was the species difference between the rat and the guinea pig in a number of parameters examined.

Lung development in the guinea pig, unlike other small laboratory animals, can be described as precocious, and as such, is similar to that of the human (Lechner & Banchero, 1982). This animal model is therefore considered suitable in research relating to pulmonary growth and development of the human infant. The newborn guinea pig was used to investigate the potential that supplementary oxygen has in suppressing lung growth. This study arose from earlier work which reported lung atrophy in the adolescent rat following hyperoxia (Kelly, 1988). Despite a reduced rate of protein synthesis in the lung of the neonatal guinea pig following hyperoxia, pulmonary growth was unaffected by the insult as a result of an accompanying fall in the rate of protein breakdown in the lung. A seemingly plausible explanation for the different response between the adolescent rat and neonatal guinea pig was the age of the animal. It is widely recognised that within a number of species, the newborn exhibit a greater survival in hyperoxia and are more resistant to oxygen-induced lung damage (Frank *et al.*, 1978; Yam *et al.*, 1978). Hyperoxia did not however inhibit protein synthesis in the lung of the adolescent guinea pig, clearly demonstrating a marked species difference with respect to oxygen-induced alterations in pulmonary protein metabolism. Clinical evidence suggests that premature infants who succumb to CLD experience suboptimal pulmonary growth and repair (Bryan *et al.*, 1973; Taghizadeh & Reynolds, 1976; Soboyna *et al.*, 1982; Tepper *et al.*, 1986). To speculate as to whether supplementary oxygen contributes to these abnormalities is not easy, and

is complicated further by the presently cited species difference. It is not possible to determine if the response of the human lung to hyperoxia resembles that of the rat or guinea pig. As a consequence, we may only conclude that infants who undergo prolonged periods of hyperoxic therapy require careful monitoring, possibly through routine pulmonary function tests, to evaluate any adverse effects of the treatment on the successful growth of the immature lung.

Investigations into the hypothesis that GSSG mediates the hyperoxic-induced inhibition of pulmonary protein synthesis began in Chapter 3. An examination of the glutathione status of the lung in neonatal and adolescent guinea pigs revealed that the concentration of GSSG did not increase following hyperoxia. Such a response initially suggested an additional species variation, since White *et al.* (1986,1987) reported an elevated GSSG content in the lungs of hyperoxic-exposed adult rats. Further studies into the inhibitory activity of GSSG on pulmonary protein synthesis under conditions of oxidative stress were described in Chapter 4. This work was not related to the clinical problems of prematurity and therefore employed the adolescent rat, since this species appeared to be more sensitive to hyperoxia with respect to both pulmonary protein synthesis and the glutathione redox status. Analogous to my findings in the guinea pig, I was unable to detect an increased concentration of GSSG in the lungs of adolescent rats following hyperoxia and thereby eliminated the original proposal of a second differential response between these two animal models. The earlier observation that GSSG accumulates in the lungs of older rats (White *et al.*, 1986,1987) most likely reflects the decreased capacity with age, to induce the enzymatic systems which efficiently reduce GSSG and subsequently maintain a high GSH/GSSG ratio in the tissue (Yam *et al.*, 1978; Crouch *et al.*, 1988).

Chapters 5 and 6 investigated the influence of pre-and postnatal steroid administration on pulmonary development. The effects of maternal dexamethasone on the fetal lung during the final period of gestation were assessed in the guinea pig. The parameters of pulmonary growth and maturation studied in this species were completely unresponsive to the treatment, a phenomena which probably reflected the absence of specific glucocorticoid receptors in the fetal lung at the time when the steroid regime was conducted (Giannopoulos, 1974). This particular

characteristic of the guinea pig is markedly different from other small laboratory animals, namely the rat and rabbit. Prenatal steroid treatment in these species not only stimulates pulmonary maturation of the fetus, but also interferes with fetal lung growth (Carson *et al.*, 1973; Frank & Roberts, 1979; Rooney *et al.*, 1979; Frank *et al.*, 1985). Such a response corresponds to the presence of specific glucocorticoid receptors in the lung of the fetus during the latter stage of gestation (Giannopoulos, 1974). The guinea pig was not a suitable animal model to investigate the potential risks and benefits of antenatal steroid therapy since cytoplasmic receptors for glucocorticoids have been reported in the lung of the human fetus (Ballard & Ballard, 1974). The efficacy of prenatal dexamethasone treatment in lowering the incidence of RDS following a premature birth (Liggins & Howie, 1972; Block *et al.*, 1977; Collaborative Group, 1981) indicates that analogous to the rat and rabbit, the human responds to glucocorticoid-induced lung maturation. Furthermore, the follow-up studies to date do not suggest that the benefits of antenatal steroid therapy are associated with an adverse effect on pulmonary growth (Wiebicke *et al.*, 1988).

Dexamethasone is administered to infants with CLD, in the hope of improving the respiratory status of the individual (Mammel *et al.*, 1983,1987; Harkavy *et al.*, 1989), and more recently, to babies with severe RDS, in an attempt to decrease the incidence of lung injury (Yeh *et al.*, 1990). An investigation into the influence of postnatal dexamethasone on protein metabolism in the lung is detailed in Chapter 6. The adolescent rat was used in these studies since pulmonary growth in this animal model is relatively rapid. Information could therefore be gained regarding the effects of steroid treatment on a growing lung. At the same time, the study enabled a valid comparison to be made between the influence of dexamethasone on the lung, with previous work conducted on the musculature and lymphoid tissues of the rat (Kelly & Goldspink, 1982,1983,1984; Kelly *et al.*, 1986). Although the study clearly illustrated the profound sensitivity of the rat lung to the growth suppressive qualities of dexamethasone, we may only speculate upon the effects of postnatal glucocorticoid therapy on pulmonary growth and repair in the premature baby. Steroid-treated infants do however experience a delay in somatic growth (Harkavy *et al.*, 1989; Noble-Jamieson *et al.*, 1989; Yeh *et al.*, 1990), indicating that the overall response of the rat and human to dexamethasone is

comparable. The future design of steroid regimes may incorporate higher doses and/or longer treatment periods, in an attempt to overcome the rather transient nature of dexamethasone therapy when employed in severe CLD. The data derived from the present work suggests that such developments require considerable caution, especially since pulmonary growth and repair is an essential requisite for the successful recovery of infants who experience acute and chronic lung disease.

It should be appreciated that the rates of pulmonary protein synthesis reported in this thesis are only the average rates for the whole lung. The lung is a heterogenous tissue, containing at least 5 major cell types, constituting approximately 9% alveolar type I cells, 15% alveolar type II cells, 37% interstitial cells, 33% endothelial cells and 6% macrophages (Barry et al., 1979). There is a high degree of specificity in the type of proteins synthesized by these cells, for example the interstitial cells are responsible for the production of collagen and elastin, while the antioxidant enzymes are synthesized by the alveolar type II cells. Furthermore, limited information suggests that protein turnover varies considerably in the different cell types of the lung. The fractional rate of protein synthesis in isolated pulmonary macrophages has been reported to be 46 to 86% (Hammer & Rannels, 1981; Oliver et al., 1984), appreciably higher than the overall rate determined in the lung in vivo (Tables 2, 14; Garlick et al., 1980; Goldspink, 1987). The experimental procedures employed in the present work, by influencing the numbers of specific cells and/or the rate of protein synthesis in a given cell type, may have exerted a differential effect on the synthesis of individual pulmonary proteins. Although oxygen toxicity induces a number of morphological alterations in the lung, the time course of these events is such, that very few changes take place within an acute exposure to a lethal dose of hyperoxia (Crapo, 1986). My investigations into the effects of hyperoxia on pulmonary protein synthesis employed lethal doses of oxygen (≥95%) for 24 or 72 hour durations. Within 72 hours of hyperoxia the inflammatory phase of oxygen toxicity is elicited, however significant increases in the number of pulmonary macrophages is a later event, and typically occurs during prolonged, sublethal (85% oxygen, 7 days) doses of hyperoxia (Crapo et al., 1978, 1980). In addition, fibroblast and alveolar type II cell proliferation and the deposition of collagen and elastin, constituting morphological changes associated with sublethal pulmonary oxygen toxicity, would not have

contributed to the results of the present study. Finally, with respect to the reduced rate of protein synthesis reported in the whole lung following dexamethasone treatment, we should not disregard a differential response to steroids by the various cells of the lung. Indeed the anti-inflammatory qualities of glucocorticoids suggests that the overall inhibition of pulmonary protein synthesis reflects a profound effect by dexamethasone on protein metabolism in the inflammatory cells of the lung.

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APPENDIX

1. Rates of Pulmonary Protein Synthesis in Adolescent Guinea Pigs Maintained in Room Air or Exposed to Hyperoxia for 24 Hours

	Fractional Rate of Synthesis (%/day)	
	21% oxygen	≥95% oxygen
	29.6	25.3
	27.5	29.2
	26.7	22.8
	26.5	33.4
	27.7	35.0
	22.4	36.9
Mean	26.7	30.4
S.D.	2.7	5.6
s.e.m.	1.2	2.3
n	6	6

Adolescent guinea pigs (approx. 250 g) were maintained in room air or exposed to hyperoxia (≥95% oxygen) for 24 hours. The fractional rate of protein synthesis was determined as described by Garlick *et al.* (1980).

2. Comparison of the Rates of Pulmonary Protein Synthesis Obtained *in vivo* and *in vitro*

Rates of protein synthesis in the lung were determined *in vivo* and *in vitro* utilizing male, Wistar rats (approx. 100 g) as the source of tissue.

Measurement of Protein Synthesis *in vivo*

Protein synthesis was measured *in vivo* after an intravenous, bolus injection of ^{3}H-phenylalanine as described by Garlick *et al.* (1980). Refer to Chapter 2, section 2.4 for full details of the methodology employed.

Ten minutes following the injection of radioisotope, the incorporation of ^{3}H-phenylalanine was typically:

38.1 dpm/mg lung protein

3.81 dpm/mg lung protein/minute

The specific radioactivity of phenylalanine in the lung was:

5.44×10^{11} dpm/mole

The rate of phenylalanine incorporation in the lung *in vivo* was:

7.0×10^{-12} moles phenylalanine/mg lung protein/minute

Measurement of Protein Synthesis *in vitro*

Protein synthesis in the lung cell-free system was monitored by following the incorporation of radiolabelled amino acid into protein. Each experiment was initiated by adding ^{3}H-phenylalanine to the postmitochondrial supernatant (PMS) of the lung and the reaction mix containing all the components required for optimal protein synthesis. Refer to Chapter 2, section 2.8 for full details of the methodology employed. At 10 minutes, the incorporation of ^{3}H-phenylalanine was typically:

7745 dpm/mg PMS protein/minute

The specific radioactivity of phenylalanine in the system was:

2.44×10^{15} dpm/mole

The rate of phenylalanine incorporation in the lung *in vitro* was:

3.2×10^{-13} moles of phenylalanine/mg PMS protein/minute

The lung cell-free system therefore synthesized protein at approximately 5% of the *in vivo* rate.

3. **Body and Lung Weights of 61 Day Gestation Guinea Pigs Following Maternal Saline or Dexamethasone Treatment**

The control and experimental pups were derived from 3 litters. The individual litter sizes were 7,3 and 4 for the control and 2,4 and 4 for the dexamethasone group. The lung weights of the second dexamethasone group were not recorded.

	Body weight (g)		Lung weight (g)	
	Maternal treatment			
	Saline	Dexa	Saline	Dexa
Litter 1	34.7	77.8	1.16	1.90
	47.5	78.3	1.23	1.78
	50.7		1.36	
	44.8		1.37	
	39.5		0.91	
	40.9		1.13	
	45.6		1.40	
Litter 2	52.8	69.1	1.29	
	69.5	66.1	1.34	
	44.5	56.7	1.25	
		64.8		
Litter 3	60.0	61.0	1.10	1.29
	54.0	54.0	1.29	1.03
	60.0	70.0	1.51	1.15
	75.0	55.0	1.18	1.19
Mean	**51.4**	**65.3**	**1.25**	**1.39**
S.D.	**11.5**	**8.8**	**0.15**	**0.36**
s.e.m.	**3.1**	**2.8**	**0.04**	**0.15**
n	**14**	**10**	**14**	**6**

Pregnant guinea pigs were injected subcutaneously with 0.9% NaCl or dexamethasone acetate (2.0mg/Kg body weight) from day 55 to day 60 of gestation, prior to fetal delivery on gestational day 61.