THE EFFECTS OF GLUCOCORTICOIDS AND OTHER AGENTS ON FETAL LUNG MATURATION

by

Mary-Gordon MacKenzie

Department of Physiology and Biophysics

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To my son, Timothy Gordon Rasmusson,

I dedicate this thesis

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ABSTRACT

One of the leading causes of mortality and morbidity in the neonate is respiratory distress syndrome (RDS). RDS occurs due to a deficiency of surfactant in the alveoli of the premature infant at birth. Glucocorticoids have been shown to be effective in decreasing the incidence of RDS when administered prenatally. Their action is to increase the production of surfactant in the immature lung. Unfortunately glucocorticoids have been found to be effective only under ideal circumstances (i.e. dependent on the dose, species, route of administration, and the time and length of exposure to these steriods). Hence investigators have attempted to find other agents which can act alone or synergistically with glucocorticoids to increase fetal lung maturation in the premature fetus.

The specific goal of these studies was to examine the effects of the glucocorticoid cortisol, given in combination with either isoxsuprine or thyrotropin releasing hormone (TRH) on fetal lung maturation. We were also interested in the individual effects of isoxsuprine and TRH on the surfactant pools. Our results indicate that a combination of glucocorticoids and isoxsuprine, administered either simultaneously or sequentially, produced no further increase in the surfactant pools above the effect of the steroid alone. In vivo experiments examining the effects of short term exposure to isoxsuprine alone have shown that this agonist may produce an inhibitory phase in surfactant secretion which is dependent on the gestational age of the fetus or newborn pup. In vitro experiments showed a stimulation of both synthesis and secretion of disaturated phosphatidylcholine (DSPC) from the alveolar type II cell treated with isoxsuprine. All combinations of glucocorticoids and TRH resulted in an increased production of surfactant over either drug given alone and the response was maximal with multiple doses of TRH. Our results indicate that the effectiveness of glucocorticoids, either alone or in combination with other agents such as TRH or isoxsuprine, is dependent on gestational age at the time of treatment and duration of prenatal drug exposure.

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
°C	degrees Celsius
^{14}C	carbon 14
Ca^{+2}	calcium
cAMP	cyclic adenosine 3',5'-monophosphate
CDP	cytidine diphosphate
cm	centimeters
CMP	cytidine monophosphate
CO_2	carbon dioxide
CP-CyT	cholinephosphate cytidylyltransferase
cpm	counts per minute
DG2BII	density gradient 2, band II
DPPC	dipalmitoyl phosphatidylcholine
DSPC	disaturated phosphatidylcholine
FCM	fibroblast conditioned medium
EDTA	ethylenediaminetetraacetic acid
FPF	fibroblast pneumocyte factor
g	gram
GTP	guanosine triphosphate
^{3}H	tritium
HBSS	Hank's Balance Salt Solution
HMD	hyaline membrane disease

IM..... intramuscular

IP..... intraperitoneal

IV..... intravenous

kDa..... kilodalton

KH_2PO_4..... potassium phosphate

kg ..... kilogram

LPC..... lyso-phosphatidylcholine

M ..... molar

µg..... microgram

µl ..... microlitre

ml ..... millilitre

MEM..... minimal essential medium

P..... phosphorus

p ..... page

ΔP..... pressure difference across a sphere (dynes/cm)

PAPase..... phosphatidic acid phosphatase

PC..... phosphatidylcholine

PE..... phosphatidyethanolamine

PG..... phosphatidylglycerol

PI..... phosphatidylinositol

PLEPs..... phospholipid exchange proteins

PS..... phosphatidylserine

r..... radius (cm)

RDS..... respirotory distress syndrome

SD..... standard deviation

SEM..... standard error of the mean

sFBS ..... carbon-stripped fetal bovine serum

SDS-PAGE..... sodium dodecylsulfate polyacrylamide gel electrophoresis

SP-A..... surfactant associated protein-A

SP-B..... surfactant associated protein-B

SP-C..... surfactant associated protein-C

SP-D..... surfactant associated protein-D

SM..... sphingomyelin

T_3..... triiodothyronine

T_4..... thyroxine

TLC..... thin layer chromatography

TPA..... 12-0-tetradecanoyl-phorbol-13-acetate

TRH..... thyrotropin releasing hormone

Tris ..... tris (hydroxymethyl) aminomethane

TSH..... thyroid stimulating hormone

v/v/v..... volume/volume/volume

ß..... Beta

τ..... surface tension

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INTRODUCTION

Respiratory distress syndrome (RDS) is a condition of the newborn characterized by acute difficulty in breathing because of lung immaturity. It is one of the leading causes of mortality and morbidity in neonates today (Crowley, 1989). In the United States alone, during 1984-85, approximately 5,000 infants a year died as a result of RDS while world-wide one in every hundred infants born will develop RDS (Nelson et al., 1985; Wegman, 1984). Thus the need to discover a cure or a means to prevent this disease is of extreme importance.

The incidence of RDS has been shown to decrease as the gestational age of the fetus at the time of birth increases. According to Jobe and Ikegami (1987), infants born at 30-32 weeks gestation have a 20% chance of developing RDS while in infants born around 26-28 weeks gestation (birth weight; 800-1,000 gram range) the incidence of RDS increases to 60-80%. In the premature infant one of the primary causes of RDS is a deficiency of surfactant at the air-liquid interface (Avery and Mead, 1959). Surfactant is a lipoprotein material composed of proteins (7-10%), neutral lipids (5-10%), and phospholipids (85%) (Possmayer, 1982). The major property of lung surfactant is its ability to lower the surface tension at the air-liquid interface thereby facilitating the infant's first and subsequent breaths by preventing atelectasis or alveolar collapse at low lung volumes. Normally the fetal lung progressively accumulates

surfactant as term approaches in preparation for extrauterine life and air breathing.

Surfactant is synthesized in the epithelial type II cells of the pulmonary alveolus and is stored in intracellular storage vesicles called lamellar bodies which are ultimately released into the alveoli. In fetal rabbits cytodifferentiation into type I and type II alveolar epithelial cells commences on the 24th gestational day (term 31 days). Alveolar type II cells are characterized by the presence of lamellar bodies which first appear on the 24th gestational day. Morphologically, lamellar bodies cannot be detected in the alveoli until the 27th gestational day, suggesting that surfactant secretion into the airways begins at this gestational age (Kikkawa et al., 1968; 1971). Thus there is a delay between initiation of synthesis of surfactant and the initiation of secretion of this material into the alveolus. Maturation of the fetal lung is associated with both an increased production of surfactant by the alveolar type II cells and an increased release of this material into the alveoli once this process has been initiated (Oulton et al., 1986).

Today investigators are examining the regulation of surfactant synthesis and secretion in an attempt to decrease the incidence of RDS. Much of this work has focused on pharmacological and hormonal agents which stimulate the type II cells to produce surfactant and increase fetal lung maturation in general. These agents include glucocorticoids, thyroid hormones, beta-adrenergic agents, estrogen, testosterone, insulin, as well as others.

Glucocorticoids are used clinically to reduce the incidence of RDS in the preterm infant but they are not always effective (Crowley, 1989). While there is no question of the importance of glucocorticoid therapy in reducing the incidence of RDS (Collaborative Group on Antenatal Steroid Therapy, 1981), this mode of treatment has not completely eliminated the problem. Hence investigators have attempted to find other agents which can act synergistically or alone to improve the outcome when compared to glucocorticoids.

One group of drugs under investigation for combined treatments with glucocorticoids are the ß-adrenergic agonists. In humans, ß-adrenergic agonists are frequently used in an attempt to interrupt preterm labor. In addition to their effects on the uterus it has been suggested that they may also influence fetal lung maturation (Wright and Dobbs, 1991). These agents appear to play both a stimulatory (Chander and Fisher, 1990; Wright and Dobbs, 1991) and inhibitory role (Gilfillan and Hollingsworth, 1980) in surfactant production. There is a strong correlation between increased endogenous glucocorticoid levels in the fetus and increased ß-adrenergic receptors in the alveolar type II cells (Mulay et al., 1973). In addition, glucocorticoids have been shown to enhance the ß-adrenergic sensitivity of the type II cell receptor by increasing the receptor-agonist interaction necessary to bring about the hormonal response in alveolar type II cells (Roberts et al., 1985). Thus interest has evolved around the possibility of a synergistic effect of the combined administration of glucocorticoids and ß-adrenergic agents

on fetal lung maturation. Cultured A549 human lung cells were observed to have enhanced ß-adrenergic agonist-stimulated secretion of surfactant after prior exposure to glucocorticoids (Smith, 1977). In another study in which a glucocorticoid was administered prior to ß-agonist, the combined treatment was shown to significantly increase the compliance of the fetal lung above that resulting from the administration of glucocorticoids alone (Ekelund and Enhorning, 1985). Although the possibility of synergism has been suggested with this combination, the actual change in surfactant pool size has not been examined.

Thyroid hormones, like glucocorticoids, have also been shown to accelerate fetal lung maturation (Douglas et al., 1970; Redding et al., 1971; 1972; Wu et al., 1973). Current attention has focused on the possibility that a combination of glucocorticoids and thyroid hormone may have a greater therapeutic value than glucocorticoids alone in the prevention of RDS (Ballard, 1984; Ballard et al., 1992; Althabe et al., 1991; Morales et al., 1989). Although several studies in both humans (Ballard et al., 1992; Morales et al., 1989) and experimental animals (Gross et al., 1984; Gonzales et al., 1986) have demonstrated additive, or indeed supraadditive effects from the combined administration of these two hormones, other studies have failed to detect any type of synergism in the action of these two agents on fetal lung maturation (Devaskar et al., 1987a; Ikegami et al., 1987).

In our laboratory, we have developed an in vivo rabbit model for studying the development of the surfactant system in the late-

gestation fetus. We have developed techniques for separately isolating the extracellular (alveolar) and intracellular (tissue-stored) surfactant pools and have established the pattern of accumulation in these two pools during the last five days of gestation. We have also established the qualitative (compositional) changes which occur in the relative proportions of the individual phospholipids during this time period. Using this system, it is possible to treat the mother and/or fetus with physiological and/or pharmacological agents at a particular point in gestation and to directly determine the effects of that treatment on the normal developmental profile of surfactant accumulation. For example, in previous work conducted by Yoon (1985) in this laboratory, it was shown that the response to the treatment with glucocorticoids was confined to a narrow window of time, namely the 24th to 25th gestational day and that glucocorticoids are capable of increasing the intracellular-stored surfactant content of 28 days gestational fetal rabbit lungs to a level equivalent to that of a 29 days gestational fetal rabbits when administered during this period. This model system was used for the in vivo studies undertaken for this thesis.

An advantage of in vitro studies compared with the whole animal model system is that the cellular environment can be regulated and confined to a single organ or cell type. In an attempt to get a better understanding of the effects of the ß-adrenergic agent, isoxsuprine, on the synthesis and secretion of surfactant phospholipids in the developing fetal lung, we also conducted in vitro studies using a pre-

type II cell culture model system.

The specific goal of these studies was to examine the effects of glucocorticoids given in combination with either ß-adrenergic agonists or thyroid releasing hormone (TRH), on the biochemical maturation of the surfactant system in the fetal rabbit lung. We were interested in determining whether either TRH or ß-adrenergic agents were capable of potentiating the efficacy of glucocorticoids on fetal lung maturation. We also were interested in the individual effects of ß-adrenergic agonists and TRH on the intracellular and extracellular surfactant pools in the fetal rabbit lung. Thus our overall goal was to find an agent that would increase the efficacy of glucocorticoids when administered in combination prenatally and would potentially be useful for decreasing the incidence of RDS in the premature newborn.

I. RESPIRATORY DISTRESS SYNDROME

Over the last decade attempts at preventing neonatal RDS have been directed towards two major areas. First, extensive research has been carried out on the effects of various pharmacological agents administered prenatally to the mother and/or fetus on the acceleration of fetal lung maturation. Secondly, after Avery and Mead, (1959) showed that one of the primary causes of neonatal RDS is a deficiency of surfactant in the alveoli, research has been conducted into the effectiveness of exogenous surfactant in the prevention and treatment of this disease.

A. Clinical, pathophysiological and pathological signs of RDS

In most cases of RDS, the infant shows clinical signs of respiratory distress within 1-3 hours of birth although in some cases the infant appears normal during the first few hours after birth but develops RDS several hours later (Farrell and Kotas, 1976). These infants have characteristic clinical signs such as nasal flaring, periods of apnea, an expiratory grunt, dyspnea with intercostal and sternal retraction, and cyanosis (Harrison et al., 1968; Walti et al., 1989). As well, babies with RDS show signs of tachypnea (respiratory rate >60 per minute) with decreased lung volumes and compliance as a result of the increased surface tension in their alveoli (Farrell, 1982b). Avery and Mead (1959) observed an elevated surface tension in the lavage

from the alveoli of premature infants with RDS as compared to the normal full term infant (RDS infants: 30.4 dynes/cm vs. normal infants: 6.7-7.6 dynes/cm).

Infants with RDS have radiographic abnormalities displaying fine reticulogranular opacities ("ground glass" appearance) and signs of pulmonary edema with diffuse microatelectasis (Humphreys et al., 1967). Histochemical examination of these lungs reveals a characteristic hyaline membrane in the alveoli (Duran-Jorda et al. 1956; Walti et al., 1989) and for this reason RDS is also referred to as hyaline membrane disease (HMD).

B. Progress in the prevention and treatment of RDS

The incidence of RDS has greatly decreased in the last two decades. Some of this improvement can be attributed to advanced obstetrical, neonatal and nursing management of the infant. Improvements in the area of neonatal management include methods to control thermoregulation, intravenous administration of fluids and drugs as well as the judicious use of oxygen. The introduction of positive end-expiratory pressure (PEEP) has also improved the treatment of RDS by maintaining lung volume in terminal conducting airways and thus oxygenation in these infants (Chakrabarti, 1989; Walti et al., 1989).

A major advance has been in the ability to accurately identify those fetuses who are at risk of developing RDS. The development of

new techniques to determine fetal maturity has resulted in a decrease in the number of deliveries of infants with immature lungs (Walti et al., 1989). One approach to the obstetrical management of pregnancies in which pre-term delivery is imminent and the fetal lungs are immature has been to accelerate the maturational process by hormones such as glucocorticoids. This idea followed very closely upon the exciting discovery by Liggins and Howie (1972) that glucocorticoids increased surfactant production in fetal sheep (Liggins, 1969) and also in humans (Liggins and Howie, 1972). Glucocorticoids have been found to significantly decrease the incidence of RDS, if administered for at least 48 hours before delivery. While there is no question of the importance of glucocorticoid therapy in reducing the incidence of RDS, this mode of treatment has not completely eliminated the problem (Collaborative Group On Antenatal Steroid Therapy, 1981; Crowley, 1989; Roberts and Morrison, 1991). As will be described in a later section (page 36), this can largely be attributed to the fact that lung development is under multi-hormonal control. Present interest in this area is focused on the concept of improving the efficacy of glucocorticoids by combining this treatment with some other hormone or agent.

With a better understanding of the cause of RDS and more information on the major constituents of surfactant, scientists have started experimenting with exogenous surfactants as treatment for RDS. Several clinical studies have been conducted using exogenous surfactants and have attempted to determine the dosage regime,

administration route, chemical makeup, morphology, turnover time and catabolism of this material (Enhorning et al., 1973 a,b; Fujiwara, 1984; Enhorning, 1985; Kendig and Shapiro, 1988; Robertson, 1989; Morton, 1989). There are three major categories of exogenous surfactant preparations: 1) human 2) bovine/porcine extracts, and 3) synthetic (Kendig and Shapiro, 1988; Robertson, 1989; Morton, 1989). Human as well as the animal exogenous surfactant is obtained from alveolar lavage or amniotic fluid while the synthetic exogenous surfactant may be synthesized in vitro. The synthetic surfactant may be a mixture of dipalmitoylphosphatidylcholine (DPPC) and alcohol or phospholipids, neutral lipids and surfactant-specific proteins (Morton, 1989). Exogenous surfactants appear to be having a positive impact on the occurrence and severity of RDS in the neonate but evidence from several clinical trials (Morton, 1989) using these supplemental surfactants has shown that these agents do not provide a (single) cure and may be more effective if administered in combination with other agents such as glucocorticoids (Liggins, 1989; Farrell and Fost, 1989).

It is clear that RDS is a disease with a complex pathophysiology and is a physiological manifestation of neonatal pulmonary immaturity (Avery and Mead, 1959; Roberts and Morrison, 1991). Thus the need to understand the normal physiological and biochemical maturation of the fetal lung is essential in order to decrease the incidence of this disease in the premature infant.

II. LUNG DEVELOPMENT

A. Morphogenesis

The respiratory system must go through a number of structural and cytomorphologic changes as the fetus prepares for extrauterine life. Lung development has been studied in many species and has been divided into four major stages by the Commission on Embryological Terminology (Avery and Mossman, 1970): (1) the embryonic stage; (2) the glandular or pseudoglandular stage; (3) the canalicular stage; and (4) the terminal sac stage.

The embryonic stage of lung development in the human begins on the 26th day after ovulation and continues to day 52 (term-280 days). During the embryonic period, the bronchial bud, which is derived from the endoderm, develops into two lung buds while a distinct separation of the trachea from the esophagus appears concurrently (Boyden, 1977).

The next stage of lung development, the glandular or pseudoglandular stage, begins around the 52nd day of gestation and terminates near the end of the 16th week in the human fetus (Boyden, 1977). Microscopically the lungs have a glandular appearance while the embryonic tree goes through a series of dichotomous branchings resulting in the formation of the terminal bronchi. In the human fetus all of the bronchial airways are formed by approximately the 16th week of gestation. Within the epithelium, undifferentiated cuboidal epithelial cells line the airways

adluminally to the basement membrane while fibroblasts are arranged peripheral to this membrane during this stage. The epithelial cells contain few cellular organelles but copious deposits of glycogen. Cilia are also visible on some of the epithelial cells lining the upper airways during this stage of development. In the rabbit fetus this stage is observed around the 19-24th gestational day (term - 31 days) (Kikkawa et al., 1968).

The canalicular stage begins in the human fetus during the 17th week and ends around the 26th week of gestation (Boyden, 1977), while in the rabbit fetus this stage occurs between the 24-27th gestational day (Kikkawa et al., 1968). One of the major events which occurs during the end of the canalicular stage is the differentiation of the epithelial cells which will line the alveoli. Type II epithelial cells become distinguishable from type I epithelial cells by the presence of osmiophilic inclusion bodies in their cytoplasm.

The terminal sac period, which is the fourth and final stage of fetal lung development, begins around the 26th week of gestation in the human fetus and continues until term. In the fetal rabbit this stage of lung development occurs between the 27th-31st gestational day (Farrell, 1982a). Alveolar type II cells begin secreting material into the alveoli around the 32nd week of gestation in the human (Oulton et al., 1982) and in the rabbit around the 27th and 28th gestational day. Unassisted respiration can now occur even though true alveoli are absent and only 10% of the adult complement of type II cells is present at birth. Development of the alveoli continues to

occur until adulthood (Thurlbeck, 1975).

B. Cytodifferentiation of the alveolar epithelial cells.

Alveolar epithelial cells are of two types, commonly referred to as type I and II. The type I cells are squamous epithelial cells which occupy a major proportion of the alveolar surface. These cells have few organelles but large nuclei. Type I epithelial cells are flat in shape and resemble a fried egg in appearance. Their surface area is larger than that of type II epithelial cells (4,000 μm^2 vs. 70 μm^2) and thus they are well suited for their function in gas exchange (Weibel and Gil, 1977).

The type II cells are cuboidal in shape and large in cross-section with a diameter between 7 and 14 μm. They are rich in endoplasmic reticulum, polyribosomes, Golgi complex and mitochondria. Type II cells also have large nuclei and multivesicular bodies in their cytoplasm. The alveolar type II cell is the site of surfactant synthesis and can be distinguished from the alveolar type I cells by the presence of lamellar bodies in the cytoplasm of the cell. Singly dispersed type II epithelial cells comprise approximately 10-15 percent of the cells found in the peripheral surface of the alveoli (Hollingsworth and Gilfillian, 1984).

In fetal rabbits cytodifferentiation of the alveolar epithelial cells commences on the 24th gestational day. Prior to 24 days gestation the alveolar epithelial cells are indistinguishable as either type I or

type II. However, with the first appearance of immature lamellar inclusion bodies on the 24th gestational day, the type II cells become distinguishable from type I cells. The appearance of these inclusion bodies within the type II cells suggests that surfactant synthesis is initiated at this time. As cytodifferentiation continues (up to the 27th gestational day) both the number and the size of the lamellar bodies within the type II cells increase. The intracellular stores of glycogen (which act as a substrate for the synthesis of fatty acids which are ultimately incorporated into surfactant phospholipids in the alveolar type II cell) have been shown to peak at 24 days gestation in the fetal rabbit and then gradually decrease as term approaches (Kikkawa et al., 1968; 1971).

At 28 days gestation cytodifferentiation appears to be complete and from 28 days until term the lamellar bodies within the type II cells continue to increase in number and size. Morphologically lamellar bodies cannot be detected in the alveoli until the 27th gestational day suggesting that surfactant secretion into the airways begins on this day (Kikkawa et al., 1968; 1971).

III. LUNG SURFACTANT

A. Properties and function of surfactant

1. Reduction of surface tension at the air-alveolar interface

The major property of lung surfactant is its ability to lower the surface-tension at the air-liquid interface in the alveoli, thus preventing atelectasis at low lung volumes. Surfactant does this by reducing the surface-tension forces at the interface to negligible levels (near 0 dynes/cm). Through the work of Laplace on the mechanics of soap bubble stability and the work of Young on the cohesive forces of liquid molecules, the Young-Laplace law was formulated to measure surface-tension in a bubble such as the alveolus. The Young-Laplace law states that:

$$\Delta P = \frac{2\tau}{r}$$

where ΔP = pressure difference across the sphere (dynes/cm)

τ = surface-tension (dynes/cm)

r = radius (cm)

The pressure difference (ΔP) is directly proportional to the surface-tension (τ); thus the greater the surface-tension of a liquid the larger the pressure required to form a bubble. The pressure required to prevent collapse of the bubble due to the surface-tension

forces is inversely proportional to the radius (r) of the bubble. In the lungs one would expect that smaller alveoli would empty into communicating larger ones resulting in atelectasis and that fluid would transudate into the alveoli due to a large collapsing pressure at low lung volumes (Possmayer, 1982). The presence of surfactant, however, prevents this phenomenon.

Today it is generally accepted that surfactant at the air-liquid interface is capable of reducing the surface-tension to 10 dynes/cm or less at low lung volumes. Furthermore, recent studies on alveolar lavage indicate that surface-tension increases with increasing lung volumes and does not vary among alveoli (Schurch, 1982). Thus the lungs become easier to distend and the work of breathing is reduced as surfactant at the air liquid interface increases the lung compliance.

2. Other functions of lung surfactant

Another suggested role for pulmonary surfactant is the prevention of transepithelial movement of fluid into the alveolar lumen which would result in the development of pulmonary edema (Guyton et al., 1984). Other functions which have been attributed to surfactant involve the pulmonary defense system and the induction of the chemotactic movement of macrophages into the alveoli (Zeligs et al., 1977; Schwartz and Christman, 1979; Juer et al., 1976; Morton, 1989).

The phospholipids phosphatidylcholine (PC-also referred to as

lecithin) and phosphatidylglycerol (PG) of normal lung surfactant have been shown to have immunosuppressive roles (Ansfield and Benson, 1980). Thus, although the primary function of surfactant is the reduction of surface tension at the air-alveolar interface, surfactant may have other important influences on the normal function of the respiratory system.

B. Composition of surfactant

1. Lipids

Surfactant is a lipoprotein material which is synthesized in the type II alveolar cell and stored in intracellular storage vesicles (lamellar bodies) which are ultimately released into the alveoli where they undergo a number of structural changes to form a lipid monolayer at the air-liquid interface. Surfactants has been isolated from both lung compartments (alveolar and tissue-stored) in a variety of species including dog (Frosolono et al., 1970; King and Clements, 1972a,b; Metcalfe et al., 1980), rat (Page-Roberts, 1972; Gil and Reis, 1973; Hallman and Gluck, 1974), rabbit (Hoffman, 1972; Di Augustine, 1974), guinea pig (Valivia, 1973), sheep (Gray et al., 1974; Harwood et al., 1975), and human (Sahu et al, 1976) and the composition has been extensively analyzed. As reviewed by Possmayer (1982) and Harwood et al., (1986) there is a remarkable interspecies consistency in the overall composition. Surfactant is

composed of proteins (5-10%) and lipids (90%). A small percentage of these lipids are neutral lipids such as unesterfied cholesterol. Phospholipids make up the remaining 83% (% by weight).

Our laboratory has examined the phospholipid composition of the surfactant isolated from both lung compartments in the human, rat, rabbit, sheep, mouse, and pig (Table 1) and, in agreement with reports by others (Possmayer, 1984; Sanders, 1982a), found an interspecies consistency in phospholipid composition. The major phospholipid found in lung surfactant is PC which accounts for 70-80% of the total lipid phosphorus in all species examined in this and other laboratories (Chandler et al., 1983; Duck Chong, 1978; Gil and Reiss, 1973; Sanders et al, 1980). The second most abundant phospholipid is PG, an anionic phospholipid whose presence is generally confined to plants and therefore occupies a unique position as a lung surfactant constituent (King and Clements, 1972b). In the adult, PG accounts for approximately 10% of the total phospholipids (Chander et al., 1983; Hallman and Gluck, 1975; Post et al., 1982; Sanders et al., 1980). This phospholipid is generally absent from fetal surfactant until late gestation but is replaced by another anionic phospholipid phosphatidylinositol (PI) (Hallman and Gluck, 1980). The relative importance of these two phospholipids and the role they play in surfactant function, though extensively researched, has never been fully clarified (Sanders, 1982a, b). The remaining phospholipids present in the adult and fetal lung surfactant represent minor constituents and include phosphatidylserine (PS), sphingomyelin

Table 1: Phospholipid compositions of surfactant isolated from various mammalian species.

Phospholipids	Human[a]	Rat[b]	Rabbit[c]	Sheep[c]	Pig[c]
		Relative percent of total lipid phosphorus			
Phosphatidylcholine	82.9	79.0	82.0	78.1	74.8
Lysophosphosphatidylcholine	-	-	0.2	-	-
Sphingomyelin	1.1	1.4	0.3	1.9	2.6
Phosphatidylinositol	2.8	2.4	3.8	1.4	7.4
Phosphatidylglycerol	7.4	6.7	6.8	11.7	7.0
Phosphatidylserine	1.4	2.3	1.1	2.1	1.2
Phosphatidylethanolamine	3.1	5.6	4.3	4.2	7.0
Unidentified phospholipids	1.4	2.5	1.5	0.7	-

a - isolated from amniotic fluid at term.

b - isolated from one-day old newborn lung tissue homogenates.

c - isolated adult lung tissue homogenates.

Surfactant fractions were isolated by a procedure employing differential and density gradient centrifugation as decribed in Materials and Method.

(SM), phosphatidylethanolamine (PE) and lysophosphatidylcholine (LPC). These phospholipids are present as constituents in most normal membranes and appear to play only a minor role in surfactant function.

The majority of the PC (75%) found in lung surfactant contains saturated fatty acids. Furthermore, approximately 50% of the PC is disaturated, i.e. saturated acyl esters occupy both the C-1 and C-2 positions on the glycerol backbone (Gross, 1986). The predominant saturated fatty acid in PC molecules is palmitic acid (16:0) (Gluck et al., 1970; Sanders, 1982b; Fisher and Chandler, 1985). Although aneniоc PC is found in all tissues of the body, lung tissue is unique in that it contains the largest percentage. The major monounsaturated fatty acids found in surfactant are oleic (18:1) and palmitoleic (16:1) acids (Hallman and Gluck, 1977).

PG was first identified as a component of surfactant in 1967 by chromatographic methods (Gray, 1967). In comarison to PC, a smaller percentage of PG exists in the saturated form and approximately 30-60% is accounted for by palmitate (Fisher and Chandler, 1985). PG may also be highly surface active, especially in the saturated form (van Golde, 1976; Rooney et al., 1974; Mendelson and Boggaram, 1991).

2. Proteins

Originally surfactant was thought to contain several different

proteins. Today investigators tend to agree on four major apoproteins which can be separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). These proteins (surfactant-associated proteins-SP) consist of two hydrophilic proteins (SP-A and SP-D) and two hydrophobic proteins (SP-B and SP-C) and have been shown to be synthesized in the alveolar type II cells (Phelps and Floros, 1988; Persson et al., 1990). Clara cells lining the bronchioli may also be responsible for the metabolism of surfactant apoproteins SP-A and SP-B (Wright and Clements, 1987; Persson et al., 1990). These apoproteins are nonserum proteins that have been considered surfactant-specific because they remain associated with highly purified surfactant when isolated and contribute to the biophysical activites of pulmonary surfactant (Gross, 1990; Possmayer, 1990).

It has been suggested that hormones such as glucocorticoids (Schellhase and Shannon, 1990; Boggaram et al., 1991), thyroid hormones (Mendelson and Boggaram, 1991) and other agents may affect the synthesis of these surfactant apoproteins in the developing lung (O'Reilly et al., 1989) and that the genes for the individual proteins are differentially regulated (Shimizuet et al., 1991; Schellhase and Shannon, 1991).

C. Surfactant phospholipids during fetal development.

Surfactant production increases during the latter part of gestation (Possmayer, 1984). In the rabbit fetus the phospholipid content of

both the intracellularly-stored and alveolar surfactant pool has been shown to increase progressively during the latter 5 days of gestation (Figure 1) (Possmayer, 1984; Oulton et al., 1986). The amount of PC and disaturated phosphatidylcholine (DSPC) in the lung lavage (extracellular surfactant) increases 10 fold in the fetal rabbit between the 27th and 31st gestational day. A further increase is also noted after labor and/or birth (Rooney, 1984). The DPPC content has also been shown to increase with gestation in the intracellular surfactant pool (Soodsma et al., 1976; Rooney et al., 1977). Developmental increases in PC and DPPC content have also been noted in other animals such as the rat (Rooney et al., 1974; Tordet et al., 1981) and the sheep (Fujiwara et al., 1968). Incorporation of radioactive choline into PC has been shown to increase significantly in the rabbit fetal lung between 26th-28th gestational days (Rooney et al., 1979a).

The increase in surfactant synthesis near term has also been shown to be accompanied by a significant increase in activity of several important enzymes involved in PC synthesis. Cholinephosphate cytidylyltransferase (CP-CyT), the enzyme involved in the conversion of choline phosphate to cytidine diphosphocholine (CDP-choline) increases either just prior to or immediately after birth as observed in the rabbit (Rooney et al., 1977;1976b), rat (Maniscalo et al., 1978) and mouse (Brehier and Rooney, 1981). The enzyme phosphatidate phosphohydrolase (PAPase), involved in the conversion of phosphatidic acid to

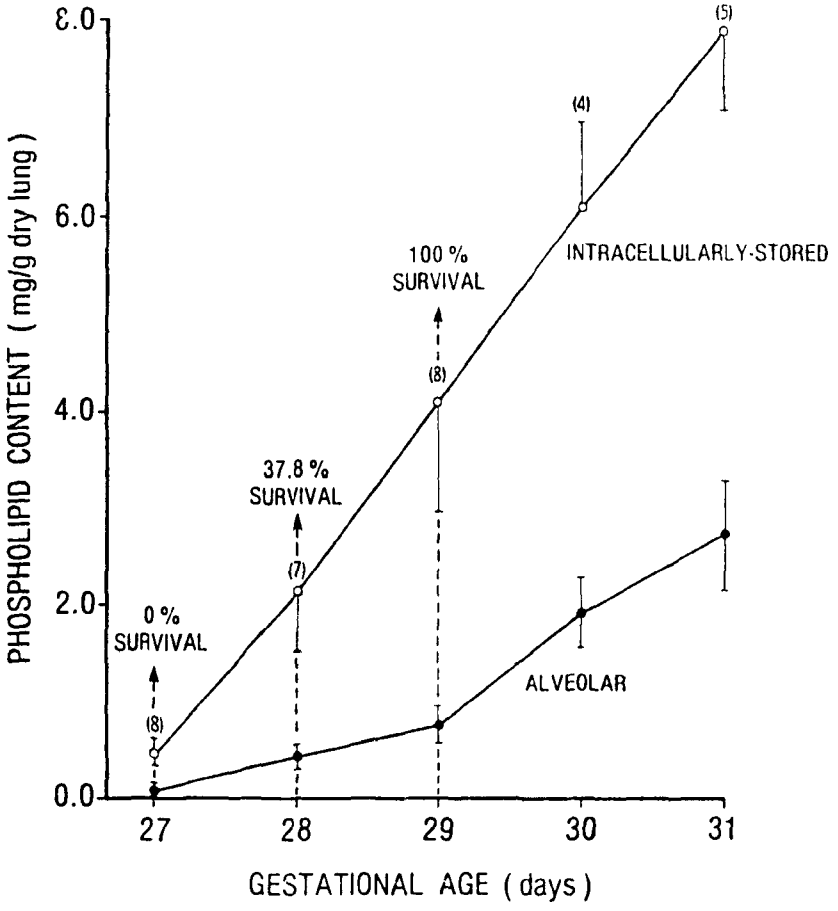

Figure 1: Correlation of survival rates of pre-term delivered newborn rabbits with mean phospholipid content of intracellularly-stored and alveolar surfactants at the gestational ages indicated (Yoon, 1985).

diacylglycerol, also increases before term in the fetal rabbit and mouse and after birth in the rat. The activity levels of the enzymes choline kinase (Rooney et al., 1976b; Maniscalco et al., 1978) and cholinephosphotransferase (Rooney et al., 1976b; Brehier and Rooney, 1981), which are involved in the de novo synthesis of PC, decrease slightly or remain constant with increasing gestation.

Given the role of the surfactant phospholipids in the adaptation of the newborn to regular air breathing, this progressive increase in phospholipid production would appear to be critically important. Indeed, in studies performed in our laboratory in which we examined the survival rate (to four hours) of fetuses delivered into a room air environment, we found that as gestation progressed from 27 days to term and the phospholipid content of both surfactant pools progressively increased, the survival rate progressively improved from 0% for fetuses delivered on the 27th gestational day to 100% for fetuses delivered on or beyond the 29th gestational day (Table 2, Figure 1)(Yoon, 1985). As the lung progressively accumulates surfactant, marked changes in the phospholipid composition have been observed not only of whole lung tissue (Rooney et al., 1979a) but also of the isolated surfactants (Oulton et al., 1986). The phospholipid composition of intracellularly stored surfactant isolated from rabbit lungs of varying gestational ages is shown in Table 3. As indicated, there is an increase in the relative proportion of PC and PG as gestation progresses towards term. There is a progressive decrease in the relative proportion of the other

Table 2: Survival rate of fetal rabbits delivered at various gestations.

Gestational Age (Days)	Total Number Delivered	% Survival to 4 hours	Range per Litter
27	37	0.0	0.0
28	98	37.8	0.0 ± 87.5
29	43	100.0	100.0
31	15	100.0	100.0

Fetuses were delivered at the gestational ages indicated and maintained in a warmed environment (32°C) with room air. Their respiratory status and survival rate was monitored for a period of 4 hours (Yoon, 1985).

Table 3: Phospholipid composition of surfactant isolated from post-lavaged lung tissue during perinatal development.*

Gestational Age	n	PS	PI	SM	PC	PG	PE	Other
days				% of total phosphorus				
27	8	5.7±1.1	9.3±0.3	3.5±1.2	66.7±3.0	n.d.†	14.0±1.5	0.7±0.7
28	7	3.8±0.7	11.2±0.6	1.4±0.7	74.2±2.0	0.3±0.2	9.1±1.0	0.1±0.1
29	8	2.7±0.4	11.8±0.4	0.8±0.2	77.4±0.9	0.3±0.2	6.7±0.5	0.3±0.2
30	4	2.4±0.3	11.7±0.3	0.3±0.4	78.1±0.9	1.2±0.4	6.1±0.3	0.2±0.3
31	5	2.1±0.3	10.9±1.8	0.9±0.5	78.4±2.2	1.5±0.9	5.6±0.7	0.5±0.4
+1	4	1.3±0.2	7.2±0.7	0.5±0.3	82.3±2.0	3.4±1.0	4.9±0.4	0.3±0.5
+2	2	1.5±0.4	6.2±0.6	0.9±0.1	82.5±0.7	3.6±0.1	4.2±0.4	1.2±0.1

* each value represents the mean ± 1 SD for the number of determinations shown.
Abbreviations used are: PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Other, unidentified phospholipid.

† n.d.- Not detectable. (Oulton et al.,1986)

phospholipids with the exception of PI, which increases between the 27th and 28th day gestation and then progressively decreases. These data are in agreement with results reported by others for fetal rabbit (Rooney et al., 1976b) and human (Hallman et al.,1980; Oulton et al., 1980) surfactant.

In fetal and newborn lung surfactant there appears to be a reciprocal relation between the relative amounts of PI and PG as gestation progresses (Hallman and Gluck, 1980; Oulton et al., 1986). Phosphatidylglycerol does not actually appear in surfactant until late gestation and increases just before term in human (Oulton et al., 1982) or shortly after birth in the rabbit (Hallman and Gluck, 1980; Oulton et al., 1986) and in the lamb (Benson et al., 1983). Although the significance of the switchover from PI to PG during the latter stage of fetal development is not known, it has nevertheless been postulated to occur via any of three possible mechanisms: 1) developmental increases in the enzyme glycerophosphate phosphatidyltransferase (Hallman and Gluck, 1980), 2) reduced serum inositol levels as term approaches resulting in a decreased availability of the substrate inositol (Hallman and Epstein, 1980), or 3) elevated levels of cytidine monophosphocholine (CMP) in type II epithelial cells due to increased PC synthesis (Bleasdale et al., 1982a). In addition to the changes observed in the overall phospholipid composition, the fatty acid composition of both PC and PG in the surfactant fraction isolated from lavaged lungs has been shown to change during fetal development (Oulton, 1986).

D. Utilization of surfactant

1. Lamellar body formation.

It is well established that lung surfactant is synthesized in the type II epithelial cells of the lung and is subsequently secreted into the alveoli. A schematic diagram of surfactant synthesis and secretion, as adapted from Hawgood and Clements (1990), is shown in Figure 2. As indicated, surfactant components are synthesized in the rough endoplasmic reticulum (RER) and transported via the Golgi apparatus (G) to lamellar bodies (LB) which are the intracellular storage vesicles for surfactant. The earliest evidence for this pathway came from autoradiographic and electron microscopy studies by Chevalier and Collet (1972) and Dickie and Massaro, (1974) in which they traced the movement of [^{3}H]-choline after its incorporation into the type II cell, from the endoplasmic reticulum through the Golgi complex, and subsequently to the osmiophilic lamellar bodies.

The precise mechanism by which lamellar bodies are elaborated is not known. One mechanism which has been explored by several investigators (Balis and Conen, 1964; Chevalier and Collet, 1972; Stratton, 1984) is growth of these lamellar bodies from multivesicular bodies (MVB). Autoradiographic and electron microscopic studies have revealed MVB detaching from the Golgi complex and migrating to the multilamellar osmiophilic bodies.

Phospholipid exchange proteins (PLEPs) may also be involved in

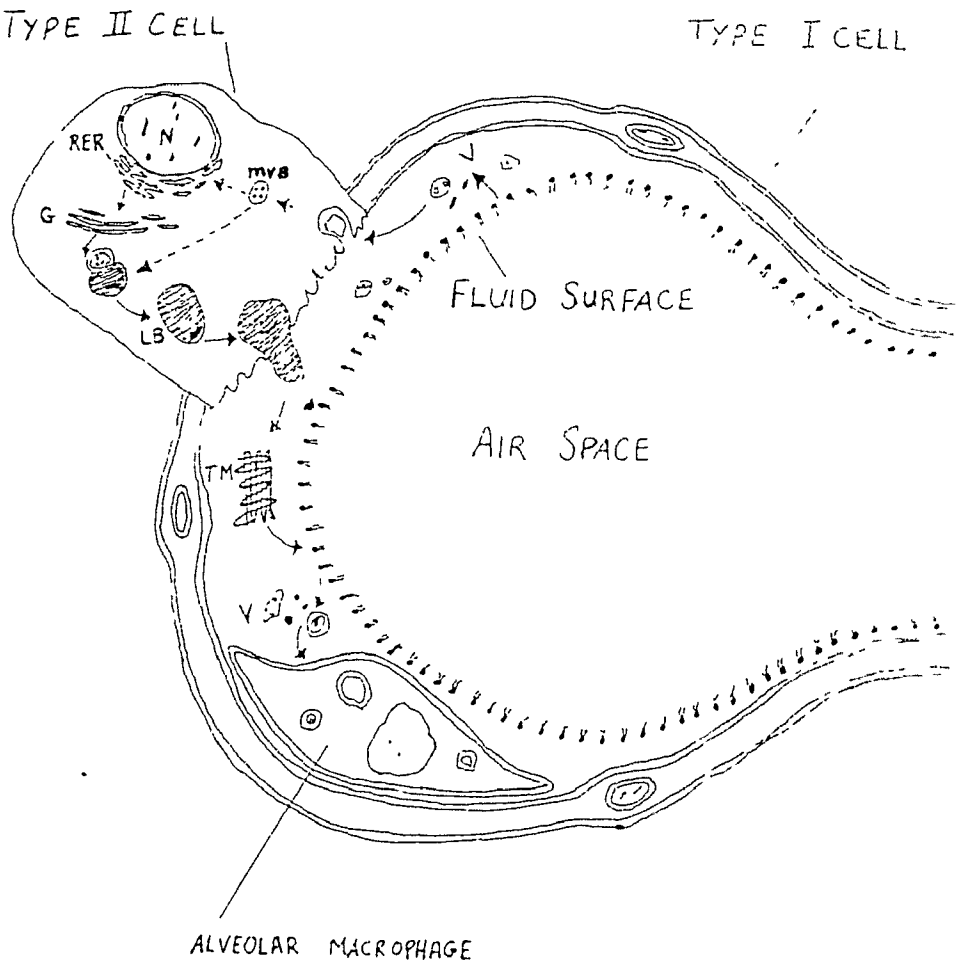

Figure 2: Schematic diagram of the metabolic pathways of surfactant production in the adult animal. A single alveolus is shown with the location and movement of surfactant components depicted. Rough endoplasmic reticulum (RER); Golgi apparatus (G); lamellar bodies (LB); tubular myelin (TM); small vesicles (V), and multivesicular bodies (MVB). The solid arrows indicate pathways that are generally accepted, while the dashed arrows are pathways that are not well substantiated (modified from Hawgood and Clements, 1990).

the exchange of phospholipids between the Golgi complex and the lamellar bodies. The presence of PLEPs in the lung was first observed by Engle et al. (1978) and Robinson et al. (1978) and they have since been detected in the lung tissue of several mammalian species (Engle et al., 1978; Robinson et al., 1978; Spalding and Hook, 1979; Tsao, 1986; Nijssen et al., 1987; Haagsman and van Golde, 1991). PLEPs are also thought to play a role in the reutilization of surfactant (Batenburg, 1984).

2. Secretion

The secretion of surfactant into the alveolar space (Figure 2) involves the exocytotic discharge of the lamellar body contents into the fluid subphase covering the alveolar surface (Wright and Dobbs, 1991). Microscopic (Sorokin, 1967), autoradiographic (Chevalier and Collet, 1972) and time-course radiolabeling (Jobe, 1977) experiments have provided evidence supporting exocytosis of lamellar bodies from type II cells into the alveolar extracellular lining layer (Wright and Dobbs, 1991).

The process of surfactant secretion has been studied in a variety of experimental models including fetal, newborn and adult animals (Wright and Dobbs, 1991) as well as in primary cultures of type II epithelial cells (Brown and Longmore, 1981). The regulation of pulmonary surfactant secretion appears to be modulated by a number of genetic, hormonal, neural, and mechanical factors. Some

suggested modulators of surfactant secretion include ß-adrenergic agonists (Enhorning et al., 1977; Walters, 1985; Warburton et al., 1988), cholinergic agonists (Corbet et al., 1978; Oyarun and Clements, 1977; Massaro et al., 1982), prostaglandins of the E series (Marino and Rooney, 1981, Oyarun and Clements, 1978; Skinner et al., 1989) and mechanical factors such as increased minute ventilation (Faridy and Naimark, 1971; Faridy, 1976; Nijjar, 1984) and labor (Marino and Rooney, 1981). Other mediators such as calcium ionophore A23187 (Dobbs et al., 1986; Sano et al., 1987) microfilaments (Tsilibary and Williams, 1983), forskolin (Rice et al., 1985), cholera toxin (Mescher et al., 1983) and tumor promotor 12-O-tetradecanyl-13-phorbol-acetate (TPA) (Mason et al., 1977a; Dobbs and Mason, 1979) have also been shown to have a stimulatory effect on surfactant secretion. These secretogues presumably act on the alveolar type II cells to stimulate surfactant secretion through second messengers such as 1) adenylate cyclase AMP (Rice et al., 1985; Lee et al., 1989; Walters et al., 1990), 2) intracellular levels of Ca^{+2} (Sano et al., 1985; Pian et al., 1988; Brown and Wood, 1989), or 3) phosphatidylinositol and diacylglcerol (Warburton et al., 1989). Although the actual mechanism involved in surfactant secretion remains unknown, it is suggested that different protein kinases are activated by these second messengers. These protein kinases catalyze the phosphorylation of specific proteins which may be involved in the facilitation of surfactant secretion (Sano et al., 1985; Warburton et al., 1989; Brown and Wood, 1989).

a) Tubular myelin

In the air-breathing animal, lamellar bodies secreted from the type II cells into the fluid subphase covering the alveolar surface have been observed to absorb water, swell and form a liquid crystal tubular lattice-like structure called tubular myelin (Figure 2) (Williams, 1977). The identification of these structures was first discovered in the alveoli by Policard (1957) and Campiche (1960) and they were later named "tubular" as a result of their rectangular tubular appearance (Weibel et al., 1966). In vitro studies have shown that the formation of tubular myelin requires calcium, phospholipids and the surfactant proteins SP-A and SP-B but the events leading to the formation of tubular myelin in vivo remains unknown (Suzuki, et al., 1989). In the fluid-filled fetal lung, tubular myelin structures are not formed but rather the secreted surfactant appears to retain its multilamellated structure (Gil, 1985; Oulton et al., 1986).

b) Monolayer

The surfactant secreted from the alveolar type II cells has been shown by autoradiograph to form a monolayer at the air-liquid interface (Chevalier and Collet, 1972). The tubular myelin present in the subphases is believed to unfold and spread along the alveolar surface (Figure 2).

As mentioned previously, the major phospholipid responsible for

reducing the surface-tension at the air liquid interface is DPPC (Clements, 1957). This phospholipid is capable of decreasing the surface-tension to near 0 dynes/cm but does not provide all of the properties attributed to surfactant (Keough, 1985). The other surfactant phospholipids as well as the apoprotein specific to surfactant are also important for its function.

The monolayer of surfactant at the air-liquid interface is believed to undergo a remodeling process during the respiratory cycle resulting in either a DPPC-rich or DPPC-poor surfactant film. Keough (1985) suggests that this remodeling of the monolayer results from the lung's ability to selectively exclude some of the surfactant components from the monolayer during compression of the alveoli thus forming a monolayer of rigid saturated molecules of DPPC. As the alveoli expand during inspiration, other surfactant phospholipids are added to the surfactant monolayer from a possible reservoir in the hypophase thereby replenishing the monolayer at the air liquid interface. As no tubular myelin is formed in the fluid-filled fetal lung, neither is the monolayer of phospholipids at the alveolar surface formed. However, with the introduction of the newborn into an air environment the surfactant system must rapidly adjust to produce these structural transitions. The actual components of surfactant which are necessary for its dynamic surface active properties remain unknown and is one of the leading questions which must be resolved if an artificial surfactant is to be fully successful in the treatment of RDS in the preterm infant.

E. Clearance and reutilization

Several mechanisms have been proposed for clearance of used or metabolized surfactant from the alveoli. These mechanisms have been recorded by Jobe and Jacob (1984) and are listed in Table 4.

Reabsorption of surfactant by the type II epithelial cells has been suggested as the major route for the clearance and subsequent reutilization of the surfactant phospholipids (Fisher et al., 1987; Rider et al., 1990; Nicholas et al., 1990). The reutilization of surfactant through the process of recycling has been demonstrated in the specific activity time curves of radioactive labeled precursor such as [^{14}C]palmitic acid (Tierney, 1974; Jobe 1979; Hallman et al., 1981). The actual mechanism involved in the reutilization of surfactant in the type II cells is unknown but appear to be a nonspecific reuptake mechanism involving degradation, possibly in the lysosome (Fisher and Chander, 1985). Reutilization appear to be very active in the lungs of neonatal rabbits, approximately 90% of PC being cleared from the alveoli versus 25% in the adult rabbit (Jacobs et al., 1984), and has been suggested as a means of preserving the intracellular and extracellular surfactant pool sizes (Jacobs et al., 1983; Nicholas et al., 1990).

Most of the studies on surfactant clearance and recycling have been performed on adult or newborn in vivo models or using various in vitro techniques. At the present time it is not known if recycling occurs in the fetus. Given that secreted lamellar bodies undergo little

Table 4: Possible mechanisms involved in the catabolism of secreted alveolar surfactant.

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1. Clearance up the airways
2. Phagocytosis by alveolar macrophages with enzymatic degradation
3. Enzymatic degradation within the alveolus
4. Reabsorption across the alveolar epithelium and reabsorption by the lymphatic system or by the pulmonary blood flow
5. Reabsorption by type I and type II epithelial cells resulting in degradation or reutilization of the surfactant components

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From Jobe and Jacobs (1984).

or no structural rearrangement in the fluid-filled fetal lung, it seems unlikely. Nonetheless the possibility cannot be ruled out but would require extensive investigation for clarification.

IV. HORMONES AND PHARMACOLOGICAL REGULATION OF LUNG MATURATION

Fetal lung maturation is a very complex process. A variety of exogenous and endogenous hormones have been shown to influence the developmental process of fetal lung maturation and therefore surfactant production. Many of these agents appear to increase surfactant synthesis or secretion or both. They may act by directly stimulating the alveolar type II cell or by using a second messenger such as fibroblast-pneumocyte factor (FPF) which is released from the fibroblasts and then stimulates the type II cells.

A. Glucocorticoids

Glucocorticoids are a group of hormones which are produced in the adrenal cortex and have been shown to have a maturational effect on various tissues of endodermal origin. The first suggestion that glucocorticoids may have an effect on fetal lung maturation was offered by Buckingham et al. (1968). They proposed that the lungs, being of endodermal origin, may be under similar glucocorticoid influences during development as had previously been observed in

the small intestine (Moog, 1953). Experimental evidence for this theory was later provided by the work of Liggins (1969) while investigating the effects of glucocorticoids on ovine parturition. These experiments consisted of injecting fetal lambs with dexamethasone which resulted in premature parturition. Unexpectedly, the premature fetal lambs that were delivered were observed to have viable neonatal lungs and displayed no signs of RDS. From these observations Liggins postulated that glucocorticoids administered prenatally are capable of accelerating surfactant production by the fetal lung. Since the work by Liggins, evidence exists which suggests that glucocorticoids may affect fetal lung maturation by altering one or more of the following; 1) the synthesis and/or secretion of surfactant (Rooney et al., 1979; Yoon, 1985), 2) the mechanical or structural properties of the fetal lung (Fiascone et al., 1987; Elkady et al., 1988; Rider et al., 1990), and/or 3) the morphological development of alveolar type II cells (Kikkawa et al., 1971; Snyder et al., 1992).

In vitro and in vivo experiments have shown glucocorticoids to increase the production of phosphatidylcholine (PC), the major phospholipid component of the surfactant (Ekelund et al., 1975; Motoyama et al., 1971; Fiascone et al., 1987). In tissue cultures glucocorticoids have also been shown to increase the incorporation of radioactive palmitate (Russell et al., 1974) and choline (Brehier et al., 1977; Rooney et al., 1979; Xu et al., 1990; Xu and Rooney, 1990) into saturated phosphatidylcholine (SPC) by fetal alveolar type II cells. In

vivo experiments conducted in our laboratory by Yoon (1985), have shown significant increases in both the intracellular and extracellular surfactant pools in fetal rabbits delivered on the 28th gestational day after receiving cortisol on the 24th gestational day.

Structural changes such as widening of air spaces, flattening of type I cells and thinning of the interalveolar septa as well as alteration in the protein ratio of collagen/elastin have been observed after glucocorticoids have been administered in the rhesus monkey (Beck et al., 1981), perinatal rat (Schenberg et al., 1987), and rabbit fetal lung (Snyder et al.,1992). Increases in lung compliance and lung volumes have been observed in preterm rabbits treated with glucocorticoids on the 25th or 26th gestational day with delivery on the 27th or 28th gestational day (Fiascone et al., 1987; Hedner et al., 1988; Ikegami et al., 1987; Gladstone et al., 1989). In the preterm monkey, structural changes in the lung, such as increased alveolarization, have been observed following glucocorticoid treatment (Beck et al., 1981: Mitzner et al., 1982; Bunton and Plopper, 1984). Many of these structural changes in fetal lung development appear to be independent of their effect on surfactant pools (Elkady and Jobe, 1987).

Morphologically glucocorticoids have been shown to enhanced differentiation in vitro of the alveolar type II cells (Post et al., 1980; Scott et al., 1983; 1986; Synder et al., 1981) as well as to increase the number of lamellar bodies within these cells compared to control animals when the glucocorticoid was administered directly to the

rabbit fetus (Kotas and Avery, 1971; Kikkawa et al., 1971). In experimental evidence shown by Scott et al. (1983), isolated pre-type II cells were unable to differentiate into type II cells in a hormone-stripped, serum-supplemented medium unless exposed to glucocorticoids. Increased differentiation of the alveolar type II cells by glucocorticoid stimulation is greatest in culture systems in which the mesenchymal-epithelial interactions are maintained (Post et al., 1980; Synder et al., 1981; Scott et al., 1986). Glucocorticoids have also been shown to accelerate morphological development of the fetal rabbit lung by approximately one and a half days (Kikkawa et al., 1971).

Clinical evidence for a role for glucocorticoids in fetal lung development has been provided by studies in which these steroids have been shown to decrease the incidence of RDS. The first clinical studies were conducted by Liggins and Howie in 1972 on 282 mothers in whom premature delivery prior to 37 weeks gestation was imminent. They observed a decrease in the incidence of RDS in the infants in which betamethasone was administered to the mother and delivery was delayed 24-48 hours. Confirmation of the success of antepartum glucocorticoid therapy in accelerating fetal lung maturation has been demonstrated by several investigators in cases in which delivery could be delayed 24-48 hours and the infant was between 28 and 32 weeks gestation (Ballard et al., 1977; Collaborative group on antenatal steroid therapy, 1981; Morton, 1989; Robertson, 1989). Glucocorticoids administered under these

optimal conditions reduce the incidence of RDS by 50-60 %. In addition to these somewhat limited circumstances in which glucocorticoids are effective in decreasing the incidence of RDS, another limitation to the usefulness is that their effectiveness appears to be dependent on the gestational age of the fetus at the time of treatment (Liggins and Howie, 1972; Collaborative group, 1981; Taeusch et al., 1981). This clinical finding is supported by both in vitro (Smith et al., 1974) and in vivo (Yoon, 1985) studies performed on fetal rabbit lung.

Normal developmental increases in the levels of free plasma corticosteroid (either cortisol or corticosterone) have been shown to correlate with the maturation of the surfactant system (Mulay et al., 1973; Kitterman et al., 1981). In the fetal rabbit increases in plasma cortisol and cortisone levels were observed between the 23rd and 25th gestational day just prior to the surge in surfactant PC synthesis (Barr et al., 1980).

The site and mechanism of action for glucocorticoids remain unknown although two major theories have been suggested. The first theory proposes that glucocorticoids act directly on the type II cells to increase the synthesis of surfactant. Glucocorticoid receptors have been identified in the cytosol (Giannopoulos, 1973) and nuclei (Giannopoulos et al., 1973) of type II epithelial cells. Receptor binding has been detected in the human fetal lung as early as 9-12 weeks gestation (Giannopoulos et al., 1975; Ballard and Ballard, 1974; Munck et al., 1990) and in the fetal rabbit as early as 18 days

gestation (Ballard, 1978). The binding capacity of these corticosteroid receptors has been shown to increase with advancing gestation (Ballard et al.,1984a).

The second theory suggests that glucocorticoids, during the latter part of gestation, act indirectly on type II cells to stimulate synthesis of surfactant. This theory suggests the involvement of mesenchymal-epithelial interaction in that the glucocorticoids bind not to the type II cells but rather to the fibroblasts (Smith, 1979; Caminiti and Young, 1991). Glucocorticoid receptors have been identified in the fibroblasts of fetal lungs (Ballard et al., 1978; Gross et al., 1983). Under the influence of the glucocorticoids, the fibroblasts have been shown to produce a polypeptide called fibroblast-pneumocyte factor (FPF) which is released and attaches to the type II cell causing an increase in cAMP levels. Monoclonal antibodies have been cloned for FPF which can inhibit the action of FPF on the type II epithelial cells when glucocorticoids are administered (Post et al., 1984).

The actions of glucocorticoids have not been fully delineated. They may increase the catalytic activity and/or synthesis of specific enzymes involved in the PC metabolic pathway such as cholinephosphate cytidylyltransferase (Rooney, 1985; Mendelson et al., 1986; Gonzales et al., 1989; Rooney et al., 1990) and phosphatidate phosphatase (Brehier et al., 1977). It has also been suggested that they increase the enzymes involved in the remodeling of unsaturated PC, lysophosphatidylcholine acyltransferase and glycerophosphate phosphatidyltransferase, although the results are

controversial (Xu and Rooney, 1989). This controversy may, in part, be due to species differences (Rooney and Brehier, 1982; Rooney, 1985; Mendelson et al., 1986). Another possibility is that these hormones accelerate fetal lung maturation by increasing the availability of substrate precursors for phospholipid synthesis. Glucocorticoids are known to induce glycogenolysis, gluconeogenesis and lipolysis in many organs including the lungs (Ballard, 1979). These hormones are also thought to accelerate fetal lung maturation by acting on other organs to increase substrates needed for phospholipid synthesis. In addition to their probable role in regulating the production of surfactant lipids, glucocorticoids have been shown to exert their influence on other aspects of lung development such as 1) synthesis of surfactant apoproteins such as SP-A (O'Reilly et al., 1989; Snyder et al., 1992), 2) type II cell differentiation (Kikkawa et al., 1971) or 3) the structural alterations within the developing lung (Rider et al., 1990).

There remains a great deal of controversy as to when glucocorticoids are most effective in stimulating fetal lung maturation because their response, whether biochemical, physiological, or morphological, appears to be dependent on the dose, species, route of administration (fetal vs. maternal) and the time and length of exposure to these agents.

B. ß-adrenergic agonists

A ß-adrenergic agonist is an exogenous substance having sympathomimetic action in the body. In humans ß-adrenergic agonists are frequently used in an attempt to interrupt preterm labor. In addition to their effects on the uterus it has been suggested that ß-agonists may influence fetal lung maturation. For instance, a decreased incidence of RDS was observed in premature infants delivered from mothers who had received a ß-agonist to suppress their imminent deliveries (Kero et al., 1973; Boog et al., 1975; Bergman and Hedner, 1978). Studies into the role of ß-agonists in fetal lung maturation began from these initial observations. In vivo studies (Wang et al., 1971; Wyszogroski et al, 1974; Enhorning et al., 1977; Hayden et al., 1977; 1979; Kanjanapone et al., 1980) as well as in vitro studies (Tsilibary and Williams, 1983; Chander, 1989) appeared to show a correlation between ß-agonists and the secretion of lung surfactant in the fetal lung. In tissues other than the lung, ß-adrenergic agonists have been shown to stimulate the secretion of cellular material into the extracellular environment (Rasmussen, 1970; Rawashdeh et al., 1988; Jones et al., 1991).

There are two major types of catecholamine receptors in the body, α-adrenergic and ß-adrenergic (Ahlquist, 1948). ß-adrenergic receptors which have been found in the lung are also further subdivided into $ß_1$ and $ß_2$ (Land et al., 1967) on the basis of their sensitivity to the catecholamine norepinephrine (Minneman et al.,

1981). The predominant ß-receptor subtype in the lung is $ß_2$ (Roberts et al., 1985; Jones et al., 1987). Although the precise mechanism of action is not known, it has been suggested that ß-agonists stimulate secretion in cells by activating the cyclic AMP (cAMP) system in these cells (Maier et al., 1989; Davis et al., 1990; Wright and Dobbs, 1991). Components of this receptor adenylate cyclase complex consist of the ß-adrenergic agonist (H), $ß_2$-adrenergic receptor (R), a coupling stimulatory guanine regulatory protein (G protein-N_S), and the catalytic moiety including the enzyme adenylate cyclase responsible for the conversion of ATP to cAMP (Figure 3). When the ß-adrenergic agonist binds to this specific membrane bound receptor found in type II cells, the receptor is altered thereby facilitating its interaction with the G protein. Guanosine diphosphate (GDP) dissociates from a binding site on the G protein and is replaced by intracellular guanosine triphosphate (GTP) as a result of the binding of the G protein with the receptor. The combining of GTP with the G protein results in an increased activity in the enzyme adenylate cyclase which is responsible for the catalytic conversion of ATP to cAMP (Kent et al.,1980; Maier et al., 1989). Intracellular levels of cAMP are known to be regulated by the degradating enzyme(s) phosphodiesterase(s) (Roberts and Musci, 1986).

Increased levels of cAMP in alveolar type II cells result in the activation of the intracellular enzyme A-Kinase (Dobbs and Mason, 1979; Brown and Longmore, 1981; Rice et al., 1985). This enzyme

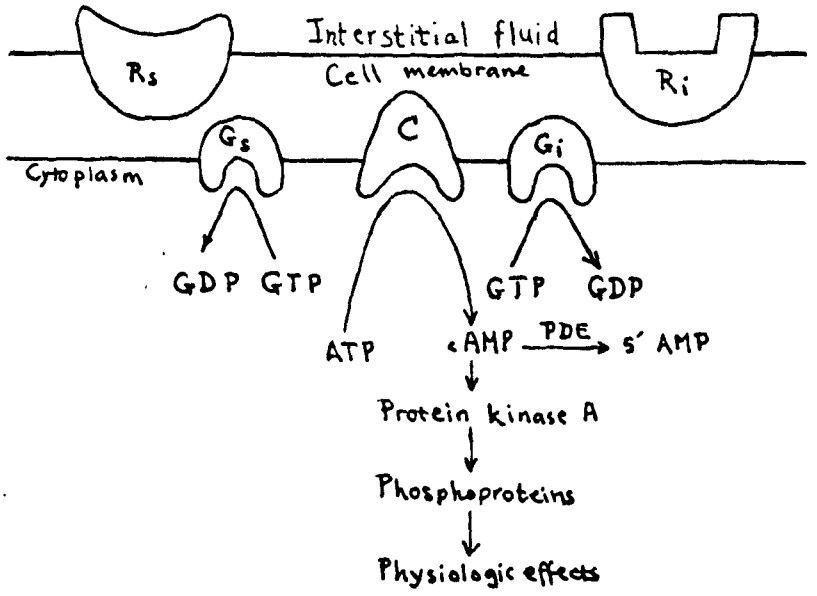

Figure 3: Schematic representation of the cyclic AMP system. Components of this receptor adenylate cyclase system consist of a stimulatory or inhibitory receptor (R_S or R_I), a coupling stimulatory or inhibitory guanine protein (G_S or G_I protein), and the catalytic moiety (C) including the enzyme adenylate cyclase responsible for the conversion of ATP to cAMP. Cyclic AMP activates protein kinase A, which then phosphorylates proteins required for physiologic effects (modified from Guyton, 1991).

appears to be responsible for the phosphorylation of specific proteins such as enzymes which may be involved in the choline-phosphate pathway. The actual role of these A-Kinase activated proteins in surfactant secretion remains unknown but any alteration or developmental delays in any steps of this complex process could result in the stimulation or inhibition of surfactant secretion from the alveolar type II cells.

Specific cell membrane ß-adrenergic receptors have been identified in the alveolar type II cells of several species (Cheng et al., 1980; Giannopoulos, 1980; Whitsett et al., 1981; 1982) and in the human fetus as early as the 16th gestational week (Engle, 1981; Roberts et al., 1985; Davis et al., 1987). In the rabbit, ß-adrenergic receptors were identified as early as the 25th gestational day (Giannopoulos, 1980; Whitsett et al., 1981) and found to double in concentration between the 28th-30th gestational day (Cheng et al., 1980). Thus ß-adrenergic receptors appear to increase exponentially in concentration with increasing gestation (Brown and Longmore, 1983; Davis et al., 1987; Lee et al., 1989).

A ß-adrenergic agonist commonly used to suppress preterm labor in the clinical situation is isoxsuprine. Isoxsuprine is a $ß_2$-adrenergic agonist of the ß-phenylethylamine group of epinephrine-like compounds. This ß-sympathico-stimulant was first synthesized in 1956 by Moed and Van Dijk and was first used clinically in cases of threatened premature labor in 1961 (Bishop and Woutersz, 1961). This ß-adrenergic agonist is also known to increase uterine blood

flow, venous tone and dilatation of the lower respiratory airways (Hayden et al., 1977). Isoxsuprine, like other catecholamines, is capable of crossing the placenta when administered to the mother (Morgan et al., 1972; Sandler et al., 1963) and is known to act by binding to membrane adrenergic receptors.

Endogenous levels of catecholamines have been shown to increase near late gestation and during labor and delivery (Callen et al., 1979). Fetal stress due to surgical manipulation, anesthetic, and/or fetal injection has also been shown to increase fetal endogenous catecholamine levels and possibly affect fetal lung maturation (Russel et al., 1974; Alexander et al., 1968; Oyarzun and Clements, 1978).

Due to their sympathomimetic action, ß-adrenergic agonists may also play a role in the reabsorption of pulmonary fluid, thereby having a dehydration effect on lung tissue (McDonald et al., 1986; Lundell et al., 1988; Barker et al., 1990). It has been suggested that epinephrine induces the "opening" or activation of the sodium channels in the apical membranes of the alveolar epithelial cells, thereby causing a net flux of this ion across the pulmonary epithelium from the alveolar lumen towards the interstitium (Olver et al., 1986; Barker et al., 1990). ß-adrenergic agonists have also been shown to induce surfactant apoproteins SP-A and SP-B in the fetal lung (Liley et al., 1989; Odom et al., 1990) as well as the enzyme fatty acid synthetase (Gonzales et al., 1989).

1. ß-adrenergic agonists and glucocorticoid interaction

There is a strong correlation between increasing endogenous glucocorticoid levels in the fetus and increasing ß-adrenergic receptors in the alveolar type II cells (Mulay et al., 1973). In vivo studies on adult rats have shown an increase in ß-adrenergic receptor concentration with the administration of glucocorticoids (Mano et al., 1979; Chander and Fisher, 1990). Similar results have been demonstrated in vitro employing tissue and organ cultures of human fetal lung exposed to glucocorticoids (Fraser and Venter, 1980; Maniscalo and Shapiro, 1983). ß-adrenergic receptor concentration has been shown to increase as much as 76% in fetal rabbits of 25 days gestation due to the administration of glucocorticoids to the doe (Roberts et al., 1985) and can be inhibited with the administration of cyclohexamide, a protein synthesis inhibitor (Maniscalo et al., 1983). Glucocorticoids are thought to enhance the ß-adrenergic sensitivity by increasing the receptor-agonist interaction necessary to bring about the hormonal response in alveolar type II cells (Roberts et al., 1985; Barker et al., 1989; Wright and Dobbs, 1991). Thus it has been suggested that glucocorticoids may respond synergistically with ß-adrenergic agonists to increase the production of surfactant in the premature lung.

C. Thyroid hormones and thyrotropin releasing hormone

In most tissues thyroid hormones promote growth and development by stimulating the synthesis of mRNA and specific proteins (Martinoli and Pelletier, 1989; Williams et al., 1991). Thyroid hormones, like glucocorticoids, have also been shown to accelerate fetal lung maturation as assessed by biochemical (Smith and Torday, 1974; Rooney et al., 1979), morphological (Wu et al., 1973; Boshier et al., 1989; Smith et al., 1990), and physiological criteria (Redding et al., 1971; 1972; Wu et al., 1973; Devaskar et al., 1987; 1991). The lung tissue from fetuses treated with thyroid hormones has been shown to have accelerated morphological maturation as evidenced by an increase in the number of lamellar bodies and by maturational changes in the alveolar structure (Wu et al., 1973; Boshier et al., 1989; Smith et al., 1990) as well as accelerated biochemical maturation as evidenced by an increase in PC synthesis (Smith and Torday, 1974; Rooney et al., 1979). It has been suggested that thyroid hormones may act directly on alveolar type II cells as observed in both fetal and adult rabbit lung (Lindenberg et al., 1978; Smith and Hitchcock, 1983) or may potentiate the effects of other endogenous hormones such as glucocorticoids (Smith and Sabry, 1983) and catecholamines (Tsai and Chen, 1978; Barker et al., 1990) in stimulating surfactant production. The actual mechanism by which thyroid hormones or their synthetic derivatives act on alveolar type II cells remains

unknown.

The two major hormones, triiodothyronine (T_3) and tetraiodothyronine (thyroxine or T_4), produced by the thyroid gland are known to increase during gestation but remain low compared to the adult (Wu et al., 1978; Devaskar et al., 1986; Costa et al., 1991). In late gestation and during the beginning of neonatal life in the human (Costa et al., 1991) and rat (Hitchcock et al., 1980), lung tissue accumulates T_3 and prior to spontaneous parturition in the sheep, endogenous levels of T_3 and thyroid-stimulating hormone (TSH) are known to increase (Fisher and Odell, 1969).

Thyroid hormone and TSH are generally unable to cross the placenta to any great extent in most species including humans and therefore must be administered to the fetus in order to study their effects on fetal lung maturation. The placentas of humans and other mammalian species have the capability of inactivating T_3 and T_4 as they pass from the maternal to the fetal circulation (Wu et al., 1973). The placentas of humans (Roti et al., 1981) and rats (Roti et al., 1982) have been found to contain active sites for the conversion of T_3 and T_4 to their inactive configurations by inner ring deiodination. However in the rat, T_3 and T_4 have been shown to cross the placenta and significantly increase PC synthesis in the fetuses if the mother is given these hormones (Hemberger and Schanker, 1978; Gross et al., 1984). Placental transfer of high doses of T_3 has also been observed to elevate fetal serum T_3 levels and increase surfactant synthesis in rabbits (Devaskar et al., 1986; Szewczyk et al., 1986; Ikegami et al.,

1987). These high maternal doses of thyroid hormones needed to stimulate the fetal lung as observed in the rat, result in an elevation of fetal plasma T_3 levels by approximately 18-fold (Gross et al., 1984). Toxic side effects such as increased blood pressure, heart rate, and possible growth retardation may occur in these fetuses as a result of these elevated levels of thyroid hormones in the plasma (Sokoloff, 1973; Brasel and Boyd, 1975). Therefore these drugs are usually administered directly to the fetus or into the amniotic fluid from where the hormone diffuses into the fetal circulation (Maschiach et al., 1978; Sack et al., 1975; Korda et al., 1984).

Another method for delivery of thyroid hormone to the fetus is to administer TRH to the mother. TRH is a tripeptide that stimulates the pituitary to release TSH which acts on the thyroid gland to release T_4 and T_3 (Moya et al., 1986). These thyroid hormones then act on the alveolar type II cells to increase fetal lung maturation. TRH has been shown to cross the placenta and to stimulate the fetal thyroid gland (via TSH) to release T_3 and/or T_4 (Tabor et al., 1990). It has yet to be determined whether TRH itself can act directly on the type II cells. TRH has also been shown to stimulate the release of prolactin from the pituitary (Wallace et al., 1979; Horita and Carinokakp, 1986; Liggin et al., 1986) and may act as a neurotransmitter regulating fetal lung maturation (Devaskar et al., 1991).

Though evidence supports the concept of a role for endogenous thyroid hormones in fetal lung maturation, the precise nature of this role has not been well established. Infants suffering from RDS have

been shown to have lower T_3 and T_4 levels than normal infants (Cuestas et al., 1976; Klien et al., 1977; Abassi et al., 1977; Amato et al., 1989). It has been suggested that these low levels of thyroid hormone in the preterm infant are the result of decreased peripheral T_4-T_3 hepatic conversion (Klein et al., 1981). Fetal animals with their thyroid glands surgically removed or animals born with hypothyroidism or no thyroid have been shown to develop RDS at birth possibly as a consequence of delayed maturation of the surfactant system (Redding et al., 1972; Erenberg et al., 1974; Cunningham et al., 1980). The lungs of such animals appeared to be biochemically (Erenberg, 1979; Cunningham et al., 1980) and morphologically immature (Erenberg, 1979).

Thyroid hormones appear to act directly on the alveolar type II cells. High affinity nuclear receptors for T_3 and T_4 have been detected in type II cells of fetal animals such as the rat and rabbit as well as in human fetuses (Lindenberg et al., 1978; Gonzales and Ballard, 1982; Smith and Hitchcock, 1983). The number of pulmonary receptor binding sites for T_3 in the fetal rabbit have been shown to increase approximately 44% between the 21st and 28th gestational day and then decrease near birth. The receptor binding sites have been shown to increase again 2-3 week after birth (Gonzales and Ballard, 1982). The number of receptors occupied by T_3 has also been shown to double between the 21st and 28th gestational day in the fetal rabbit (Gonzales and Ballard, 1982). In the fetal rabbit lung a temporal relationship between increasing thyroid receptor binding

and increasing fatty acid synthesis was reported (Das, 1980). Thyroid hormones appear to bind to high affinity nuclear receptors in the alveolar type II cells inducing the production of mRNA and the subsequent de novo synthesis of protein such as enzyme(s) (Das et al., 1983; Gross et al., 1984; Mukherjee et al., 1990) directly involved in the choline incorporation pathway. Inhibitors of protein and RNA synthesis, actinomycin D and cycloheximide, have been shown to inhibit the effects of T_3 mediated stimulation of mRNA synthesis (Ballard et al., 1984b; Das et al., 1984). Unlike glucocorticoids, thyroid hormones do not appear to enhance the surfactant apoproteins but instead have been suggested to decrease SP-A and SP-B mRNA in the rat (Whitsett et al., 1987; Nichols et al., 1990).

Thyroid hormones also seem to play a role in potentiating the effects of FPF on alveolar type II cells (Viscardi et al., 1989). Glucocorticoids have been shown to stimulate fibroblasts to release FPF which subsequently binds to alveolar type II cells and increases intracellular levels of cAMP. T_3 is thought to act directly on type II cells to enhance the responsiveness of these cells to FPF and thus potentiate the effects of glucocorticoids on type II cells (Smith and Sabry, 1983).

1. Interaction of glucocorticoids and thyroid hormones.

Studies involving the exposure of tissues other than lung to the combined treatment of thyroid hormones and glucocorticoids have

revealed an additive or synergistic effect of these agents on tissue development (Evans et al., 1982; Kriz et al., 1982). Both glucocorticoids and thyroid hormones, when administered alone, have been shown to accelerate fetal lung maturation in animals as well as in humans (refer to page 36). The possibility of additive or supra-additive effects in accelerating fetal lung maturation with the combined treatment of these agents is presently being investigated.

A possible link between cortisol and thyroid hormones in facilitating fetal maturation was first observed by Thomas et al. (1978) who demonstrated an increase in T_3 metabolism as a result of the infusion of cortisol into fetal sheep. Similar results were observed by Liggins et al., (1988). In other organs, the rat pancreas for example, glucocorticoids appear to play a modulating role in T_3 receptor binding capacity (Lee et al., 1991). Several investigators have since shown in both <u>in vivo</u> and <u>in vitro</u> studies that combinations of cortisol and thyroid hormones or TRH may result in an additive or synergistic effect on fetal lung maturation (Gonzales et al., 1986; Warburton et al., 1988; Boshier et al., 1989; Tabor et al., 1990; Ikegami et al., 1991).

<u>In vitro</u> studies involving organ cultures of fetal rabbit lungs exposed to T_3 and dexamethasone demonstrated an increased incorporation of choline as well as glucose, glycerol and acetate precursors into PC (Ballard et al., 1984b). Organ and monolayer cultures of fetal rat (Gross and Wilson, 1982; Smith and Sabry, 1983; Gross et al, 1984), mouse (Smith et al., 1990) and human (Gonzales et

al., 1986) fetal lung tissue exposed to this combined hormonal treatment have also demonstrated an effect on choline incorporation.

In vivo studies on the effects of the combined treatment of glucocorticoids and thyroid hormones on the PC synthesis in fetal lung have also been investigated. Pregnant rats were injected with betamethasone and T_3 24-48 hours prior to delivery. The combined treatment of these two hormones resulted in an additive effect on the incorporation of choline into PC in alveolar type II cells of fetal rats (Gross et al., 1984). Although several studies have demonstrated an additive or supra-additive effect from the combined treatment of glucocorticoids and thyroid hormones or TRH, there is some controversy as to whether there is indeed a synergistic relationship between these two hormones in their effect on fetal lung maturation. In vivo studies by Devaskar et al. (1987) and Ikegami et al. (1987) were unable to demonstrate a synergistic effect between glucocorticoids and thyroid hormones on fetal lung maturation. This conflict suggests that further investigation is required determine if there is indeed an additive effect of these hormones on surfactant production and if so, whether this effect is dependent on gestational age.

V. TECHNIQUES FOR STUDYING LUNG DEVELOPMENT

In addition to the use of whole animals, several in vitro model systems have been developed for studying lung development and surfactant production. It is well-recognized that each model system affords distinct advantages and disadvantages. These will be disc ssed in the following sections.

A. In Vitro techniques

In vitro model systems include the isolated and perfused lung (Bassett and Fisher, 1974; Godinez and Longmore, 1973; O'Neil et al., 1977; Rhoades, 1974), tissue slices (Engelbrecht and Maritz, 1974; Massaro et al., 1971; O'Neil et al., 1977; O'Neil and Tierney, 1974), organotypic cell culture (Douglas and Teel, 1976), and alveolar type II cell culture (Scott et al., 1987). The advantages that the in vitro methods have over whole animal model systems are that the cellular environment can be regulated and confined to a single organ or cell type and thus allows for the study of the direct effect of hormones and other agents in a controlled environment. Some of the disadvanteges of these various in vitro techniques result from the difficulty encountered in trying to maintain differentiated cells in primary culture without degeneration, transformation and overgrowth by mesenchymal elements and other contaminants (Douglas and Smith, 1982).

In weighing the advantages and disadvantages of the various in vitro techniques for studying fetal lung development and surfactant production, it is apparent that the pre-type II cell culture model system would allow for the most direct study of these processes and how they may be regulated or modulated by hormones and other agents. In the present study this model system was used to study the effects of the ß-adrenergic agent, isoxsuprine, on the synthesis and secretion of surfactant phospholipids.

B. In Vivo models

In contrast to using an isolated type II (or pre-type II) cell model system, the use of the whole animal does not allow for the study of direct effects of individual physiological or pharmacological agents on this cell type. It does, however, have the distinct advantage of providing a model in which cell interactions both within the lung and among various organs are preserved. Also, in considering the application of a particular treatment to a clinical situation, it is well-recognized that an in vivo model system provides the closest approximation of all model systems to this situation.

In vivo models have been developed to examine fetal lung maturation in many mammalian species including the rat (Hitchcock, 1979), sheep (Ikegami et al., 1987), and rabbit (Oulton et al., 1986). Various end points have been used to assess surfactant production. These include biochemical assays of surfactant phospholipids or the

enzymes involved in their production as well as surfactant proteins and/or their specific mRNAs, morphological examination of lung tissue, and assessment of the biophysical properties of the surfactants isolated from saline washings of the lung. In our laboratory, we have developed an in vivo rabbit model for studying the development of the surfactant system in the late gestation fetus. We have developed techniques for separately isolating the extracellular (alveolar) and intracellular (tissue-stored) surfactant and have established the pattern of accumulation in these two pools during the last five days of gestation. We have also established the qualitative (surfactant phospholipid composition) changes which occur in the relative proportions of the individual phospholipids during this interval. Using this system, it is possible to treat the mother and/or fetus with physiological and/or pharmacological agents at a particular point in gestation and to determine directly the effects of that treatment on the normal developmental profile of surfactant accumulation. For example, in previous work conducted by Yoon (1985) in this laboratory, it was shown that the response to the treatment of glucocorticoids was confined to a narrow window of time, namely the 24th to 25th gestational day. This model system was used for the in vivo studies undertaken for this thesis.

VI. SUMMARY

The regulation of fetal lung maturation is poorly understood.

Glucocorticoids are frequently administered to pregnant women in an attempt to accelerate fetal lung maturation when a preterm delivery cannot be avoided. Unfortunately glucocorticoids have been found to be effective only under ideal circumstances. Several lines of evidence have shown that agents such as β-adrenergic agonists and thyroid hormones may also influence fetal lung maturation and possibly potentiate the effects of glucocorticoids. Further examination of the effects of β-adrenergic agonists and TRH in combination with glucocorticoids on the individual surfactant pool size is merited and is the subject of this thesis. Such combination therapy may result in increased efficacy of steroids in reducing the incidence of RDS.

OBJECTIVES

1. To determine in vivo the effects of the combined treatment of cortisol and isoxsuprine administered simultaneously (cortisol + isoxsuprine both on the 24th gestational day) or sequentially (cortisol-24th gestational day + isoxsuprine-26th gestational day) on the intracellular and extracellular surfactant pool size. All fetuses were delivered on the 27 gestational day

2. To determine the effects of short term isoxsuprine exposure on intracellular and extracellular surfactant pool size. Also, to determine whether the effects of this β-adrenergic agent, if any, are dependent on the gestational age at the time of treatment or breathing status of the animal, i.e. whether the fetus was maintained in utero or delivered by hysterotomy and allowed to breathe room air. These experiments were performed on either the 28th (fetus) or 30th (neonate) gestational day.

3. To determine in vivo the effects of the combined treatment of cortisol and TRH on the intracellular and extracellular surfactant pool size in 27 gestational day fetal rabbits.

4. To determine in vitro the effects of isoxsuprine on surfactant production in differentiating alveolar type II cells isolated from fetal rabbit lung.

MATERIALS AND METHODS

I. MATERIALS

A. Chemicals, thin layer chromatography plates and cell culture supplies

Thin layer silica gel G Redi-Plates were obtained from Fisher Scientific (Dartmouth, N.S.) while the LK5D silica gel plates used in the in vitro studies were obtained from Terochem Laboratories (Rexdale Ontario). Ammonium molybdate was purchased from BDH (Toronto, Ontario) while 1-amino-2-naphthol-4-sulfonic acid (ANSA) was obtained from Sigma Chemical Co., (St. Louis, Mo. USA). All culture materials and enzyme solutions used for the in vitro studies were purchased from Gibco Laboratories (Burlington, Ontario).

Carbon stripped fetal bovine serum (sFBS) was prepared as described by Yoshizato et al. (1980). Fetal bovine serum was thawed and 6.5 gm of Norit neutral carbon (Fisher Scientific, Toronto, Ontario)/100 ml of serum was added and the mixture stirred constantly at 4°C for 24 hours. The carbon was removed by centrifuging the mixture for 60 minutes at 10,000 x g and the fetal bovine serum was resterilized by membrane filtration (0.22 mm filter). Culture plates were obtained from Falcon Plastics (Oxnard, California) or Gibco Laboratories. Radioactive isotopes were purchased from New England Nuclear (Lachine, Quebec). All

chemicals and reagents for the marker enzyme analyses, with the exception of ^{3}H-AMP (which was purchased from New England Nuclear, Lachine, Quebec) were purc'.ased from either Fisher Scientific (Dartmouth N.S.) or Sigma Chemical Co. (St. Louis, Mo, USA). All other chemicals, including organic solvents and mineral acids, were obtained from Fisher Scientific, Dartmouth, Nova Scotia.

Tris buffered-saline used in these experiments contained 0.145 M NaCl, 0.01 M Tris, 0.001 M disodium ethylenediamine tetraacetate salt (EDTA), (pH 7.4) and will be referred to as Tris buffer. In the in vivo studies the method used for phospholipid visualization after thin layer chromatography consisted of a modified molybdenum blue spray prepared according to Gotell: et al. (1978). A standard phosphorus solution (10 µg P/ml), used as a reference in these experiments (refer to Section II.A.5, p.70), was prepared from a stock standard solution of KH_2PO_4 (100 µg P/ml).

B. Experimental animals.

Pregnant New Zealand white albino rabbits were purchased from Rieman's Fur Ranch, St. Agatha, Ontario. The time of copulation was known to within one hour and was considered the zero point of gestation. The pregnant does arrived in the animal care facilities on the 19th or 20th gestational day and were housed under controlled lighting conditions (lights on 0700-1900 hours) and constant temperature (21°C) until the time of the experiment unless otherwise

indicated. Each doe was housed in a separate cage with food and water available ad libitum. The number of fetuses per litter ranged from one to fifteen therefore the lung tissues and alveolar lavage returns were pooled. Five to seven pups were used for each experimental data point .

C. Drugs

Cortisol (hydrocortisone-21-hemisuccinate) was purchased from Sigma Chemical Co.(St. Louis, Mo.), dissolved in 0.9% NaCl at a concentration of 10 mg/ml and sterilized by filtering through a Millex-GS 0.22 µm filter unit (Millipore Corp. Bedford, Ma.). Isoxsuprine hydrochloride (Vasodilan 5 mg/ml) was purchased from Bristol Myers, Candiac, P.Q.. Thyrotrophin releasing hormone (Relefact TRH); 0.2 mg/ml was obtained from Hoechst Pharmaceuticals (Hoechst Canada Inc., Montreal, P.Q.). The vehicle for both isoxsuprine and TRH is sterile water.

II. METHODS

A. In vivo experiments

1. Drug Administration

The protocols describing the agents administered as well as the route and time of administration for each experiment performed are

presented in the result's section. In experiments involving the administration of cortisol, isoxsuprine and saline on the 24th gestational day, these agents were given intraperitoneally to the fetus. This required an extensive surgical procedure that is described below.

a) Injection to the fetus

On the 24th gestational day the doe was anaesthetized with 3.0-3.5% halothane and anaesthesia was maintained at a concentration of 2.0-2.5% halothane administered by open mask. The doe's abdomen was shaved, scrubbed with Hibitane (Ayerst, Montreal P.Q.), washed with ethanol and then coated with betadine. Under sterile conditions, a midline laparotomy was performed and the uterine horns exposed. Each fetus was injected IP through the intact uterine wall.

The uterus was kept moist and warm during the entire operational procedure by applying warmed saline-soaked surgical sponges. The peritoneum was closed with a simple continuous suture while the ventral abdominal fascia was closed with a simple interrupted suture. The suturing techniques used to close the subcutaneous layer consisted of a simple continuous suture while the skin was closed with a simple interrupted suture (2-0 Novafil). Variton ointment was applied to the incision line to prevent the doe from eating the sutures. The doe was allowed to recover in a warmed

incubator (27°C) for approximately one hour and then returned to her cage for three days.

The use of littermates as untreated controls has been used by several researchers in an attempt to verify their results (Enhorning et al., 1977; Ekelund et al., 1983). The rationale proposed for this is that these fetuses are exposed to similar environmental influences as the treated fetuses, therefore eliminating any external variables from the results. However some studies have shown that these untreated littermates can actually be influenced by the drugs injected into their neighboring fetuses(Wyszogrodski et al., 1974; Kotas and Avery, 1971). In the present study we did a few experiments in which fetuses of one uterine horn were injected with cortisol while the fetuses in the other horn were left untreated. We found that the untreated fetuses had increased intracellular surfactant pools size which were similar to those in the cortisol-treated fetuses (results not shown). We therefore decided to use separate litters for each treatment group as well as for the untreated controls in order to eliminate the possibility of this "neighbor effect".

2. Hysterotomy and Fetal Delivery

In all experiments, on the day on which the fetuses were to be delivered, the does was anaesthetized with sodium pentobarbital (30 mg/kg) delivered intravenously through the lateral ear vein. The doe's abdomen was shaved and a laparotomy was performed, under

clean but not sterile conditions, exposing the uterine horns. If a laporatomy had been performed on the 24th gestational day to administer the drug IP to the fetus, the sutures were removed and the abdominal incision re-opened. After hysterotomy the fetal rabbits were removed with intact fetal membranes to prevent air breathing and euthanized by an IP injection of sodium pentobarbital (13-23 mg/fetus). The amniotic sac was removed and the umbilical cord clamped and cut and the placenta removed.

3. Collection of bronchoalveolar lavage and fetal lung tissue.

Following euthanization with sodium pentobarbital, a midline incision was made on the ventral surface of each fetus' neck to expose the trachea. The trachea was cannulated using a Jelco intracatheter (22 gauge, 25 cm.; Johnson and Johnson, Markham, Ontario) which was secured in place with 3-0 silk suture. The lungs were lavaged with 0.15 M NaCl a total of seven times, each time with a volume of 0.7 or 1.0 ml depending on the individual fetal rabbit weight. Fetuses weighing less than 30 gm were lavaged with 0.7 ml of NaCl while fetuses weighing more than 30 gm. were lavaged with 1.0 ml. Our laboratory has previously shown that at least 90% of the total phospholipid present in 12 consecutive lavages is recovered in the first seven (Oulton et al., 1986). The lavage from five to seven pups was pooled and stored on ice until analyzed. Following the

lavage, the lungs from each fetus were excised, pooled (as for the lavage return) and immersed in ice-cold 0.01M Tris buffer.

4. Isolation of surfactant fractions.

a) Extracellular (alveolar) surfactant

The extracellular surfactant was isolated as follows: After removal of aliquots for total phospholipid analysis, the pooled alveolar lavage returns were centrifuged for 30 minutes at 10,000 xg in an IEC B-20A centrifuge. The isolated surfactant fraction contained in the resultant pellet was suspended in 3.0ml of 0.15M NaCl for lipid extraction and analysis.

b) Intracellular (tissue-stored) surfactant pool

The isolation procedure adopted for routine use for the present studies (Figure 4) was adapted from the procedures of Frosolono et al. (1970) as modified by Oulton et al. (1986). It involves: 1) preparation of a tissue homogenate 2) a series of differential and density gradient centrifugations and 3) washing the final surfactant preparation.

Prior to homogenization, the excised lung tissue was finely minced, patted dry between layers of paper towel and then weighed. A representative portion of the minced tissue (0.2-0.3 gm) was

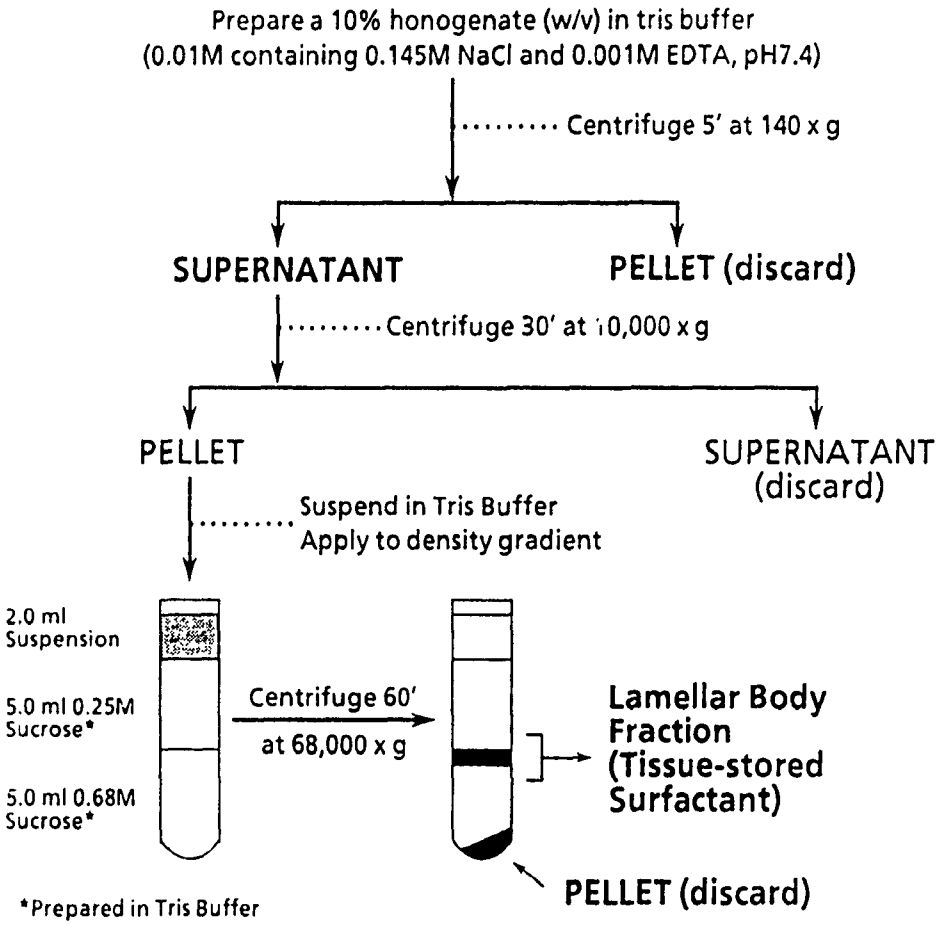

Figure 4: Outline of procedure for isolating the intracellularly stored surfactant from post-lavage lung tissue. Sucrose solutions were prepared in Tris buffer (0.01 M, containing 0.145 M NaCl and 0.001 M EDTA, pH 7.4). In studies performed on fetuses on the 27th gestational day, the lamellar body fraction was further purified by repeating the density gradient procedure.

removed and dried to constant weight at 120°C. A 10% homogenate (w/v) in 0.01 M Tris buffer was prepared from the remainder using a Brinkman polytron homogenizer. A 20 mm generator was used in the polytron and the speed was set at 4.0 throughout these experiments. The tissue was homogenized for 20 seconds in two 10 second bursts. The homogenate was centrifuged at 140 xg (750 rpm) for 5 minutes in an IEC DPR-6000 centrifuge using a swing bucket. The supernatant was collected and centrifuged at 10,000 xg for 30 minutes in an IEC B-20A centrifuge using a fixed angle rotor (IEC-870).

The 10,000 xg pellet was resuspended in Tris buffer and 2.0 ml aliquots were layered over a discontinuous density gradient consisting of 5.0 ml each of 0.25 M and 0.68 M sucrose (prepared in Tris buffer) (Frosolono et al., 1970). The gradient was centrifuged for 60 minutes at 65,000 xg in an IEC B-60 ultracentrifuge using a swinging bucket rotor (Type SB-283). The material banding at the interface between the two sucrose layers was removed and the extraneous sucrose removed by suspending the sample in approximately 15 ml Tris buffer and centrifuging it for 30 minutes at 10,000 xg. The washed surfactant material was recovered as a pellet following this centrifugation step. In experiments performed on the 27th gestational day, the pelleted surfactant material was reconstituted in Tris buffer and subjected to a second density gradient centrifugation. The banded material was washed once with Tris buffer as described above and the final surfactant preparation

was suspended in 3.0ml of 0.15M NaCl for phospholipid extraction and analysis. For studies done on the 28th and 30th gestational day only one density gradient step was employed and the washed surfactant fraction from this gradient was suspended in 0.15M NaCl for phospholipid extraction.

5. Phospholipid analyses

Lipids were extracted from the intracellular and extracellular surfactant fractions using 1 volume of methanol and 2 volumes of chloroform. After vigorously mixing for one minute, the samples were centrifuged for 5 minutes at 1,500 x g (2,000-2,500 rpm) in an IEC DPR-6000 to separate the layers. The upper aqueous layer, containing non-lipid constituents, was discarded and the lower organic layer, containing the phospholipids, was dried under a stream of air at 37°C and reconstituted with chloroform : methanol (2:1) to 1.0 ml.

Phosphorus analysis was performed according to the method of Bartlett (1959). Appropriate aliquots were taken from the 1.0 ml sample and dried under a stream of air while placed in a water bath kept at approximately 37°C. One ml of 70% perchloric acid (Anachemia, Lachine, Quebec) and several glass beads were added to the dried samples which were then heated for 10 minutes at 110°C in a Micro-Kjeldahl digestion rack (Labconco Corp., Kansas, USA). After cooling, the digested sample was diluted with 6.5 ml of distilled

water, 0.4 ml of 5% ammonium molybdate and 0.2 ml ANSA. The mixture was vortexed, covered with tin foil and heated for 15 minutes at 115°C in a block heater (Lab-Line Instruments, Inc. Illinois, USA) to develop the colour. Ammonium molybdate and ANSA form a blue-colored complex with inorganic phosphate (Pi) which can be quantified spectrophotometrically. After cooling, the absorbence of the sample was read at 830 nm on a Pye Unican SPC-500 UV spectrophotometer and compared to a sample blank containing 6.5 ml distilled water, 0.75 ml 72% perchloric acid, 0.4 ml 5% ammonium molybdate and 0.2 ml ANSA. Duplicate reference samples containing 2 μg of phosphorus (stock KH_2PO_4 - 10 μg P/ml) were run with each assay. Periodically, standard curves were prepared using various aliquots of the stock KH_2PO_4 solution to verify linearity and reproducibility of the curve. For routine analysis, the reference samples consisted of 0.2 ml stock KH_2PO_4 solution, 6.3 ml water, 0.75 ml perchloric acid, 0.4 ml 5.0% ammonium molybdate and 0.2 ml ANSA. The blank and reference samples were covered with tin foil, vortexed and heated on the block heaters in the same manner as the samples and then read at 830 nm on the spectrophotometer. The standard readings were averaged and divided by two to give the absorbence per μg phospholipid phosphorus. This value was then used to calculate the phosphorus content of each sample. Assuming that phospholipids contain 4% phosphorus by weight, total phospholipid values were estimated by multiplying the phosphorus content by 25. The total phospholipid

content of the individual surfactant preparations was calculated on the basis of dry lung weight and expressed as milligram phospholipid per gram dry lung.

Individual phospholipids were separated using a two dimensional thin layer chromatography (TLC) system. An aliquot containing approximately 21 μg of lipid phosphorus was dried under a stream of air at 37°C, reconstituted in 40-50 μl of chloroform : methanol (2:1) and applied to thin layer silica gel G Redi-plates which had been previously activated by heating at 120°C for one hour. The first solvent consisted of chloroform : methanol : water (65:25:4, v/v/v) while the second contained butanol : acetic acid : water (90:30:30, v/v/v). The plates were developed in the first solvent for one hour or until the solvent rose to approximately 18 cm from the bottom and then dried for 10 minutes with warm air blowers. The plates were rotated 90° and placed in the second solvent for approximately 90 minutes or until the solvent rose 14-16 cm. The plates were dried for 20 minutes using warm air blowers and the phospholipids were visualized by spraying the plate with the molybdenum blue spray reagent. This reagent was prepared according to the procedure of Gotelli et al. (1978) as follows: to 200 ml of 12.5 M H_2SO_4 were added 8.0 g molybdic anhydride (MoO_3) and the solution was heated with stirring until the solvent dissolved. Powdered molybdenum (250 mg) was then added and the solution slowly heated again until dissolved. After cooling to room temperature, the solution was then added to 400 ml distilled water. Stored in a dark bottle at room

temperature, the reagent is stable for at least two months. Each individual phospholipid, which turned blue on exposure to the spray reagent, was scraped from the plate and the phosphorus analysis was performed as described above. Silica gel blanks were also removed from each plate and subjected to phosphorus analysis. The absorbance reading for the silica gel blank sample was subtracted from that of each phospholipid sample. The phosphorus content of each individual phospholipid was calculated as described above. The result for each phospholipid was expressed as a percentage of the total lipid phosphorus present in each individual sample.

B. In vitro experiments

1. Cell culture

The in vitro studies were done using undifferentiated alveolar type II cells. These cells were isolated from fetal rabbit lungs on the 24th gestational day as described by Scott et al. (1983, 1986) (Figure 5). The pregnant rabbits were euthanized by a lethal dose of sodium pentobarbital (500 mg) given intravenously into the lateral ear vein. The fetuses were removed by hysterotomy, killed by decapitation and immersed in sterile ice-cold Hank's balanced salt solution (HBSS). Under sterile conditions, the fetal lungs were removed from the thorax, rinsed and placed in fresh HBSS. The lungs were chopped, on a Sorval tissue chopper (Sorval Instruments, Newton, Conn.), into

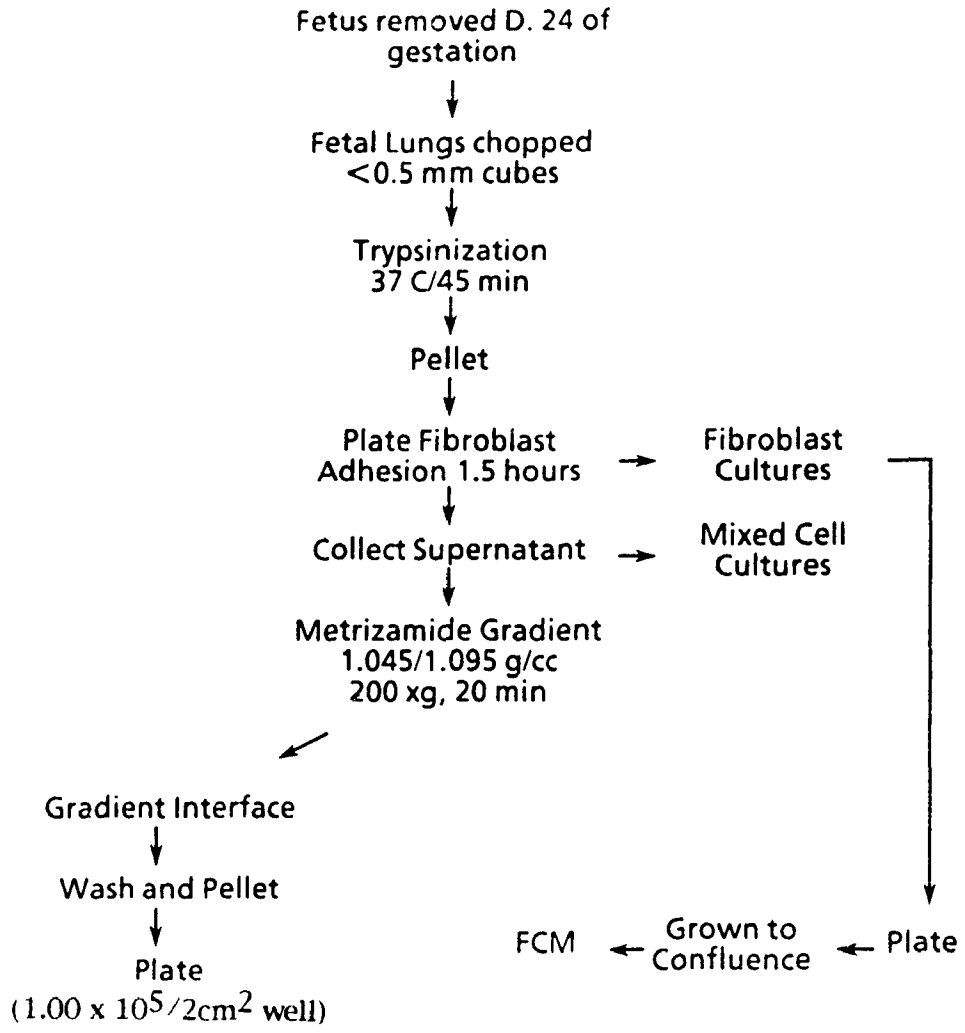

Figure 5: Method of isolation of a homogeneous population of pre-type II alveolar cells from the fetal rabbit lung on the 24th day of gestation.

pieces approximately 0.5 mm in diameter. The tissue pieces were dispersed in 150 ml of a solution of trypsin/EDTA (0.05%/0.02%) with 50 mg of deoxyribonuclease I (Sigma Chemical Co., St. Louis, Mo.) and stirred at 37°C for 45 minutes. The cell suspension was filtered through four layers of sterile gauze to remove tissue fragments. The filtered material was centrifuged for 10 minutes at 400 xg to collect the cells.

After resuspending the cells in serum-free minimum essential medium (MEM) containing 10% carbon-stripped fetal bovine serum (sFBS), they were plated into 80 cm^2 flasks and held in an atmosphere of 5% CO_2 in air for 1 hour to allow the fibroblasts to adhere. The supernatant was collected and centrifuged as described above to collect the cells. The undifferentiated type II cells were separated by a method modified from Mason et al. (1977c). The cells were resuspended in 10 ml of MEM and placed over a discontinuous metrizamide gradient (10 ml of 0.099 M over 10 ml of 0.218 M metrizamide). The gradient was prepared by dissolving metrizamide in HBSS to give a final density of 1.045 g/ml (0.099 M) and 1.095 g/ml (0.218 M). The gradient was centrifuged at 200 x g for 20 minutes. Cells collecting at the interface between the two metrizamide layers were aspirated into a sterile vessel and plated into 24-well plates (2 cm^2/well) at a concentration not less than 1.00×10^5 cells/well. Each well was filled with culture medium consisting of MEM supplemented with 10% sFBS and antibiotics (2,000 units penicillin/ml, 2000 µg streptomycin/ml and 3 µg

amphotericin B/ml). The culture medium was replaced every 48 hours. The sFBS was prepared as described by Tanswell et al. (1983). All cultures were maintained in an atmosphere of 5% CO_2 and 95% room air at 37°C. Under these conditions cells reached confluence in 2-3 days.

2. Preparation of fibroblast conditioned medium (FCM)

FCM has been shown to induce differentiation of fetal lung type II alveolar cells. For use in the present studies, FCM was prepared by incubating the cell suspension as described in Section II.B. (page 75) for one hour to allow fibroblasts to attach (Scott et al., 1983; 1986). These cells were grown to confluence in MEM supplemented with 10% unstripped fetal bovine serum in an atmosphere of 5% CO_2 in room air at 37°C. The fibroblast monolayer was washed with serum-free MEM and incubated in fresh serum-free MEM for 48 hours. The supernatant was collected, sterilized (using a 0.22 μm membrane filter) and stored at -70°C. Throughout the experiment FCM was always used at a concentration of 20% (v/v).

3. Incorporation studies

Incorporation studies were performed with differentiating type II alveolar cells isolated as described in Section II.A. (page 75). Cell cultures of the type II cells were exposed to FCM for 24 hours to

initiate differentiation (Rasmusson et al., 1988) as assessed by the level of DSPC synthesis. At the end of 24 hours the medium was replaced with fresh serum-free medium or serum-free medium containing isoxsuprine (1 μM, 5 μM, or 10 μM) plus 0.5 μCi of [^{3}H]choline which will be incorporated into phosphatidylcholine, the major component of surfactant. Samples of each exposure level were removed at 12, 24, 48 or 96 hours after medium replacement. The medium was collected, the cells were washed with HBSS and released from the attachment substrate with the 0.5% trypsin solution (Freshney, 1983) and collected. Duplicate aliquots from each cell suspension were counted on a Coulter cell counter (Coulter Electronics, Hialeah, Florida).

4. Chemical analyses

Phospholipids present in the alveolar type II cells and media were extracted according to Bligh and Dyer (1959). Aliquots of the organic phase were spotted on LK5D silica gel plates (Terochem Laboratories, Rexdale Ontario) and developed in a solvent system (modified from Skipski and Barclay, 1969) of chloroform : methanol : acetic acid : water (75:25:1:3). Internal standards of phosphatidylcholine (PC) and sphingomyelin (SM) were also run with alternate samples. Phospholipids were visualized by spraying the plate with iodine, the appropriate spots were marked and the iodine was allowed to evaporate. The spots were scraped into scintillation vials and 5 ml of

scintillation cocktail (Beckman Instruments, Palo Alto, Ca.) was added. Radioactivity was determined on a Beckman LS5801 using H# for quench compensation (Beckman Instruments, Palo Alto, Ca.). DPM were converted to picomoles/10^4 cells using the initial ratio of radioactive to nonradioactive choline in the culture medium as the conversion factor.

Incorporation of tritiated choline into disaturated phosphatidylcholine (DSPC) was estimated by the method of Mason et al. (1976). Phospholipid aliquots were reacted with osmium tetroxide (3.28 mg/ml in carbon tetrachloride), dried under nitrogen and the residue redissolved in chloroform : methanol (20:1, v/v). Disposable poly-prep chromatography columns (Bio-Rad, Mississauga, Ontario) were used for chromatography. They were packed with glass wool, 200 mg Celite and 1.0 gm of neutral alumina. The samples were applied to these alumina columns and eluted with 10 ml chloroform : methanol 20:1 (v/v), and 5 ml chloroform : methanol : 7 M ammonium hydroxide (70:30:2). The latter eluant was collected in scintillation vials and allowed to evaporate before scintillation cocktail was added. Radioactivity was measured as described above.

III. STATISTICAL ANALYSIS

All data are presented as mean ± standard deviation (S.D.) unless otherwise indicated. Differences between means were evaluated statistically using Student's t-test (Mendenhall,1975) or Duncan's

New Multiple Range Test (Duncan, 1955; Ott, 1977) as indicated in the tables. Measurements were considered to be significantly different at 95% confidence interval ($p<0.05$) .

RESULTS

I. PRELIMINARY EXPERIMENTS TO ESTABLISH A ROUTINE PROCEDURE FOR ISOLATION OF TISSUE-STORED SURFACTANT

Preliminary studies were conducted to establish optimum procedural conditions for isolating the intracellular surfactant pool from the lungs of fetal rabbits in the earlier gestational age groups (i.e. 27 and 28 days). As mentioned previously, the initial isolation procedure used in this study was developed by Frosolono et al. (1970) and modified by Oulton et al. (1986). While this procedure had been explored in some detail in previous studies (Oulton et al., 1986), it had largely been used for fetal rabbits at 29-31 days gestation. It was therefore desirable to determine the conditions under which the procedure could be optimally adapted for the 27 and 28 day gestation fetus.

Experiments were performed to assess the effect of modifications to the original procedure on the yield and/or purity of the final preparation. The various conditions examined were: A) extent of homogenization of the lung tissue B) number of gradient purifications required and C) extent of washing of the final preparation. Purity of the preparation was assessed by phospholipid analysis (composition), electron microscopy and marker enzyme analysis. Each of the preliminary studies is described below.

A. Extent of homogenization

Tissue homogenization is a procedure used to rupture the cell membrane for subsequent isolation of organelles. Ideally the cell organelles, including the lamellar bodies, should remain intact. In previous studies (Oulton et al., 1986), we used two or three 10 second bursts using a Brinkman polytron homogenizer (Brinkman Instruments, Rexdale, Ontario) with a speed setting at 4.0. In these studies we found that greater than 85% of a ^{3}H-labelled lamellar body preparation, added to a tissue sample and subjected to the isolation procedure, was recovered in the final preparation.

The following study was undertaken to determine if variations in the homogenization time would have any effect on the recovery of phospholipid in this fraction. The range of homogenization time studied was from 16-80 seconds. Three experiments were performed on lung tissue obtained on the 28th gestational day (Table 5). In each, the entire tissue was homogenized for the shorter time period shown, then one half of the sample was removed and homogenized for the required time to bring the total homogenization time up to the longer time period shown. The number of density gradient centrifugations (two) and washes of the final surfactant preparation (one) as used in the original procedure, were employed for these experiments. The phospholipid content of each the final surfactant preparations was examined as described in Methods p.70.

As shown in Table 5, the overall yield of lamellar body

Table 5: Effect of varying the homogenization time on the phospholipid content of intracellularly stored surfactant. For each experiment, lung tissue obtained from 10-12 fetuses on the 28th gestational day was pooled, divided and homogenized for the time indicated. The surfactant fraction was collected after consecutive purification on two sucrose gradients. The sample was washed once with Tris buffer. Results are expressed as mg of phospholipid/gm dry lung.

Experiments number	Homogenization time (sec.)	Phospholipid content of intracellularly stored surfactant (mg/gm dry lung)
1 A	20	2.77
B	40	3.17
2 A	40	2.54
B	80	3.38
3 A	16	1.01
B	20	1.25

phospholipid appeared to be reasonably independent of homogenization time between 16 and 80 seconds. A 20 second exposure consisting of two 10 second bursts was selected for routine use. On visual examination of the preparation obtained under these conditions, the tissue appeared to be adequately homogenized after this time.

B. Number of density gradient purifications.

Lamellar bodies can be isolated from fetal lung tissue by density gradient centrifugation. Due to their density, lamellar bodies have been shown to accumulate at the interface between sucrose layers with similar isopynic densities (0.25M and 0.68M sucrose) when centrifuged at 65,000 xg (Frosolono et al., 1970). Previous studies in this laboratory have shown that a lamellar body fraction obtained in this manner from fetal rabbits of 29 days gestation following one gradient step was approximately 15% contaminated by phospholipids from non-surfactant sources, i.e. mitochondria, but this contamination could be removed by subjecting the sample to a centrifugation on a second density gradient. Because we planned to use younger pups in the present study, preliminary experiments were performed to assess the yield and purity of the fractions obtained from these younger pups following centrifugation of the sample on one as compared to two density gradients. Lung tissue from fetal rabbits of 27 or 28 days gestation as well as tissue from

cortisol-treated fetuses (treated on the 24th gestational day and delivered on the 27th gestational day) were used in this study.

After lavage the lung tissue was finely minced with scissors, a 10% (w/v) homogenate was prepared as described above. Two 10 second bursts were used, as in the original procedure as described in Methods p.67. The washed surfactant band, recovered as a pellet following the first 68,000 xg centrifugation step was reconstituted in an appropriate volume of Tris buffer and one half was centrifuged on a second density gradient, as described on p.67, while the other half was extracted for phospholipid analysis. In these experiments the surfactant material obtained from the second density gradient was washed five times with Tris buffer, while that recovered from the first gradient was subjected to only one wash with Tris buffer.

The phospholipid content and composition of the surfactant fraction collected after both the first and second centrifugation of the sucrose gradients was determined. Phospholipid analysis was also performed on the supernatant (refers to fractions obtained from above and below the surfactant fraction) and pellet fractions resulting from centrifugation of the sample on the second density gradient.

The results are shown in Tables 6 and 7. The material obtained from the 27th gestational day untreated fetuses after the first sucrose gradient contained 1.06±0.48 mg phospholipid/g dry lung (Table 6). Of this amount approximately 25% (0.23±0.04 mg phospholipid/g dry lung) was recovered at the interface (washed

Table 6: Phospholipid content of fractions produced by centrifugation of fetal rabbit lung homogenate on sucrose density gradients. Lung tissue was obtained from fetuses of either 27 (both with and without prior cortisol treatment) or 28 days gestation. In each experiment an aliquot of the surfactant fraction collected from the first gradient was centrifuged on a second gradient and each of the resultant fractions from the second gradient was collected for phospholipid analysis. Phospholipid content is expressed in mg. phospholipid/g. dry lung and all results are expressed as the mean ± 1 S.D. for the number of determinations shown in the parenthesis (n=2, mean plus range given).

Fraction	**Gestational Age: Treatment:**	27 days: none (n=5)	27 days: cortisol (n=2)	28 days: none (n=2)
		mg phospholipid/g. dry lung		
First Gradient Surfactant		1.06 ± 0.48	2.96 ± 0.70	2.68 ± 0.59
Second Gradient Surfactant(washed x5)*		0.23 ± 0.04	1.43 ± 0.08	1.48 ± 0.97
Combined Supernatants		0.21 ± 0.06	0.59 ± 0.17	0.33 ± 0.29
Pellet		0.45 ± 0.06	0.43 ± 7.07	0.39 ± 0.08

* the phospholipid content of the combined wash supernatants are shown in Table 8.

Table 7: Phospholipid composition of fractions obtained by centrifugation of homogenized fetal rabbit lungs on sucrose gradients. Fractions DG1 SURF. (surfactant band from first density gradient, washed x1); DG2 SURF. (surfactant band from second density gradient, washed x5); DG2 PELL. (pellet from second density gradient) and DG2 SUPT. (combined supernatants from second density gradient) were prepared from the lungs of untreated as well as cortisol-treated fetal rabbits of 27 gestational days and untreated rabbits of 28 days gestation as indicated. The result for each phospholipid is expressed as the percent of the total lipid phosphorus and represent the mean ± 1 S.D. for the number of determinations shown (n .

Fraction	n	PC	PG	PI	PS	PE	SM	Other
DG1Surf								
27-Untreated	5	53.35 ± 5.29	n.d.	6.59 ± 1.14	6.18 ± 2.11	22.15 ± 2.59	9.38 ± 2.38	1.78 ± 3.00
27-Cortisol	2	67.60 ± 2.27	0.28 ± 0.35	8.72 ± 0.37	5.11 ± 0.54	13.63 ± 0.51	4.01 ± 0.26	1.24 ± 0.66
28-Untreated	2	67.32 ± 0.03	0.33 ± 0.46	8.96 ± 0.13	4.96 ± 0.68	13.81 ± 1.03	4.15 ± 0.16	0.56 ± 0.42
DG2 Surf								
27-Untreated	5	64.10 ± 6.11	0.22 ± 0.49	8.68 ± 1.11	5.32 ± 1.31	15.11 ± 2.31	6.22 ± 5.04	0.72 ± 1.11
27-Cortisol	2	74.68 ± 2.17	0.28 ± 0.35	10.02 ± 0.31	2.78 ± 0.52	10.27 ± 0.21	2.19 ± 1.24	0.30 ± 0.52
28-Untreated	2	67.32 ± 0.03	0.33 ± 0.46	9.18 ± 0.83	4.11 ± 0.21	13.81 ± 1.02	4.15 ± 0.16	0.56 ± 0.42
DG2 PELL.								
27-Untreated	3	51.19 ± 2.59	0.25 ± 0.38	5.99 ± 0.64	6.87 ± 0.88	25.20 ± 1.11	10.35 ± 0.73	0.21 ± 0.24
27-Cortisol	2	56.19 ± 1.70	0.23 ± 0.09	5.45 ± 1.68	7.24 ± 0.06	24.28 ± 3.01	6.62 ± 2.74	n.d.
28-Untreated	1	50.13	n.d.	6.91	8.35	23.30	6.97	n.d.
DG2 SUPT.								
27-Untreated	4	50.85 ± 1.57	0.50 ± 0.49	6.75 ± 0.52	9.56 ± 1.21	22.64 ± 1.65	9.17 ± 0.82	0.66 ± 0.78
27-Cortisol	2	65.61 ± 6.52	0.21 ± 0.35	8.91 ± 0.85	7.39 ± 0.97	13.86 ± 3.57	4.04 ± 3.11	n.d.
28-Untreated	1	69.89	n.d.	11.04	3.15	10.01	5.93	n.d.

Abbreviations used for phospholipids are: PC. phosphatidylcholine: PG. phosphatidylglycerol: PI. phosphatidylinositol: PS. phosphatidylserine: PE. phosphatidylethanolamine: SM. sphingomyelin: Other. unidentified phospholipids. n.d. - not detectable.

five times) from the second sucrose gradient while another 20% was recovered in the combined supernatants and approximately 50% was recovered in the pellet after the second gradient. In the cortisol-treated groups the material from the interface of the first gradient contained 2.96±0.70 mg phospholipid/g dry lung and, of this, nearly 50% was recovered at the interface of the second gradient. About 35% was recovered in the other fractions from the gradient. The pattern observed for the 28th gestational day fetuses was similar to that for the cortisol-treated group (27th gestational day). In all three groups approximately the same amount of phospholipid was recovered in the pellet obtained from the second sucrose gradient centrifugation. Also shown in these preliminary experiments, is an increase in intracellular surfactant total phospholipid in the cortisol-treated pups.

The results of the analyses of phospholipid composition of these fractions are shown in Table 7. In comparing the interface fraction obtained from the first sucrose gradient (DG1 Surf), to that obtained from the second sucrose gradient (DG2 Surf), in all three groups the second sucrose gradient fraction contained relatively more phosphatidylcholine and phosphatidylinositol with concomitantly less phosphatidylethanolamine and sphingomyelin. This suggests an enrichment of lamellar bodies in the interface fraction following the second sucrose gradient. Also, the pellet fraction obtained from each group after the second sucrose gradient revealed a composition that was somewhat different from the gradient interface material and in

fact is almost identical to that previously reported by this laboratory for fetal rabbit lung mitochondria (Oulton and Dolphin, 1988). With regards to the supernatant resulting from the second gradient, the composition of that obtained from the lungs of untreated fetuses of 27th gestational days was similar to that of the pellet fraction suggesting removal of non-surfactant material. On the other hand, the phospholipid composition of this fraction from the cortisol-treated fetuses (27th gestational day) and the 28th gestational day untreated fetuses more closely resembled that of the material obtained from the sucrose gradient interface suggesting possible (but small) losses of surfactant material on the second gradient in these particular groups.

In conclusion, we found that the interface material obtained following centrifugation of lung tissue from 27th gestational day fetuses (untreated) on the first density gradient was 75% contaminated with phospholipids from a non-surfactant origin and a second gradient was necessary to remove this contamination. With lung tissue from the 28th day gestational fetuses, the material banding at the interface was only 15% contaminated with phospholipids of non-surfactant origin, which could also be removed by a second density gradient. In this group, however, the second gradient resulted in losses of material of surfactant origin. It was therefore decided to use two density gradient centrifugations for experiments performed on the 27th gestational day but only one for experiments done on the 28th (and later) gestational day. Although

this preliminary study shows that the pattern obtained for the 27th gestational day (cortisol-treated) group more closely resembles that of the 28th gestational day untreated than the 27th gestational day untreated group, to maintain consistency, in all studies done on the 27th gestational day two density gradient steps were employed.

C. Number of washes of the final surfactant preparation.

A surfactant fraction was "washed" by resuspending it in Tris buffer and recollecting it by centrifuging at 10,000 x g for 30 minutes. Under these conditions the surfactant fraction was recovered as a pellet while any residual sucrose and non-surfactant phospholipids or proteins remaining from the density gradient centrifugation were removed in the supernatant. We have previously shown that up to five washes may be required to remove extraneous protein (Oulton et al., 1980). For the present study, several experiments were performed to determine the minimum number of washes required to remove extraneous phospholipid without compromising the yield. In all these experiments homogenization was for 20 seconds and the homogenates were centrifuged on two consecutive sucrose gradients, as indicated in the original procedure. The surfactant material collected from the second sucrose gradient was washed five times in Tris buffer. The phospholipid content of the final surfactant pellet and each resultant wash supernatant was determined as described on page 70. The phospholipid composition

was also determined for each of the final washed surfactant pellets. Not enough material was present in each of the wash supernatants for analysis of phospholipid composition. Therefore, after removal of aliquots for determination of the total phospholipid content, the remainder of each of the wash supernatants from each preparation was pooled and the phospholipid composition determined on the pooled sample. Results are shown in Tables 8 and 9.

For simplicity, the phospholipid content (Table 8) of the first wash supernatant obtained from each preparation is shown separately while that for each of the remaining four wash supernatants were combined for presentation. As indicated, for each experimental group similar amounts of phospholipid (0.16 - 0.18 mg/g dry lung) were removed by the first wash and the total amount of phospholipid removed by the next four washes was not much greater than that removed by the first wash alone. The results of the compositional analysis (Table 9) show that the washed surfactant fraction obtained from 27th gestational day animals is enriched in PC and PI and has less SM in comparison to the supernatant fractions from the same animals. These results suggest that the washing procedure does indeed remove extraneous (i.e. non-surfactant) phospholipid but since the bulk of phospholipid was removed by the first wash there appeared to be little value in further washing. In the few experiments that were done on tissue obtained from the 28th gestational day fetuses, there was little difference in the phospholipid composition of the washed surfactant and the combined

Table 8: Phospholipid content of washed surfactant fractions and resultant wash supernatants. The surfactant fraction was collected after the second sucrose gradient and washed five times with Tris buffer. For convience, the phospholipid content of the first wash supernatant (Wash Supt.1) is recorded separately while that of the remaining four washes (Wash Supt. 2-5) were combined and their sum recorded. Results are expressed as the mean ± S.D. for the number of determinations shown.

Treatment Group	n	Washed Surfactant	Wash Supt. 1	Wash Supt. 2,3,4,5
27-Untreated	5	0.23 ± 0.04	0.18 ± 0.14	0.25 ± 0.03
27-Cortisol	2	1.43 ± 0.08	0.16 ± 0.06	0.36 ± 0.03
28-Untreated	2	1.49 ± 0.97	0.18 ± 0.14	0.21 ± 0.04

Table 9: Phospholipid composition of the washed surfactant fraction and corresponding wash supernatants. For each group the surfactant fraction was collected after the second sucrose gradient and washed five times with Tris buffer. In each experiment all five wash supernatants were combined for determination of the phospholipid composition. Results are expressed as a % of total lipid phosphorus and represent the mean ± 1 S.D. for the number of determinations shown (range given for n = less than 3).

Treatment	n	PC	PG	PI	PS	PE	SM	Other
Fractions:								
Washed Surfactant								
27-Untreated	5	64.10 ± 6.11	0.22 ± 0.49	8.68 ± 1.11	5.32 ± 1.31	15.11 ± 2.31	6.22 ± 5 04	0.72 ± 1 11
27-Cortisol	2	74.68 ± 2.17	0.28 ± 0.35	10.02 ± 0.31	2.78 ± 0.52	10.27 ± 0.21	2.19 ± 1.24	0.30 ± 0.52
28-Untreated	2	67.32 ± 0.03	0.33 ± 0.46	9.18 ± 0.83	4.11 ± 0.21	13.81 ± 1.02	4.15 ± 0.16	0.56 ± 0 42
Wash Supt. 1-5								
27-Untreated	2	53.18 ± 9.80	n.d.	4.25 ± 0.33	6.58 ± 1.02	15.63 ± 2.91	20.38 ±11.38	n.d
27-Cortisol	2	67.56 ± 1.85	0.48 ± 0.68	9.65 ± 0.49	4.23 ± 0.95	12.16 ± 1 78	5.09 ± 0 13	0.95 ± 1 34
28-Untreated	1	67.95	0.80	8.77	3.99	14.04	4.47	n.d

Abbreviations used are. PC, phosphatidylcholine. PG, phosphatidylglycerol. PI, phosphatidylinositol, PS, phosphatidylserine. PE, phosphatidylethanolamine. SM, sphingomyelin. Other, unidentified phospholipids n d - not detectable.

wash supernatant fraction. Thus, extensive washing of these preparations also seemed to be of little value. It was therefore decided to incorporate a single washing step into the routine procedure for the isolation of the intracellularly stored surfactant.

D. Further assessment of purity of intracellular surfactant.

In addition to phospholipid compositional analyses, the purity of the intracellular surfactant fraction was assessed by marker enzyme analyses and electron microscopy. Both of these procedures were performed on samples prepared from the lungs of pups of 27 days gestation. In all experiments, homogenization was for 20 seconds, two consecutive density gradients were employed and the final surfactant preparation was washed once with Tris buffer.

1. Electron Microscopy

Intracellular surfactant fractions obtained from the lungs of 27th gestational day fetal rabbits, were prepared for electron microscopy (EM) by collecting the material from the sucrose gradient interface, washing once with Tris buffer, and fixing the pellet for 2 hours at 4° C in 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.3). The pellets were then postfixed for 2 hours at 4° C in 1% osmium tetroxide in the same buffer. The specimens were stained overnight in 0.25-0.5%

aqueous uranyl acetate. The remaining steps were performed by Dr. G. Faulkner in the Department of Microbiology. These steps consisted of dehydration with acetone, embedding in Taab resin, sectioning and double staining with 2% aqueous filtered uranyl acetate and lead citrate. Observations were made using a Phillips E.M. 200 (Oulton et al.,1980).

A low power electron microscopy photomicrograph of the final surfactant fraction is shown in Figure 6. Lamellar body structure appears to be well preserved. The sample consisted mainly of these well-preserved structures with only small amounts of contaminating membranous constituents present.

2. Marker enzyme analyses

Aliquots of intracellular surfactant preparations isolated from the lungs of 27th gestational day fetuses as described on page 67.II.A.4.b. (page 67) were removed and analyzed for succinate dehydrogenase (EC 1.3.99.1) a mitochondrial marker enzyme as described by Pennington (1961), NADPH-cytochrome c reductase (EC 1.6.2.4.), a microsomal marker enzyme by the method of Omura and Takesue (1970), and 5'-nucleotidase, a plasma membrane marker enzyme, according to Avruch and Wallach (1971). For comparative purposes, enzyme analyses were also performed on aliquots of mitochondrial and microsomal-enriched (though not purified) fractions, prepared as described by Oulton and Dolphin (1988). These

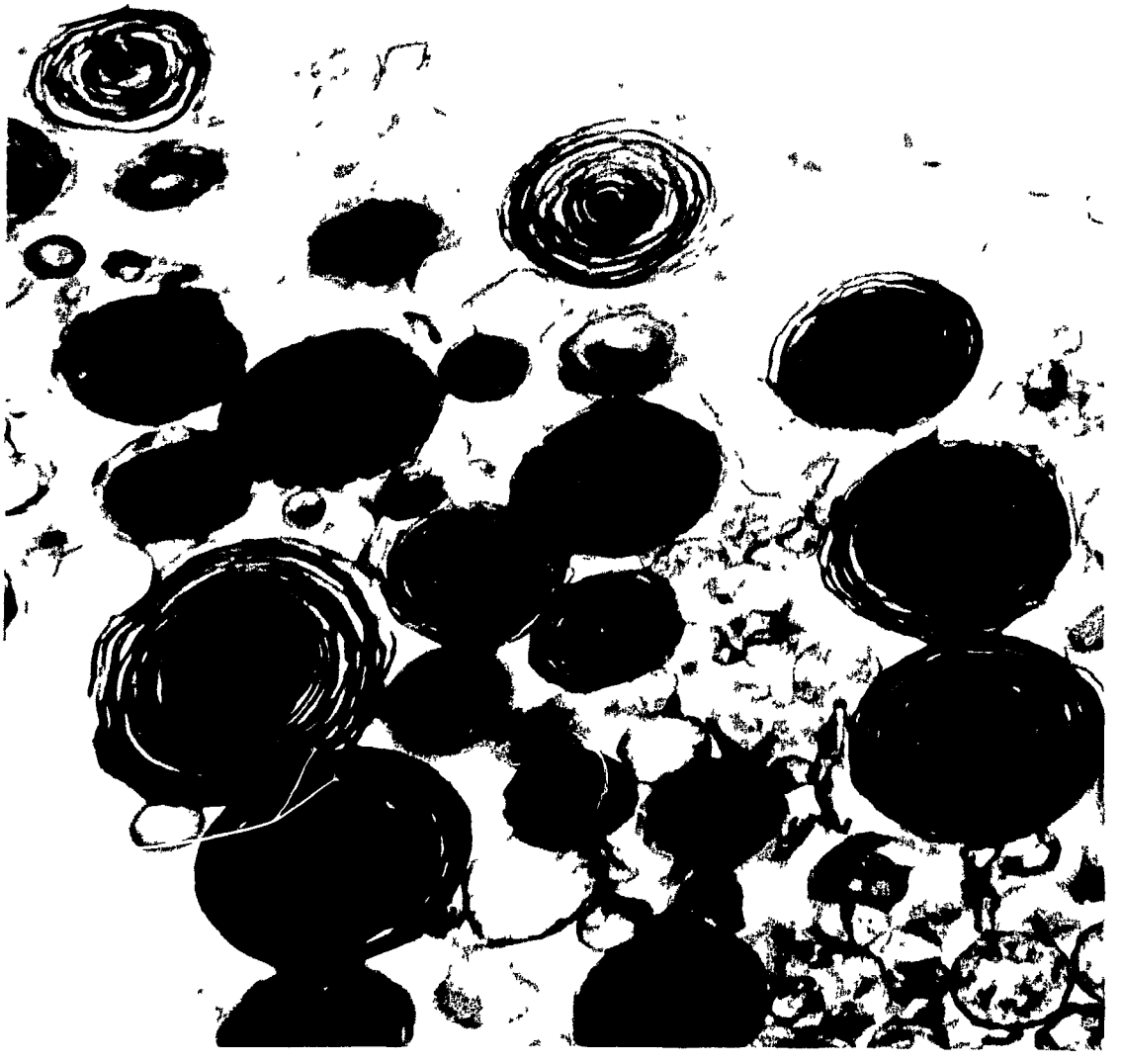

Figure 6: Electron micrograph of surfactant isolated from post-lavaged lung tissue at 27 days gestation demonstrating the presence of mainly intact lamellar bodies with minimal contamination of extraneous membrane components; x39,000.

analyses were performed by the technical staff in our laboratory and the results are included in this thesis as support documentation of the purity of the fraction we refer to as the intracellular surfactant.

The results are shown in Table 10. Of the total activity of succinate dehydrogenase, a mitochondrial marker enzyme, 88.5% was recovered in the mitochondrial-enriched fraction while only 0.17% was recovered in the lamellar bodies. Given that the specific activity of this enzyme in the lamellar body fraction is 15% of that of the mitochondrial-enriched fraction, suggests a 15% contamination of the lamellar body preparation with mitochondria. However, as the mitochondrial preparation was not purified, the actual contamination of the lamellar body fraction by mitochondria would be even less than this value. The percentage of total activity recovered in the microsomal fraction for a microsomal marker enzyme, NADPH: cytochrome$_c$ reductase, was 43.3%. Only 0.1% of the total activity was recovered in the lamellar body fraction. Another 34.5% was in fact recovered in the mitochondrial-enriched fraction. Only 0.1% of the total activity was recovered in the lamellar body fraction. The specific activity of NADPH: cytochrome$_c$ reductase, in the lamellar body fraction was approximately 8% of that in the microsomal-enriched fraction, suggesting some contamination of the lamellar body preparation with microsomes. Again, as the microsomal-enriched preparation was not purified, the actual contamination of the lamellar bodies by this organelle fraction would be less than this value. Only 0.08% of the total activity of 5' nucleotidase, a plasma

Table 10: Marker enzyme analysis of subcellular fractions obtained from fetal rabbit lung on the 27th gestational day.

Fraction	Succinate Dehydrogenase	NADPH: Cytochrome C Reductase	5'- Nucleotidase
	unit/mg protein		
Tissue homogenate	3.9 ± 0.6*	5.4 ± 0.6	4.0 ± 0.9
Mitochondrial - enriched	26.1 ± 4.9 (88.7%)+		
Microsomal - enriched		23.8 ± 0.7 (43.3%)	
Lamellar bodies	3.9 ± 2.6 (0.17%)	1.9 ± 1.8 (0.1%)	1.3 ± 0.8 (0.08%)

Each value represents the mean ± 1 S.D. from two to six determinations.

* One unit, 1 nmol/min

+Values in parenthes represent the percentage of total activity recovered in each fraction.

membrane marker enzyme, was recovered in the lamellar body fraction. The specific activity in this fraction was less than that for the tissue homogenate. We did not prepare plasma membranes from the lungs of fetuses of 27 gestational days but in previous studies, we did prepare this fraction from lungs of fetuses of later gestational ages (namely 30-31 days) and found the specific activity of this fraction to be 55.5 nmol/min per mg protein (constituting 73% of the total activity) (Oulton et al., 1986). At the same gestational age, the specific activity of the lamellar body fraction was 1.9 nmol/min per mg protein (0.5% of total activity). Based on the specific activity of these fractions an approximately 3% contamination of the lamellar body preparation with plasma membranes was calculated for preparations obtained at this later gestational age. The values reported above for the lamellar body preparation obtained on the 27th gestational day are similar to those for the later gestational age groups suggesting that the extent of contamination would be in the same range.

In summary, the optimal procedural conditions for isolating the intracellular surfactant pool, as described on page 67, involves: i) a 20 second homogenization time ii) a second density gradient centrifugation on fetal lung tissue obtained on the 27th gestational day but only one for older fetuses and iii) one washing of the final intracellular surfactant fraction. This procedure provided us with an optimal yield and a relatively pure surfactant fraction as assessed by compositional, marker enzyme and electron microscopical analyses.

II. IN VIVO EXPERIMENTS

A. Combination of Cortisol and Isoxsuprine

Our intent was to examine in vivo the effect of the combined treatment of cortisol and isoxsuprine administered: 1) simultaneously or 2) sequentially on the intracellular and extracellular surfactant pool size of fetal rabbits. The treatment protocol is shown in Table 11.

In the first series of studies (Table 11), cortisol and/or isoxsuprine were administered to the fetus on the 24th gestational day for subsequent delivery on the 27th gestational day. Each fetus was injected intraperitoneally (IP) through the intact uterine wall with one of the following agents: saline (0.1 ml/fetus), cortisol (1.0 mg/fetus), isoxsuprine (0.5 mg/fetus) or cortisol (1.0 mg/fetus) + isoxsuprine (0.5 mg/fetus) (Table 11). The rationale for the dosage of cortisol used in these studies was based on the work of Yoon (1985) where he injected cortisol (1.0 mg/fetus) IP to the fetus on the 25th gestational day with subsequent delivery on the 28th gestational day. Yoon observed a significant increase in the intracellular surfactant stores in the cortisol treated fetuses above the untreated and saline treated fetuses. The dosage of isoxsuprine (0.5 mg/fetus) administered IP to the fetus in these studies has been recommended by others where this agonist was administered to 28th gestational day fetal rabbits and increased

Table 11: Treatment protocol to examine the effects of cortisol and/or isoxsuprine on surfactant levels in the fetal rabbit. All fetuses were delivered by hysterotomy on the 27th gestational day.

Treatment	Gestational age at time of treatment	n	Route	Injection to Fetus or Doe	Dosage
Series 1					
1) Saline	24	6	IP*	Fetus	0.1 ml/fetus
2) Cortisol	24	5	IP	Fetus	1.0 mg/fetus
3) Isoxsuprine	24	8	IP	Fetus	0.5 mg/fetus
4) Cortisol +	24	7	IP	Fetus	1.0 mg/fetus
Isoxsuprine	24		IP	Fetus	0.5 mg/fetus
Series 2					
5) Isoxsuprine	26(10:00hrs) 26(22:00hrs)	5	IM†	Doe	5.0 x 2 mg/kg
6) Cortisol +	24	5	IP	Fetus	1.0 mg/fetus
Isoxsuprine	26(10:00hrs) 26(22:00hrs)		IM	Doe	5.0 x 2 mg/kg

n Number of determinations
* Intraperitoneal
† Intramuscular

surfactant secretion was observed compared to the untreated fetuses (Enhorning et al., 1977; Wyszogrodski et al., 1974). Although this dosage is relatively high for fetal rabbits at 24 days gestation, no observable side effects were noted in these fetuses. Saline-injected fetuses were included as sham experiments.

In the second series of studies cortisol (1.0 mg/fetus) was administered IP to the fetus on the 24th gestational day as described above (Table 11). Two days later (26th gestational day) the pregnant doe was treated with isoxsuprine (5.0 mg/kg) intramuscularly (IM) at 1000 and 2200 hours. In this series, some does received isoxsuprine (5.0 mg/kg) on the 26th gestational day (1000 and 2200 hours) without prior glucocorticoid treatment (Group 5, Table 11). All fetuses were subsequently delivered on the 27th gestational day. Due to the volume of isoxsuprine required for IM injection, the dosage was divided and administered to both hips at each treatment time. Untreated fetal rabbits delivered by hysterotomy on the 27th gestational day were studied as controls, for both series. For these studies, five to seven pups were used for each experimental data point and at least five data points were collected for each treatment protocol (Table 11).

1. Phospholipid content of the intracellular surfactant pool

The effect of saline, cortisol and/or isoxsuprine on the size of the intracellular surfactant pool is shown in Figure 7. Compared to

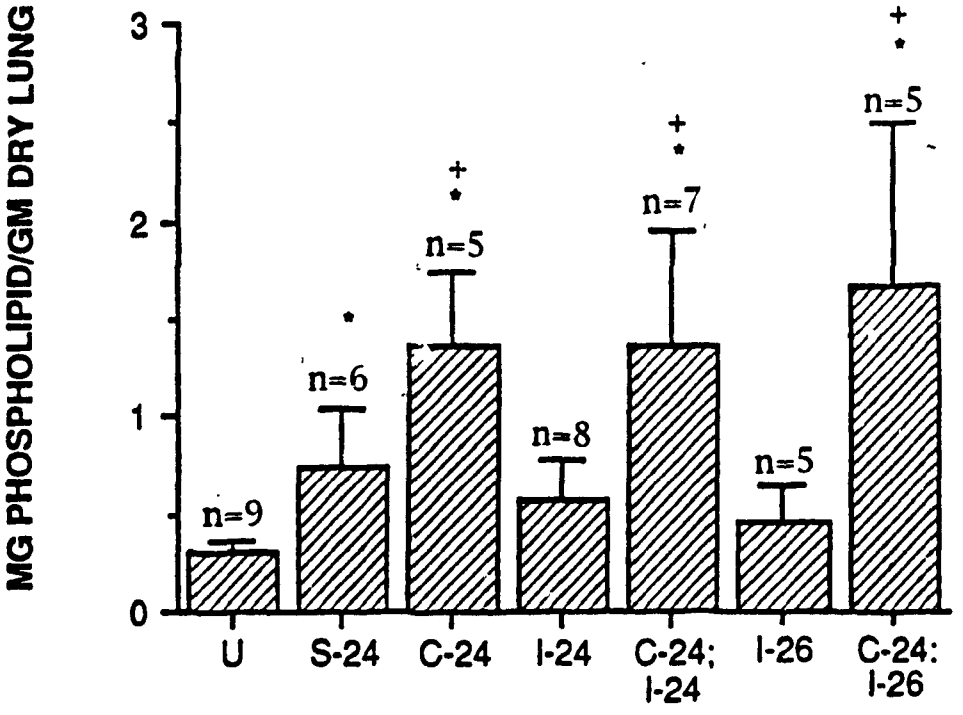

Figure 7: Effect of cortisol (C) and/or isoxsuprine (I) on the phospholipid content of intracellularly-stored surfactant from fetal rabbits of 27 gestational days. Both untreated (U) and saline-treated (S) fetuses served as controls. Gestational age at time of treatment is shown below the abscissa. Each bar represents the mean ± 1 S.D. for the number of determinations shown. Asterisk (*) indicates significantly different from untreated fetuses ($p<0.05$). Plus symbol (+), significantly different from saline-treated fetuses ($p<0.05$ by Duncan's multiple range test).

untreated controls, treatment of fetal rabbits with saline on the 24th gestational day also significantly increased ($p<0.05$) the intracellular surfactant pool size on the 27th gestational day. In fetuses treated with cortisol on the 24th gestational day the mean intracellular pool size (1.39±0.18 μg phospholipid/dry lung) was approximately four times that obtained from the untreated fetuses (0.34±0.12 μg phospholipid/dry lung) and twice that of the saline-treated fetuses (0.77±0.35 μg phospholipid/dry lung). Isoxsuprine administered alone on the 24th day of gestation did not significantly alter the mean size of the intracellular surfactant pool. However, the combination of isoxsuprine with cortisol on the 24th gestational day resulted in a significant increase ($p<0.05$) in the intracellular surfactant pool size compared to that of both the untreated and saline treated fetuses. However, this increase was not significantly greater than the effect observed with cortisol alone.

No effect was observed when isoxsuprine was administered on the 26th gestational day without prior glucocorticoid treatment but the combination of cortisol on the 24th gestational day followed by isoxsuprine treatment on the 26th gestational day resulted in a significant increase ($p<0.05$) in the intracellular surfactant pool size compared to that of the untreated or saline treated fetuses. This latter effect, however, was not significantly greater then that observed in the fetuses treated only with cortisol on the 24th gestational day.

Thus, while both saline and cortisol administered on the 24th

gestational day increased the mean size of the intracellular surfactant pool recovered from the lungs of 27th gestational day fetuses, the effect observed with cortisol was significantly greater than that observed with saline. Isoxsuprine on the other hand, had no effect on the pool sizes when administered alone on either the 24th or 26th gestational day and had no additive effect when administered in combination with cortisol.

2. Phospholipid content of extracellular (alveolar) surfactant pool.

The effect of saline, cortisol and/or isoxsuprine on the extracellular surfactant pool size of fetal rabbits is shown in Figure 8. While saline treatment slightly increased the size of this pool compared to that of the untreated fetuses, the increase was not statistically significant ($p>0.05$). Cortisol administration to the fetus on the 24th gestational day resulted in a significant increase ($p<0.05$) in the mean extracellular pulmonary surfactant pool size compared to that of both the untreated or saline-treated fetal rabbits. In contrast, isoxsuprine administered on the 24th gestational day or on the 26th gestational day did not significantly alter the extracellular surfactant pool sizes compared to that of the untreated or saline-treated fetuses (Figure 8). Treatment of fetuses with both cortisol and isoxsuprine on the 24th gestational day significantly increased ($p<0.05$) the extracellular pool size compared to that of the untreated

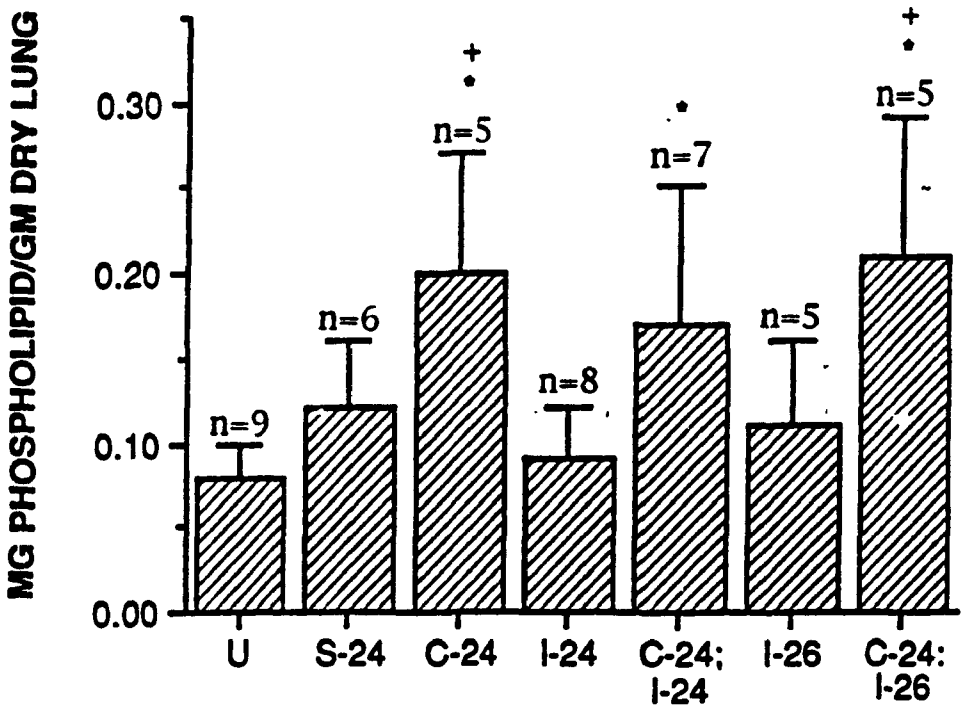

Figure 8: Effect of cortisol (C) and/or isoxsuprine (I) on the phospholipid content of alveolar surfactant from fetal rabbits of 27 gestational days. Both untreated (U) and saline-treated (S) fetuses served as controls. Gestational age at time of treatment is shown below the abscissa. Each bar represents the mean ± 1 S.D. for the number of determinations shown. Asterisk (*), significantly different from untreated fetuses ($p<0.05$). Plus symbol (+), significantly different from saline-treated fetuses ($p<0.05$ by Duncan's multiple range test).

fetuses but the increase was not greater than that produced by cortisol alone. Administration of cortisol to the fetus on the 24th gestational day followed by isoxsuprine to the doe on the 26th gestational day also resulted in a significant increase ($p<0.05$) in the extracellular surfactant pool size compared to that of the untreated fetuses. Again this increase was not significantly different from the effect observed in the fetuses treated only with cortisol. Thus, as with the intracellular surfactant pools, the extracellular pools were significantly increased by cortisol treatment, but no effect was observed with isoxsuprine treatment and no additive effect was found with the combination of cortisol and isoxsuprine.

3. Phospholipid composition

The effects of cortisol and/or isoxsuprine on the intracellular surfactant phospholipid composition are shown in Table 12. Phospholipid analysis of the intracellular surfactant pool obtained from the fetal lungs of all the groups studied indicated that phosphatidylcholine (PC) accounted for the greatest percentage of the surfactant phospholipids. Fetuses exposed to saline, cortisol or cortisol plus isoxsuprine showed a significant increase ($p<0.05$) in the relative proportion of PC within this pool compared to that obtained from the lungs of untreated fetuses. The percentage of PC was also significantly increased ($p<0.05$) in the intracellular surfactant pool in the lungs of fetuses treated with isoxsuprine on the 24th gestational

Table 12: Effect of cortisol and/or isoxsuprine on the phospholipid composition of intracellularly stored surfactant isolated from fetal lung tissue. Results are expressed as a mean percentage of total lipid phosphorus ± 1 S.D. for the number of determinations shown. *

Treatment (Gestational day)	n	PC	PG	PI	PS	PE	SM	Other
Untreated	8	63.83 ± 5.81	0.16 ± 0.38	8.68 ± 1.01	4.85 ± 1.41	15.67 ± 2.79	6.12 ± 4.12	0.72 ± 1.11
Saline (24)	3	71.72 ± 0.38†	0.05 ± 0.09	10.15 ± 1.20	4.56 ± 0.80	11.41 ± 0.99†	1.76 ± 0.54†	0.30 ± 0.52
Cortisol (24)	5	73.02 ± 2.55†	0.32 ± 0.38	9.73 ± 0.78	3.53 ± 0.88	10.96 ± 0.96†	2.19 ± 0.86†	0.00 ± 0.58
Isox. (24)	3	71.56 ± 3.52†	0.11 ± 0.12	9.47 ± 0.63	3.79 ± 0.83	11.73 ± 1.98†	3.00 ± 1.33	0.33 ± 0.57
Cortisol (24) + Isox. (24)	7	74.05 ± 3.97†	0.27 ± 0.36	9.83 ± 0.96	3.39 ± 1.37†	9.11 ± 3.08†	3.41 ± 2.47	0.57 ± 0.81
Isox. (26)	4	65.73 ± 1.56	n.d.	9.92 ± 2.55	5.02 ± 1.34	15.45 ± 2.04‡	3.90 ± 1.33	n.d.
Cortisol (24) + Isox. (26)	4	75.20 ± 1.82†	0.46 ± 0.74	9.45 ± 1.27	2.98 ± 0.82†	9.39 ± 1.15†	1.79 ± 0.50†	0.29 ± 0.39

Abbreviations used are: PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; SM, sphingomyelin; Other, unidentified phospholipids. n.d. - not detectable.

* Each value represents the mean ± 1 S.D. for the number of determinations shown for each experimental group.

† Indicates significantly different ($p<0.05$) compared to the untreated fetuses.

‡ Indicates significantly different ($p<0.05$) compared to the saline-treated fetuses.
(as determined by Duncan's Multiple Range Test)

day compared to that in the lungs of the untreated fetuses. However isoxsuprine administered to the doe on the 26th gestational day did not alter the relative proportion of PC in the intracellular surfactant pool compared to that in the lungs of the untreated fetuses.

There was no significant change in the relative proportions of the acidic phospholipids, PG and PI within the intracellular surfactant pool following any of the treatments. However, small but significant decreases were found in the relative proportion of PS in both groups treated with a combination of cortisol plus isoxsuprine compared to the untreated, but not the saline-treated fetuses. Furthermore, in comparison to untreated fetuses, the proportion of PE was significantly decreased ($p<0.05$) in all treatment groups except those which received isoxsuprine on the 26th gestational day. Finally, the relative proportion of SM was significantly decreased ($p<0.05$) compared to the untreated control group, in fetuses treated with saline, cortisol and cortisol followed by isoxsuprine on the 26th day while no changes were found in the unidentified phospholipids. Overall it can be seen that each treatment except for isoxsuprine administration to the doe on the 26th gestational day resulted in a more mature phospholipid composition compared to that of the untreated fetuses.

4. Fetal body and lung weight

The mean body and lung weights of fetal rabbits delivered on the

27th gestational day after being treated with either saline, cortisol, isoxsuprine, or cortisol plus isoxsuprine are shown in Table 13. Treatment with cortisol on the 24th gestational day (Group 3) or cortisol on the 24th day followed by isoxsuprine on the 26th gestational day (Group 7) significantly decreased mean fetal body weight ($p<0.05$) compared to that of the untreated fetuses. In the latter group the mean fetal body weight was also significantly less ($p<0.05$) than that of the saline-treated fetuses. None of the other treatments produced significant changes in the mean fetal body weights. Both saline and cortisol significantly decreased ($p<0.05$) the mean fetal lung weights compared to those of the untreated fetuses. These decreases were not found in any of the groups which received isoxsuprine.

Conclusion

In conclusion, our findings have shown that, in agreement with previous findings by Yoon, using the same model system (1985), treatment with cortisol on the 24th gestational day significantly increased the phospholipid content of both surfactant pools in the 27th gestational day fetus and accelerated the maturational profile of the phospholipid composition of these pools. We have also shown that the combination of cortisol and isoxsuprine administered either simultaneously or sequentially produced no further effect on either pool than that observed with cortisol alone. Finally, we did not find

Table 13 : Effects of cortisol and/or isoxsuprine on mean fetal rabbit body and lung weights. All fetuses were delivered by hysterotomy and sacrificed on the 27th gestational day without breathing. The gestational day at the time of treatment is indicated in the parenthesis following description of the treatment. Cortisol and saline were administered by direct fetal injection and isoxsuprine by intramuscular injection to the doe as described in page 64

Treatment	Number of determinations	Body Weight (gm)	Lung weight (gm) Fetus
1) Untreated	9	28.20 ± 2.45*	0.76 ± 0.08
2) Saline (24)	6	26.78 ± 2.64	0.66 ± 0.08†
3) Cortisol (24)	5	23.76 ± 2.78†	0.61 ± 0.08†
4) Isoxsuprine (24)	8	25.96 ± 3.80	0.79 ± 0.06‡
5) Cortisol (24) + Isoxsuprine (24)	7	26.95 ± 2.00	0.84 ± 0.09‡
6) Isoxsuprine (26)	5	26.84 ± 1.52	0.74 ± 0.11‡
7) Cortisol (24) + Isoxsuprine (26)	5	22.98 ± 3.16†‡	0.68 ± 0.11

* Each value represents the mean ± 1 S.D. for thr number of determinations shown.
† Indicates the value is significantly different ($p<0.05$) compared to the untreated fetuses.
‡ Indicates the value is significantly different ($p<0.05$) compared to the saline-treated fetuses. (as determined by Duncan's Multiple Range Test)

any effect of isoxsuprine on either surfactant pool size when administered alone on the 24th or 26th gestational day.

B. Auxiliary isoxsuprine studies

1. Varying the route and time of isoxsuprine

As a result of the unexpected finding from the previous experiments that isoxsuprine had no effect on the accumulation of surfactant in either the intracellular or extracellular pools when administered alone or in combination with cortisol, several experiments were undertaken to determine if this lack of effect may somehow be related to the route of administration of isoxsuprine, the gestation at the time of administration or the duration of exposure to isoxsuprine. Fetal rabbits delivered on the 28th or 30th day of gestation were used for these experiments. The treatment protocols are shown in Table 14. All fetuses were delivered by hysterotomy as described in Methods Section (page 65). From two to four analyses were performed for each treatment protocol. In the first series, all fetuses were delivered on the 28th gestational day. Isoxsuprine was administered by IM injection to the doe, as described on page 64, in either two (5.0 mg/kg) or three (3.33 mg/kg) consecutive doses, starting twenty-four hours prior to delivery. Isoxsuprine (0.5 mg/fetus) was also administered IP to the fetus, three hours prior to delivery. In these experiments the doe was anesthetized with sodium

Table 14: Treatment protocol for auxilliary studies to examine the effects of various isoxsuprine treatment regimens on surfactant levels in fetal rabbits delivered on the 28th (Series 1) or 30th (Series 2) gestational day.

Gestational age at time of treatment	Treatment times	n	Route	Injection to Fetus or Doe	Dosage
SERIES 1					
27	1000, 2200hrs.	2	IM†	Doe	5.0 mg/kg
27	1000, 1800, 0200hrs.	3	IM	Doe	3.33 mg/kg
28	3hrs. prior to delivery	3	IP*	Fetus	0.5 mg/fetus
28	4hr. infusion prior to delivery	4	IV‡	Doe	10.0 mg/kg
SERIES 2					
30	2hr. infusion prior to delivery	3	IV	Doe	10.0 mg/kg
30	3hr. infusion prior to delivery	2	IV	Doe	10.0 mg/kg
30	4hr. infusion prior to delivery	3	IV	Doe	10.0 mg/kg

n Number of determinations
† Intramuscular
* Intraperitoneal
‡ Intravenous

pentobarbital, a laparotomy was performed and the uterine horns exteriorized as described on page 64. The fetuses were injected with isoxsuprine (0.5 mg/fetus) through the intact uterine wall. The uterus was returned to the abdominal cavity, as described on page 65, and the doe was allowed to recover until delivery of fetuses three hours later. For the final group of this series isoxsuprine was administered intravenously (IV) to the doe. On the 28th gestational day isoxsuprine (2.5 mg/kg/hr in 50 ml of 5% glucose) was infused IV, into the lateral ear vein of the doe beginning four hours prior to delivery.

In the second series of experiments all the fetuses were delivered on the 30th gestational day and all isoxsuprine treatments were by IV infusion to the doe. For these experiments each doe received 10.0 mg/kg isoxsuprine in 50 ml of 5% glucose and the infusion were started at either 2, 3, or 4 hours prior to delivery. In all experiments involving IV infusions, the does were removed to a rabbit restraining box 15 minutes before the infusion and allowed to relax prior to and during the actual infusion.

Intramuscular administration of isoxsuprine either in two doses twelve hours apart or every eight hours for three consecutive doses on the 27th gestational day had no significant effect on either the size of the intracellular or extracellular surfactant pools of fetuses delivered on the 28th gestational day compared to the untreated control fetuses delivered on the same gestational day. Similarly in fetuses treated with isoxsuprine IP three hours prior to delivery on

the 28th gestational day, no significant effect on the surfactant pools were observed in the isoxsuprine treated fetuses compared to the untreated fetuses. Administration of isoxsuprine by intravenous infusion to the doe over a 2-4 hour period prior to delivery also had no significant effect on the intracellular or extracellular surfactant pool sizes in either the 28th or 30th gestational day fetuses. Thus, varying the route or time of administration of isoxsuprine had no significant effect on fetal lung maturation in 28th and 30th gestational day fetal rabbits.

C. Isoxsuprine - Short term effects of isoxsuprine.

Given the lack of effect observed when fetuses were exposed to isoxsuprine for approximately 2-4 hours, we then wanted to determine if perhaps a shorter exposure time i.e., 30 minutes, might produce an effect on either the intracellular or the extracellular surfactant pool size. We were also interested in determining if such an effect could be observed, might it be dependent on the gestational age of the fetus or the breathing status of the animal i.e., whether the fetus was maintained in utero or delivered by hysterotomy and allowed to breathe room air. Our rationale for using a shorter exposure time, i.e. 30 minutes was based on the work of Wyszogrodski et al. (1974) where 28 gestational day fetal rabbits were injected with isoxsuprine and allowed to breath for 30 minutes. They observed improved pressure-volume curves in these treated fetuses suggesting an increase in surfactant secretion in the

isoxsuprine treated fetuses The treatment protocol for this study is shown in Table 15. These experiments were performed on either the 28th (preterm) or 30th (term) gestational day. Isoxsuprine was administered by direct fetal injection and exposure time was 30 minutes. Five to seven determinations were performed for each treatment group on the 28th gestational day (Series 1) and from five to thirteen for the 30th gestational day groups.

For these experiments the doe was anesthetized with sodium pentobarbital as described on page 65. A laparotomy was performed and the uterine horns exteriorized. Each fetus was intraperitoneally injected with isoxsuprine (0.5 mg/fetus) through the intact uterine wall of the doe and was either left in utero for 30 minutes or delivered immediately and allowed to breathe room air for 30 minutes. The fetuses remaining in utero for 30 minutes were kept warm and moist by applying warmed saline-soaked surgical sponges to the exteriorized uterus. The delivered fetuses were promptly removed from their amniotic sacs, and placed in a warmed box (approximately 27° C) to breathe room air. All fetuses were delivered on the 28th or 30th gestational day by hysterotomy as described on page 65. Concurrent controls consisted of animals on which a laparotomy was performed and the uterus exteriorized and maintained in this condition with warming for 30 minutes or delivered and allowed to breathe for 30 minutes. After 30 minutes the fetuses were killed with sodium pentobarbital IP (13-23

Table 15: Treatment protocol to examine the short term effect of isoxsuprine on surfactant levels in preterm (28th gestational day) and term (30th gestational day) fetal and/or newborn rabbits.

Gestation (days)	Treatment	n	Breathing† / No Breathing‡ (B/NB)
Series 1			
28	Untreated	7	NB
28	Untreated	7	B
28	Isoxsuprine (0.5 mg/fetus -IP*)	7	NB
28	Isoxsuprine	5	B
Series 2			
30	Untreated	5	NB
30	Untreated	13	B
30	Isoxsuprine (0.5 mg/fetus -IP)	5	NB
30	Isoxsuprine	9	B

n Number of determinations
† breathing (B) - allowed to breathe room air for 30 minutes.
‡ no breathing (NB) - left in utero for 30 minutes.
* Intraperitoneal

mg/fetus).

1. Phospholipid content of intracellular and extracellular surfactant pools.

The effect of isoxsuprine administration (short term exposure) on intracellular and extracellular surfactant pool sizes in fetuses which were either maintained in utero or were delivered and allowed to breathe for 30 minutes is shown in Table 16. Results are shown separately for the two surfactant pools and are also expressed as a ratio of the extracellular to the intracellular pool size. Significantly greater ($p<0.05$) amounts of phospholipid were present in both pools in the lungs of the untreated 30th gestational day fetuses maintained in utero compared to the lungs of a comparable group of 28th gestational day animals. In fact, the intracellular pool of the 30th gestational day group was approximately six times greater (12.11±3.75 vs. 1.84±0.39 mg phospholipid/g dry lung) than that of the 28th gestational day group while the extracellular pool was approximately 13 times greater (1.65±0.68 vs. 0.12±0.03 mg phospholipid/g dry lung.

Isoxsuprine treatment had no effect on the intracellular pool size of any of the groups of fetuses. Also there was no effect of isoxsuprine treatment on the intracellular surfactant pool size in the animals (either on the 28th or 30th gestational day) which were allowed to breathe. In fact, breathing itself had no effect on the

Table 16: Effects of isoxsuprine administration on intracellular and extracellular surfactant pool sizes in fetal rabbits which were either maintained in utero (no breathing) or delivered and allowed to breathe for 30 min. (breathing). Isoxsuprine administration was as described in Table 15. Results for each pool are expressed as the mean mg phospholipid/g dry lung ± S.D. for 5 to 12 determinations. The ratio of the phospholipid content in the extracellular and intracellular pool is also shown.

Treatment	Intracellular pool size	Extracellular pool size	$\frac{\text{Extracellular}}{\text{Intracellular pool size}}$ x100
28 day - no breathing			
Untreated*	1.84 ± 0.39	0.12 ± 0.03	6.63 ± 1.93
Isoxsuprine	3.05 ± 1.56	0.19 ± 0.08†	6.80 ± 1.93
28 day - breathing			
Untreated	2.71 ± 1.17	0.37 ± 0.19‡	13.71 ± 2.95
Isoxsuprine	2.04 ± 0.97	0.17 ± 0.09†	7.96 ± 0.99†
30 day - no breathing			
Untreated	12.11 ± 3.75	1.65 ± 0.68	17.50 ± 4.45
Isoxsuprine	8.50 ± 0.94	0.58 ± 0.19†	6.98 ± 2.44†
30 day - breathing			
Untreated	9.92 ± 2.57	2.42 ± 0.95‡	22.30 ± 5.70
Isoxsuprine	10.06 ± 1.91	2.59 ± 0.82	26.14 ± 7.71

* Control values for both surfactant pools as well as the ratio of the two pools in the 28th gestational day group were significantly different ($p<0.05$) compared with the corresponding control values in the 30th gestational day group (Statistics- Duncan's Multiple Range Test).

† Indicates significantly different ($p<0.05$) from corresponding values of untreated animals.

‡ Indicates significantly greater ($p<0.05$) than corresponding nonbreathing untreated values.

intracellular pool size.

In contrast to the lack of effect on the intracellular surfactant pool size, significant effects of this drug were observed on the size of the extracellular pool. In the 28th gestational day fetal rabbits maintained in utero for 30 minutes, a small but significant increase ($p<0.05$) was observed in this pool. No difference was observed in the ratio of the extracellular to intracellular pool size. Breathing significantly increased ($p<0.05$) the extracellular pool over that of the non-breathing group (ie. 0.37±0.19 mg phospholipid/g dry lung in the breathing group vs. 0.12±0.03 mg phospholipid/g dry lung for the non-breathing group) but this increase was abolished by isoxsuprine treatment. The ratio of the extra- to intracellular pool was also significantly decreased (ie. from 13.71±2.95 to 7.96±0.99).

The effect of isoxsuprine on the extracellular surfactant pool size in the 30th gestational day fetal animals was opposite to that observed in the 28th gestational day animals. In the 30th gestational day animals maintained in utero, isoxsuprine treatment significantly decreased ($p<0.05$) the extracellular pool size as well as the ratio of the extracellular to intracellular pool. Furthermore, as was the case with the 28th gestational day animals, breathing significantly increased ($p<0.05$) the extracellular pool size. However, in contrast to the findings in the 28th gestational day fetuses, isoxsuprine treatment did not abolish the breathing-induced increase in the phospholipid content of the extracellular surfactant pool size in the 30th gestational day fetuses. The extracellular to intracellular pool

size ratio was also unaltered. Thus short term isoxsuprine treatment had no effect on the intracellular pool size under any of the circumstances examined. It did, however, affect the size of the extracellular pools and in this case appeared to be related to both the gestational age of the fetus and its breathing status in that at 28 days gestation isoxsuprine had an inhibitory influence on secretion after birth while at 30 days gestation this inhibitory effect was observed prior to birth.

2. Phospholipid composition

The effect of short-term isoxsuprine exposure on the phospholipid composition of intracellularly stored surfactant is shown in Table 17. Significant differences were observed in the 30th gestational day untreated non-breathing fetal animals when compared to the corresponding control animals of 28 gestational days. These included a significantly greater ($p<0.05$) percentage of PC (76.35 ± 1.09 vs. 66.40 ± 3.62) and PG (0.92 ± 0.47 vs. undetectable levels) and significantly less ($p<0.05$) PE (6.66 ± 0.38 vs. 13.80 ± 1.47) and SM (0.89 ± 0.13 vs. 3.68 ± 0.79). No significant changes in phospholipid composition were observed following isoxsuprine treatment on the 28th gestational day in either those fetuses which were left in utero or those which were allowed to breathe.

In contrast to the findings with the 28th gestational day rabbits, isoxsuprine treatment of fetuses of 30 days gestation left in utero did

Table 17: Effect of isoxsuprine administration on intracellularly-stored composition in breathing or non-breathing pre-term-delivered rabbits. Isoxsuprine administration was as described in Table 15 Results are expressed as the mean ± S.D. for 5-13 determinations.

	% of Total Phospholipid						
Treatment	PS†	PI	SM	PC	PG	PE	Other
			28 Day - No breathing				
Control	4.15±1.91	11.96±2.25	3.68±0.79[a]	66.40±3.62[a]	n d	13.80±1.47[a]	nd
Isoxsuprine	4.19±2.52	11.80±2.13	2.95±0.71	68.70±2.33	0.06±0.11	12.30±1.67	n d
			28 Day - 30 min. breathing				
Control	4.12±0.80[a]	11.08±0.80	2.58±0.64[a]	72.44±4.50[a]	0.29±0.27	10.39±0.63[a]	0.45±0.52
Isoxsuprine	5.27±1.50	10.31±0.69	3.47±0.78	68.20±3.05	0.22±0.30	11.38±2.22	1.15±1.07
			30 Day - No breathing				
Control	1.93±0.77	13.17±1.68	0.89±0.13	76.35±1.09	0.92±0.47[b]	6.66±0.38	0.08±0.15
Isoxsuprine	1.13±0.77[c]	15.93±0.45[c]	1.87±1.40	77.44±5.21	0.18±0.13[c]	9.21±3.73	0.09±0.19
			30 Day - 30 min. breathing				
Control	2.69±0.49	11.72±0.67	1.17±0.57	77.27±1.65	0.69±0.46	6.45±0.49	0.01±0.03
Isoxsuprine	2.91±0.53	10.94±0.77	1.43±0.45	77.09±1.44	1.04±0.85	6.63±0.91	n d

[a] Indicates significantly different ($p<0.05$) compared to the corresponding control value on the 30th gestational day.

[b] Indicates significantly different ($p<0.05$) from isoxsuprine-treated animals.

[c] Indicates significantly different from the corresponding 30th gestational day isoxsuprine-treated animals which were allowed to breathe.

† Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine. n.d. - not detectable

induce significant changes but these were confined to the acidic phospholipids. For example, PG levels (expressed as a relative proportion of the total phospholipids) were significantly decreased ($p<0.05$) within the intracellular surfactant pool after isoxsuprine treatment in those fetuses which did not breathe. The relative proportion of PS was also significantly reduced ($p<0.05$) after treatment with the drug. These decreases were accompanied by a concomitant significant increase ($p<0.05$) in the relative proportion of PI in the same group of animals. Breathing appeared to negate these effects on the acidic phospholipids.

3. Fetal body weight and lung weight

The effects of short term exposure to isoxsuprine on mean body and lung weights of 28th and 30th gestational day fetal rabbits which were either left in utero or delivered by hysterotomy and allowed to breathe for 30 minutes are shown in Table 18. Mean fetal body and lung weights were significantly decreased ($p<0.05$) in 28th gestational day fetal rabbits treated with isoxsuprine and left in utero for 30 minutes compared to the mean fetal body and lung weights of the untreated fetuses. No significant alterations in fetal body or lung weights were detected in 28th gestational day neonates (allowed to breathe for 30 minutes) treated with isoxsuprine. Neither of the groups which received isoxsuprine on the 30th gestational day showed any significant alterations in their mean fetal body or lung

Table 18: Effects of short-term isoxsuprine exposure on mean body and lung weights in fetal rabbits which were delivered by hysterotomy on either the 28th or 30th gestational day. After isoxsuprine administration the fetuses were either left in utero or immediately delivered and allowed to breathe for 30 minutes. Results are expressed as the mean ± 1S.D. for the number of determinations shown.

Treatment	Number of determinations	Body Weight (gm)	Lung weight (gm) Fetus
28 day, no breathing			
Untreated	7	34.65 ± 2.69	1.33 ± 0.17
Isoxsuprine	7	30.26 ± 4.98†	1.06 ± 0.14†
28 day, 30 min. breathing			
Untreated	7	33.78 ± 4.65	1.25 ± 0.15
Isoxsuprine	5	31.71 ± 3.55	1.08 ± 0.16
30 day, no breathing			
Untreated	5	44.57 ±10.40	1.29 ± 0.34
Isoxsuprine	5	45.17 ± 3.03	1.37 ± 0.07
30 day, 30 min. breathing			
Untreated	13	42.96 ± 5.64	1.32 ± 0.43
Isoxsuprine	9	44.40 ± 5.50	1.30 ± 0.17

† Indicates significantly different ($p<0.05$) from corresponding control value (as determined by Duncan's Multiple Range Test).

weights when compared to those of the untreated fetuses.

Conclusion

In conclusion our findings have shown that short term exposure to isoxsuprine had an inhibitory effect on surfactant secretion that appeared to be dependent on the gestational age of the fetus and the breathing status of the animal.

D. Auxiliary study to examine the effect of increasing the exposure time to isoxsuprine in the neonatal lung.

This auxiliary study was designed to see if the inhibitory effect observed in previous experiments could be reversed over time. On the 30th gestational day the doe was anesthetized with sodium pentobarbital, a laparotomy performed and the uterine horns exteriorized as described on page 65. Thirtieth gestational day fetal rabbits were given isoxsuprine by IP injection (0.5 mg/fetus) and the fetuses were left in utero for 1.5-2 hours. Concurrent untreated controls for this gestational age was also included in this study. All fetuses were delivered by hysterotomy.

Increasing the length of time the fetuses were exposed to isoxsuprine (0.5mg/fetus - IP) while still in utero to a period greater than 30 minutes (1-2 hours) did not produce any significant change in the size of the intracellular (10.2±1.57) or extracellular surfactant

(1.89±1.23) pools or in the ratio of extracellular to intracellular surfactant (19.54±13.99) compared to the untreated controls (intracellular: 12.11±3.75; extracellular: 1.65±0.68; extracellular/intracellular: 17.50±4.45). Each value represents the mean ± 1S.D. of three to five determinations and is expressed as mg phospholipid/gm dry lung. Thus increasing the length of time the fetal rabbits were exposed to isoxsuprine in utero to a period of time greater than 30 minutes, appears to abolish the inhibition on surfactant secretion observed when the fetuses are exposed to isoxsuprine in utero for only 30 minutes.

E. Combination of Cortisol + TRH

Given the lack of effect of isoxsuprine (administered alone or in combination with cortisol) in accelerating surfactant production using our in vivo model system, we were interested in determining if another agent which has been shown to influence lung maturation, namely TRH would affect either the intracellular or extracellular surfactant pool size and if so would the effect be i) dependent on gestational age at the time of treatment and ii) additive or synergistic with the cortisol effect.

The treatment protocols for these studies are shown in Tables 19 and 20. As a first part of the study the effect of TRH administration was compared to that of cortisol. Cortisol was administered IP to the fetus on the 24th gestational day as previously described (refer to

Table 19: Treatment protocol to compare the effect of exogenous glucocorticoid and TRH on surfactant in the fetal rabbit. Glucocorticoid (cortisol) was administered IP to fetus on the 24th gestational day. TRH was administered IV to the doe in single or multiple doses beginning on the 24th gestational day and ending on the 26th. Untreated fetuses and fetuses treated IP with saline on the 24th gestational day served as comparison groups. All fetuses were subsequently delivered by hysterotomy on the 27th gestational day.

Treatment	Gestational age at time of treatment	Number of determinations	Route	Injection to Fetus or Doe	Dosage
1) Saline	24	6	IP*	Fetus	0.1 ml/fetus
2) Cortisol	24	7	IP	Fetus	1.0 mg/fetus
3) TRH	24	4	IV‡	Doe	40 µg/kg
4) TRH	25	4	IV	Doe	40 µg/kg
5) TRH	26	3	IV	Doe	40 µg/kg
6) TRH	25.26	6	IV	Doe	40 (x 2) µg/kg
7) TRH	24.25.26	5	IV	Doe	40 (x 3) µg/kg

* Intraperitoneal
‡ Intravenous

Table 20: Treatment protocol to examine the effect of the combination of cortisol and TRH on surfactant production in the fetal rabbit. Treatment was administered at the gestational ages indicated and the fetuses were subsequently delivered by hysterotomy on the 27th gestational day.

Treatment	Gestational age at time of treatment	Number of determinations	Route	Injection to Fetus or Doe	Dosage
1) Cortisol +	24	4	IP*	Fetus	1.0 mg/fetus
TRH	24		IV‡	Doe	40 µg/kg
2) Cortisol	24	5	IP	Fetus	1.0 mg/fetus
TRH	25,26		IV	Doe	40 (x 2) µg/kg
3) Cortisol +	24	5	IP	Fetus	1.0 mg/fetus
TRH	24,25,26		IV	Doe	40 (x 3) µg/kg

* Intraperitoneal
‡ Intravenous

page 64). TRH (40 μg/kg) was administered intravenously to the doe into the lateral ear vein on the 24th, 25th, 26th, 25th + 26th or 24th + 25th + 26th gestational days. Concurrent controls consisted of untreated fetuses delivered on the 27th gestational day as well as fetuses treated with saline on the 24th gestational day.

For the second part of the study the effect of the combined treatment of cortisol and TRH was examined (Table 20). Glucocorticoid treatment was administered on the 24th gestational day. For these experiments the doe was anesthetized with halothane, a laparotomy performed, and cortisol (1.0 mg/fetus) was injected IP to the fetus through the intact uterine wall as described previously on p.64. Following recovery from the surgical procedure, TRH (40 μg/kg) was administered intravenously into the lateral ear vein of the doe on the 24th, 25th + 26th or 24th + 25th + 26th gestational days. For both parts of the study, fetuses were delivered on the 27th gestational day by hysterotomy as described previously on p.65. From three to seven analyses were performed per treatment group.

1. Phospholipid content of intracellular surfactant

The effects of treatment with cortisol and/or TRH on the intracellular surfactant pool size is shown in Figure 9. In agreement with results shown previously on p.101, cortisol administered to the fetus on the 24th gestational day significantly increased ($p<0.05$) the intracellular surfactant pool size compared to that of either the

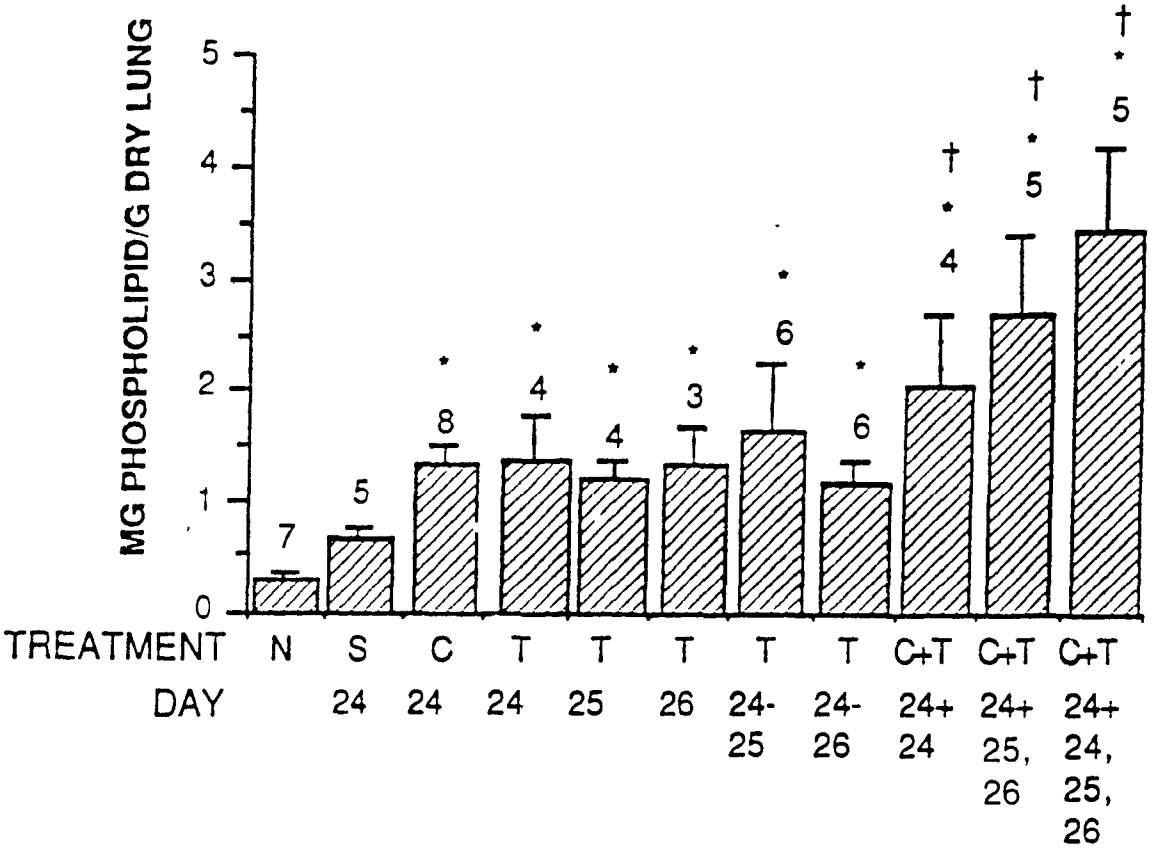

Figure 9: Effect of TRH (T) administered alone or with cortisol (C) at various gestational ages on the phospholipid content of the intracellular surfactant pool in fetal rabbit lungs of 27 days gestation. Gestational age at the time of treatment is indicated below the abscissa. Untreated (N) and saline treated (S) controls were included in the study. Each value represents the mean ± S.D. for the number of determinations shown above each bar. Asterisk (*) indicates significantly different from both the untreated and saline-treated animals ($p<0.05$). Dagger (†) indicates significantly different from both the cortisol and corresponding TRH treatment ($p<0.05$ by Duncan's multiple range test.

untreated or saline treated fetuses.

TRH administered to the doe on the 24th, 25th or 26th gestational day or on two or more of these days, significantly increased ($p<0.05$) the mean surfactant pool sizes compared to that of either untreated or saline-treated fetuses. There were no significant differences in the size of the intracellular pools amongst the various TRH treatment regimens or between any of these TRH treatments and the administration of cortisol to the fetuses on the 24th gestational day.

All combinations of cortisol and TRH significantly increased ($p<0.05$) the intracellular surfactant pool size compared to that obtained by treatment with only cortisol or TRH. The greatest effect was observed following treatment with cortisol on the 24th day plus TRH on the 24th, 25th, and 26th gestational day. This combination resulted in a greater than ten fold increase (3.40 ± 0.78 vs 0.29 ± 0.07 mg phospholipid/gm dry lung)in the phospholipid content of the intracellular surfactant pool compared to that of the untreated fetuses (Figure 9).

2. Phospholipid content of extracellular surfactant

The effect of the administration of cortisol and/or TRH on the extracellular surfactant pool is shown in Figure 10. Cortisol administered on the 24th gestational day resulted in a significant increase ($p<0.05$) in the mean extracellular surfactant pool size compared to that of the untreated fetal rabbits but not to that of the

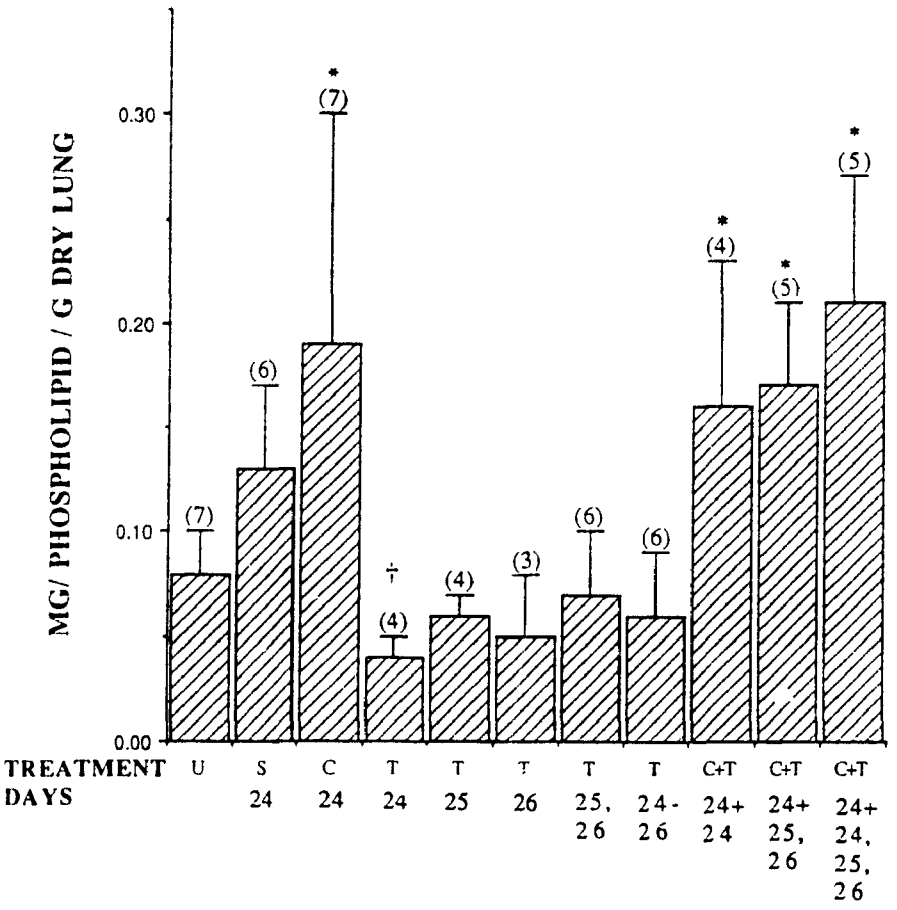

Figure 10: Effect of TRH (T) administered alone or with cortisol (C) at various gestational ages on the phospholipid content of the extracellular surfactant pool in fetal rabbit lungs of 27 days gestation. Gestational age at the time of treatment is indicated below the abscissa. Untreated (U) and saline treated (S) controls were included in the study. Each value represents the mean ± S.D. for the number of determinations shown above each bar. Asterisk (*) indicates significantly different from the untreated animals ($p<0.05$). Dagger (†) indicates significantly different from the saline-treated fetuses ($p<0.05$ by Duncan's multiple range test.

saline-treated fetuses. TRH administration on the 24th, 25th or 26th gestational day or two or more of these days did not significantly alter the extracellular surfactant pool size compared to that of untreated fetuses. TRH on the 24th gestational day was shown to significantly decrease the extracellular pool size compared to the saline treated fetuses while it had no effect at the other gestational age. There were also no significant differences in the mean extracellular surfactant pool size amongst the group of fetuses treated with the various TRH regimens.

All combinations of cortisol and TRH resulted in a significant increase ($p<0.05$) in the extracellular pool size compared to that of the untreated animals; pool sizes in these treatment groups were not significantly different from those of fetuses receiving only cortisol.

3. Phospholipid composition

Compositional analyses of the lamellar body fraction (intracellular surfactant) in untreated fetuses and in fetuses treated with cortisol on the 24th gestational day plus TRH on the 24th, 25th, and 26th gestational days are shown in Table 21. Treatment with cortisol and TRH produced a significant increase ($p<0.05$) in the relative proportion of PC and PI and a concomitant decrease in PS, PE and SM. Though the magnitude of the change was not as large, similar results were observed for each group in which the treatment increased the extracellular surfactant pool size (results not shown). The results in

Table 21: Effect of the combined treatment of cortisol and TRH on the phospholipid composition and content of intracellularly stored surfactant obtained from fetal rabbit lung at 27 days gestation. In the treatment group cortisol was administered at 24th gestational day and TRH on the 24th, 25th, and 26th gestational day.*

Treatment	n	PC	PG	PI	PS	PE	SM	Other
Untreated	6	58.3 ± 3.8	n.d.	8.8 ± 0.4	7.2 ± 0.8	19.2 ± 2.8	5.7 ± 1.4	0.9 ± 1.1
Cortisol (day 24); + TRH (days 24,25,26)	5	68.8 ± 3.6†	n.d.	10.7 ± 0.7†	4.9 ± 0.8†	12.2 ± 2.0†	3.1 ± 0.8†	0.1 ± 0.2

Abbreviations used are: PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Other, unidentified phospholipids. n.d. - not detectable

* Each value represents the mean ± 1 S.D. for the number of determinations shown for each experimental group.
† Indicates significantly different ($p<0.05$) from that of the untreated fetuses as determined by Student's *t* test.

Table 21 illustrate this trend.

4. Fetal body and lung weight

The effects of exogenous cortisol and TRH on mean fetal body and lung weights are shown in Table 22. In this series of experiments, while both body and lung weights were slightly less in the saline and cortisol-treated fetuses than in the untreated group of animals, these decreases were not statistically significant. Also in this series of experiments, none of the TRH treatments significantly altered either mean fetal body or lung weight. The combination of cortisol and TRH on the 24th gestational day or cortisol on the 24th gestational day plus TRH on the 24th, 25th and 26th gestational days resulted in a significant decrease ($p<0.05$) in both the mean fetal body and lung weights compared to those of the untreated animals. In the latter group the mean fetal lung weight was also significantly less ($p<0.05$) than that of the untreated fetuses as well as those groups of fetuses treated with saline or cortisol. There was no significant difference in the mean fetal body or lung weights in the fetuses treated with cortisol on the 24th gestational day followed by TRH on the 25th and 26th gestational days compared to the untreated animals.

Conclusion

In conclusion TRH administered alone in either single or multiple

Table 22 : Effect of cortisol and/or TRH on mean fetal rabbit body and lung weights. All fetuses were delivered by hysterotomy and sacrificed on the 27th gestational day without breathing.

Treatment	Number of determinations	Body Weight (gm)	Lung weight(gm) Fetus
Series 1			
1) Untreated	7	30.10 ± 0.44$^{\Delta}$	1.09 ± 0.05
2) Saline (24)	6	26.42 ± 2.71	0.69 ± 0.11
3) Cortisol (24)	7	25.46 ± 3.69	0.74 ± 0.23
4) TRH (24)	4	28.73 ± 3.43	1.08 ± 0.12
5) TRH (25)	4	28.45 ± 1.84	1.06 ± 0.14
6) TRH (26)	3	31.32 ± 3.30	1.14 ± 0.14
7) TRH (25,26)	6	28.97 ± 0.77	1.09 ± 0.09
8) TRH (24, 25, 26)	5	28.56 ± 1.48	1.00 ± 0.07
Series 2			
9) Cortisol (24) + TRH (24)	4	25.29 ± 4.74*	0.91 ± 0.16*
10) Cortisol (24) + TRH (25,26)	5	30.04 ± 3.68	0.98 ± 0.17
11) Cortisol (24) + TRH (24,25,26)	5	26.29 ± 0.92*	0.80 ± 0.08*†‡

$^{\Delta}$ Each value represents the mean ± S.D. for the number of determinations shown.

* Indicates the value is significantly different ($p<0.05$) compared to the untreated fetuses.

† Indicates the value is significantly different ($p<0.05$) compared to the saline-treated fetuses.

‡ Indicates the value is significantly different ($p<0.05$) compared to the cortisol-treated fetuses.

(as determined by Duncan's Multiple Range Test)

doses significantly increased the intracellular pool compared to the untreated fetuses but had no effect on the extracellular surfactant pool. All combinations of cortisol and TRH resulted in an increase in the intracellular pool size over that observed with either drug given alone. The maximal response appears to be related to the timing of the combined treatments.

III. IN VITRO EXPERIMENTS

As a result of our previous in vivo experiments showing that short term exposure to isoxsuprine had an inhibitory effect on fetal secretion we were interested in whether this inhibition was also displayed by isolated type II cells exposed to different concentrations of isoxsuprine. Using a method described by Scott et al. (1983) (refer to page 73), a homogeneous population of undifferentiated cells from fetal rabbit lung was obtained and induced to differentiation by exposing these cells to FCM for either 24, 48, or 96 hours. Differentiation was assessed by the levels of DSPC synthesis by this monolayer cell culture.

The effects of 20% medium (v/v) conditioned by fetal lung fibroblast culture on [^{3}H]choline incorporation into intracellular DSPC by monolayer cultures of undifferentiated type II alveolar cells are shown in Table 23. All groups exposed to FCM for 24, 48 or 96 hours showed a significant increase ($p<0.05$) in the mean incorporation of [^{3}H]choline into DSPC per 10^4 cells. We therefore concluded that 24

Table 23: Effect of Medium Conditioned by Confluent Monolayer Cultures of fetal rabbit lung fibroblasts on [^{3}H] choline incorporated into intracellular disaturated phosphatidylcholine (DSPC).

	Picomoles DSPC/10^4 cells at time (hrs.)					
Treatment	24hrs	n	48hrs	n	96hrs	n
Control	17.52 ± 0.10	16	34.67 ± 1.87	16	75.44 ± 9.11	12
Conditioned medium, 20% (v/v)	35.11 ± 3.13[b]	16	47.12 ± 4.29[b]	16	100.27 ± 4.03[b]	12

[a] Results are expressed as mean ± SEM.

[b] Indicates significantly different ($p<0.05$) from the corresponding control value as determined by Student's t-test.

hours of exposure to FCM was adequate to induce differentiation and incorporated this time period into our procedure.

The effect of isoxsuprine on the incorporation of [^{3}H]choline into intracellular DSPC by type II cells which had been previously exposed to FCM for 24 hours is shown in Figure 11. Compared to untreated alveolar type II cells, cells exposed to isoxsuprine at a concentration of 1, 5, or 10 μM for 12 hours showed no significant change in the incorporation of [^{3}H]choline into DSPC. Exposure of alveolar type II cells to the drug for 24 hours showed no effect with 1 or 5 μM isoxsuprine but a significant decrease ($p<0.05$) in intracellular radioactively labelled DSPC was observed with isoxsuprine at a concentration of 10 μM. After 48 hours type II cells initially exposed to either 5 or 10 μM of isoxsuprine contained significantly more ($p<0.05$) radioactively labelled DSPC compared to those cells not in contact with the drug. This trend was reversed by 96 hours at which time those cells exposed to all concentrations of isoxsuprine showed a significant decrease ($p<0.05$) in the intracellular content of radioactively labelled DSPC (Figure 11).

The appearance of radioactively labelled DSPC from the type II cells in the culture media is shown in Figure 12. Cells exposed to 10 μM isoxsuprine for 12 hours showed a significant increase ($p<0.05$) in the media content oflabelled DSPC compared to that of the untreated alveolar type II cells. This trend continued such that at 24 hours all three concentrations of isoxsuprine significantly increased ($p<0.05$) the content of labelled DSPC in the culture medium. This effect was

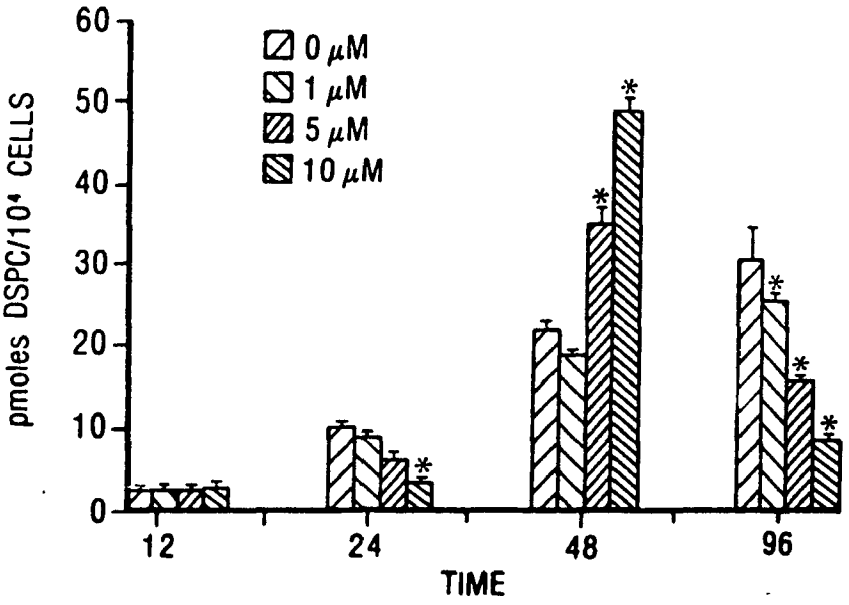

Figure 11: Effect of isoxsuprine on the incorporation of [^{3}H]choline into intracellular DSPC. Type II alveolar cells were exposed to conditioned medium for 24 hours, the medium was removed and fresh medium containing the indicated concentrations of isoxsuprine was added. At the appropriate times (12-96 hours) the medium was collected and the cells washed and removed. DSPC was extracted and radioactivity measured. Each point represents the mean ± SEM of replicates (generally six) from two preparations run concurrently. Asterisk (*) indicates significantly different ($p<0.05$) from corresponding control values as determined by Duncan's multiple range test.

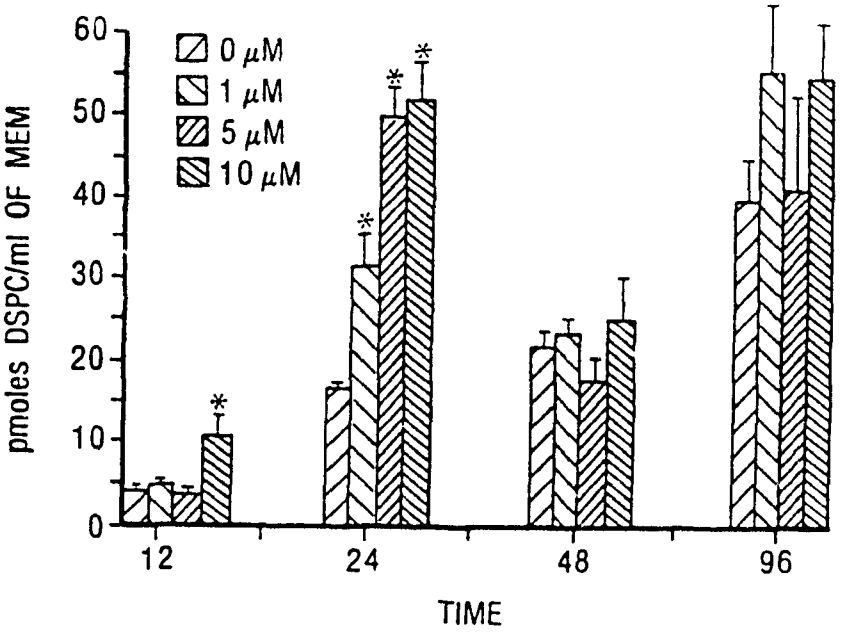

Figure 12: Effect of isoxsuprine on secretion of radio labeled DSPC by type II alveolar cells. DSPC was isolated from the culture medium and the radioactivity determined. Asterisk (*) indicates significantly different ($p<0.05$) from the corresponding control values as determined by Duncan's multiple range test.

dose dependent and inversely related to the level of labeling of the intracellular DSPC. After 48 hours of exposure a decrease in the labelled DSPC secreted into the culture medium was observed as compared to the labelled DSPC present at 24 hours after exposure to all three isoxsuprine concentrations. The level of secreted radioactive DSPC into the culture media increased after 96 hours.

Conclusion

Thus in conclusion isolated alveolar type II cell exposed to isoxsuprine respond by stimulating synthesis and secretion of radiolabelled DSPC and this response appears to be phasic in nature.

DISCUSSION

Although progress continues to be made in the development of treatments for RDS and the survival rate of preterm newborns is steadily increasing, the incidence of RDS remains high. In an attempt to reduce the number of newborns potentially exposed to this risk, glucocorticoids continue to be routinely used as a means of accelerating fetal lung maturity. There is, however, documented evidence of the limitation of their effectiveness (Crowley, 1989; Liggins, 1989) and hence their confinement to use only in selected cases of preterm labor. These problems have led researchers to explore and evaluate the usefulness of other agents which could be used alone or in combination with glucocorticoids to further decrease the incidence of RDS.

The specific goal of this thesis was to examine the effects of glucocorticoids given in combination with other agents such as ß-adrenergic agonists (isoxsuprine) and TRH, on the size of the intracellular and extracellular surfactant pools in the fetal rabbit lung. We were interested in determining whether either isoxsuprine or TRH was capable of potentiating the efficacy of glucocorticoids on accelerating fetal lung maturation and also the effects these agents, when given individually, had on the size of the surfactant pools in the premature lung.

Our findings have shown that in the fetal rabbit : 1) treatment with cortisol on the 24th gestational day is effective in increasing

surfactant synthesis and secretion in the 27th gestational day fetus; 2) a combination of cortisol and isoxsuprine, administered either simultaneously or sequentially, produced no further increase in either the intracellular or extracellular pools beyond the effect observed with cortisol alone; 3) short term exposure to isoxsuprine appeared to have an inhibitory effect on fetal surfactant secretion and this inhibition appeared to be dependent on the gestational age of the fetus or newborn and the presence or absence of breathing; 4) in vitro, isoxsuprine stimulated synthesis and secretion of radiolabelled DPPC by isolated alveolar type II cells; 5) TRH, whether administered alone in single or multiple doses, increased the intracellular pool, but had no effect on the extracellular surfactant pool; 6) all combinations of cortisol and TRH resulted in an increased response in the intracellular surfactant pool size over either drug given alone and the maximal response produced by their combination was observed with multiple TRH doses.

I. METHODOLOGICAL CONSIDERATIONS

A. In Vivo methodology

1. Intracellular surfactant fraction

The basic methodology described in this thesis for the sequential isolation of surfactant from the alveolar lavage returns and post-

lavage lung tissue has been extensively evaluated in the 29th-31st gestational day fetal rabbit by our laboratory (Oulton et al., 1986; Oulton and Dolphin, 1988). Oulton et al., (1986) observed that no surfactant is present in the alveolus and very little is present in the tissues at 26 days gestation and therefore this technique could not be feasibly used to isolate any surfactant at this early gestational age. On the 27th gestational day, however, recognizable lamellar bodies could be isolated by this technique. Nevertheless, as the surfactant pools are relatively small at this gestational age, compared to later in gestation (Oulton et al., 1986) we were concerned as to whether our isolation technique would provide us with enough, and sufficiently pure material, to accurately assess surfactant pool size at this gestational age.

The extent of homogenization of the lung tissue could potentially affect lamellar body yield. Under-homogenization could result in failure to disrupt the cell membranes of the alveolar type II cells whereas over-homogenization could result in rupture of the lamellar bodies themselves. We previously examined the homogenate resulting from homogenizing the fetal lung tissue for 20 seconds (in 10 second bursts) by electron microscopy and found no evidence of unbroken alveolar type II cells or of ruptured or broken lamellar bodies (Oulton - unpublished). While we did not examine our homogenate by EM, we did find consistency in the yield (phospholipid content) of the isolated surfactant fraction with the

same 20 second exposure. Thus the homogenizing time of 20 seconds was used in this thesis work.

Density gradient centrifugation of our crude intracellular surfactant fraction (10,000 xg pellet) has been previously examined in 31 gestational day fetal rabbits by Oulton et al. (1986). This fraction was subjected to density gradient centrifugation by both upward flotation according to Duck-Chong (1978) and downward sedimentation, according to Frosolono et al., (1970) through linear sucrose gradients ranging from 0.9 to 0.2M sucrose. Under both conditions the intracellular surfactant material was recovered as a single distinct band in the region corresponding to 0.4M sucrose (density - 1.055g/ml) (Oulton et al., 1986). Therefore, in the present studies the crude surfactant fraction (10,000 xg pellet) was layered over a discontinuous density gradient composed of 0.68M and 0.25M sucrose.

Our preliminary experiments demonstrated the necessity of using a second density gradient step to enhance the purity of the intracellular surfactant fraction obtained from the lung tissue of 27 gestational day fetal rabbits. Our data suggest that approximately 75% of the banded material obtained after the first gradient was of non-surfactant origin and that this material was effectively removed by the second sucrose gradient. Thus the phospholipid composition of the final surfactant fraction was found to closely approximate the actual phospholipid profile of surfactant material obtained at this gestational age by others (Oulton et al., 1986).

At 28 days gestation the banded material obtained following the first gradient was only 15% contaminated with non-surfactant phospholipids. Though this contamination was removed by the second gradient, this additional gradient also resulted in considerable loss (approximately 20%) of the surfactant material. We therefore decided to use only one sucrose gradient to isolate the surfactant at this and later gestational ages (i.e. 30th gestational day). The phospholipid composition of the intracellular surfactant fractions obtained by these techniques at each of the gestational ages examined are in agreement with those reported previously by our laboratory (Oulton et al., 1986) and by others (Sanders, 1982; Hallman and Gluck, 1980).

In previous work in our laboratory (Oulton et al., 1986) electron microscopy and marker enzyme analyses were used to assess the purity of surfactant fractions isolated from the 28th gestational day fetus and adult rabbit. In the present study the electron micrographs of the intracellular surfactant fraction obtained from the 27th gestational day fetal lung were similar to those observed by Oulton et al. (1986) in the 28th gestational day fetal lung tissue in that the preparations consisted mainly of well-preserved lamellar bodies. Extraneous material which was visible following the first gradient was largely removed by the second gradient. Thus on the basis of electron microscopy, the amount of contamination by membranous material from other subcellular sources was considered to be relatively minor as compared with the number of well preserved

organelles. This finding was supported by our marker enzyme studies.

From the foregoing, it is concluded that the methods that we have developed for the isolation of the intracellular surfactant fraction in the 27th and 28th gestational day fetuses are highly suitable for studies involving quantitative aspects of the developing surfactant system in that they yield fractions of acceptably high purity, and of consistent and reproducible yield.

2. Extracellular surfactant fraction.

The lavage technique which we used to collect the extracellular surfactant fraction does not seem to damage cells. This conclusion was based on electron micrographs (Oulton et al., 1986) as well as ultrastructural studies conducted by others (Nicholas and Barr, 1981; Oyarzun and Clements, 1977). The electron micrographs of fetal (31 days gestation) alveolar lavage pellet showed the presence of mainly multilamellated structures and low levels of lactate dehydrogenase, a cytoplasmic enzyme, in the return material (Oulton et al., 1986). Also, unlike adult rabbits (Kurland et al., 1988; Hayakawa et al., 1990) and newborns (at least 24 hours of age) there were no alveolar macrophages or other cells present in this pellet fraction. Furthermore while the 10,000 xg supernatant obtained from newborn and adult lavage contains a variety of sub-types of alveolar surfactant (Stevens et al., 1987; Spain et al., 1987; Bruni et al., 1988)

the lavage supernatant obtained from fetus and early neonate, has been shown to contain phospholipids of non-surfactant origin (Oulton et al., 1986). This is particularly so for earlier gestation fetuses (Oulton et al., 1986) such as those examined in this study. Therefore, for the purpose of the present study, the 10,000 xg pellet is the most precise representation of the extracellular (alveolar) surfactant pool.

B. Rabbit model

Many investigators have used the 28th gestational day rabbit to study pre-term pulmonary adaptation. Fetuses at this gestational age are large enough to allow tracheotomy and alveolar lavage but, more importantly, intracellular and extracellular surfactant pools are easily measurable at this time (Oulton et al., 1986).

In a previous study in our laboratory (Yoon, 1985) in which the fetuses were examined on the 28th gestational day, it was found that prior treatment with glucocorticoids on the 24th gestational day accelerated fetal lung maturation by one day (as assessed by increases in both the intracellular and extracellular pool size). To determine if we could enhance this accelerating effect of glucocorticoids, we delivered the fetuses one day earlier, i.e. on the 27th gestational day. To our benefit we found that not only were the fetuses at this early age large enough to allow tracheotomy, thereby eliminating technical problems related to alveolar lavage, but also that we were able to isolate measurable quantities of surfactant from

both lung compartments. The development of this new and valuable model has therefore enabled us to examine the possible synergistic effects of glucocorticoids in combination with other agents on fetal lung maturation.

II. GLUCOCORTICOIDS AND FETAL LUNG MATURATION

Glucocorticoids have been the focus of many investigations of RDS and its prevention because of their ability to accelerate fetal lung maturation (Oulton et al., 1988; Rider et al; 1990) and the maturation of many other tissues (Lee et al., 1991). In vivo and in vitro experimental evidence has demonstrated that glucocorticoids accelerate 1) the biochemical (Torday et al., 1975; Oulton et al., 1989); 2) the physiological or structural (Seidner et al., 1988; Elkady and Jobe; 1987; Rider et al., 1990); and 3) the morphological (Kikkawa et al., 1971; Scott et al., 1983; 1986; Snyder et al., 1992) development of the fetal lung.

The objective of our experiments was first to determine the effects of glucocorticoids on the surfactant pool size when administered to fetal rabbits on the 24th gestational day and delivered 72 hours later on the 27th gestational day and second to determine if these effects could be enhanced through the combined administration of these steroids with other agents such as ß-adrenergic agonists or TRH.

Experimental evidence showing the effects of glucocorticoids on surfactant pool size has been very controversial. This controversy can be explained in part by the different gestational ages at which treatment was given and in part by the various end points that were used for assessing surfactant pool size. In the rabbit, for example, glucocorticoids had very little effect on either intra- and extracellular surfactant pool size when administered after the 25th gestational day (Motoyama et al., 1971; Beck et al., 1981; Elkady and Jobe, 1987; Ikegami et al., 1989a; 1989b). In fact, a comprehensive study by Yoon, (1985) found that there was a small window in time in which the fetal lung responded to glucocorticoids by increasing the surfactant pool size (Yoon, 1985). This represents the time at or during which the alveolar epithelium of the fetal rabbit lung begins to differentiate and start producing surfactant (Kikkawa et al., 1971). It is also the time during which the circulating levels of endogenous cortisol reach a peak in the fetal rabbit (Barr et al., 1980). The data of Yoon suggest that the effect of glucocorticoids on surfactant production (at least with respect to the phospholipid constituents) may be to initiate the process and that they may have no effect on modulating the process initiated. This concept is supported by <u>in vitro</u> studies done with both fetal rabbits (Mendelson et al., 1982) and human fetal lung tissue (Smith et al., 1973; 1974).

In the present study, glucocorticoids were administered on the 24th gestational day (one day earlier than in Yoon's study) and there was still evidence of accelerated maturation. This provides further

support for the concept of glucocorticoid regulation in the initiation of the process of surfactant production.

With respect to the other point of controversy, namely the end point used for assessing surfactant pool size, many investigators have measured total lavage DSPC as an index of alveolar pool size and total lung tissue DSPC as a measure of the intracellular pool, given that DSPC is the major constituent of the surfactant complex. While this is not so problematic for the alveolar pool, as few constituents other than surfactant are present in this pool (Oulton and Dolphin, 1988), it does represent a problem for assessing the tissue stores. Several investigators (Young et al., 1981; Engle et al., 1980; Oulton and Dolphin, 1988) have shown that DSPC, while enriched in the lamellar body pool, is localized not specifically in this subcellular organelle but is present in all other subcellular fractions as well as plasma membranes and the cytosolic fraction. It has also been shown to be present in fetal lung tissue even before lamellar body assembly is initiated. Clearly, measurement of this constituent in whole lung tissue does not provide a very accurate index of the actual lamellar body pool size and this may account for failure by some investigators to observe an effect of glucocorticoids on this measurement even when given at an optimum time in gestation (Ikegami et al., 1989). To circumvent this problem, Oulton et al. (1986) and Yoon (1985) used procedures for isolating the lamellar body fraction and direct analysis of its phospholipid content and composition. This allowed for a more sensitive assessment of the lamellar body fraction. Using this

same technique, our present results indicate that glucocorticoid treatment increases surfactant pool size but only when administered on the 24th or 25th gestational day in agreement with previous work by Yoon (1985).

While it is apparent that glucocorticoids have no effect on surfactant pools, at least with respect to the phospholipid constituent, when given during late gestation there is mounting evidence to suggest that they may affect some other aspect of lung maturation, such as structural development (Beck et al., 1981; Schenberg et al., 1987; Snyder et al., 1992). In fact, a recent study by Yeh and co-workers (1990) has shown that, even in the newborn, exogenous glucocorticoids were very effective at promoting alveolarization.

Recent studies also suggest that glucocorticoids may play an important regulatory role in the production of the various surfactant-specific proteins, at least SP-A, SP-B, and SP-C (O'Reilly et al., 1989; Boggaram et al., 1991; Snyder et al., 1992). It appears that the developmental profiles for the accumulation of these proteins, as well as their response to glucocorticoids (Mendelson and Boggaram, 1991), are different from one another (Liley et al., 1989; Hawgood and Shiffer, 1991). Although there have been no studies to date correlating the response of the phospholipid and protein constituents of lamellar bodies to glucocorticoids, it has nevertheless been shown that the effect of glucocorticoids on the production of the surfactant specific proteins is also dependent on the gestational age at the time of treatment (Liley et al., 1989).

The action of glucocorticoids on surfactant synthesis remains unknown but it has been suggested that these steroids increase the production of DPPC by altering the activity and/or synthesis of specific proteins involved in the de novo synthesis of this phospholipid. Glucocorticoids have been shown to increase the production of the enzyme fatty acid synthetase, a key enzyme involved in fatty acid synthesis (Gonzales et al., 1990; Xu et al., 1990) and the activity of choline-phosphate cytidylyltransferase (Rooney et al., 1990), the rate-limiting enzyme involved in PC production in the alveolar type II cells (Rooney, 1985). Since this latter enzyme has recently been purified (Rooney et al., 1990), further insight into the mechanisms involved in this regulation should be forth-coming.

Smith (1978) has provided considerable evidence that suggests that glucocorticoids may not act directly on the surfactant-producing alveolar type II cells but rather at the level of the interstitial fibroblast. These cells produce a low molecular weight peptide termed fibroblast-pneumocyte factor (FPF). FPF augments DPPC production by increasing the activity of the rate-limiting enzyme, CTP: cholinephosphate cytidylyltransferase (Post et al., 1986). Smith and Sabrey (1983) have also demonstrated that FPF is produced in fibroblasts during the same critical period in fetal development in which the lung is responsive to glucocorticoids. With regard to the surfactant proteins, glucocorticoids are thought to exert their effect at the level of transcription, though some post-translational

modification has been suggested for SP-A (Floros et al., 1989; Whitsett et al., 1987; Odom et al., 1988; Liley et al., 1989).

In addition to their effect on acceleration of lung maturation, glucocorticoids have been shown to inhibit cell growth in many organs, especially during the period of rapid cell proliferation (Adam, 1968; Snyder et al., 1992). In the first set of experiments in the present study in which cortisol was administered to the fetus we observed a significant decrease in body and lung weight per fetus in the cortisol-treated fetal rabbits compared to the untreated and saline-treated fetus. The 24th gestational day fetal lung undergoes several morphological changes as it matures and develops prior to birth; this is the period in gestation of the rabbit fetus in which the alveolar type II cells differentiate (Kikkawa et al., 1971). Glucocorticoids have been shown to impede growth in the fetal lung by retarding cellular mitosis and significantly diminishing cell number, thereby decreasing fetal lung weight (Carson et al., 1973; Ikegami et al., 1989; Rider et al., 1990). A recent study by Fussell and Kelly (1991) demonstrated that the decreased protein synthesis which they observed in young rat pups exposed to dexamethasone could be attributed to a loss of polyribosomes and an elevated ribosomal monomer pool, indicating the blockage of translation at the site of peptide-chain initiation. Whatever the mechanism for glucocorticoid inhibition of lung growth, it is presently unknown whether or not it can be reversed.

The runting effect of cortisol on fetal lung and body weight was suggested in the present experiments in which glucocorticoids were administered in combination with TRH but this decrease was found not to be statistically significant when compared to the untreated controls in this study. Significance would likely have been reached with a larger sample size, as was observed in the first series of studies with isoxsuprine (Table 13) as well as in previous studies in our laboratory (Oulton et al., 1988).

Summary

In summary, glucocorticoids have been shown to significantly increase ($p<0.05$) the intracellular and extracellular surfactant pools in 27th gestational day fetal rabbits when these steroids are administered on the 24th gestational day to the fetus, thus confirming e. .er studies by Yoon (1985) that showed that surfactant synthesis and secretion can be induced by the administration of glucocorticoids at this early stage of development. The actual mechanism involved in surfactant production remains unknown but possibly glucocorticoids are altering the synthesis or activity of specific proteins involved in fetal lung surfactant production such as fibroblast pneumocycte factor (Floros et al., 1985), fatty acid synthetase (Xu et al., 1990; Gonzales et al., 1990), and choline-phosphate cytidylyltrasnferase (Rooney et al., 1990)

and/or by interacting with tl.e surfactant specific proteins (Floros et al., 1989).

III. ISOXSUPRINE AND FETAL LUNG MATURATION

Several previous studies have suggested a possible role for ß-adrenergic agonists in fetal lung maturation (for review see Chander and Fisher, 1990). Isoxsuprine and other ß-adrenergic agents have been shown to stimulate surfactant release by the fetal lung (Bergman et al., 1981; Brown and Longmore; 1981; Corbet et al., 1983; Ekelund et al., 1981; Chander, 1989). The present studies employed both an in vivo and an in vitro model system to extend these investigations and to determine possible developmental influences in fetal rabbits. Specifically, the goal of the present studies was to determine the effect of these agents on the intracellular and extracellular surfactant pools when administered alone and in combination with glucocorticoids. The latter are thought to increase the number of ß-adrenergic receptors by inducing one or more of the proteins that make up the receptor complex in the alveolar type II cells (Cheng et al., 1980; Roberts et al., 1985). Therefore it was of interest to determine whether prior exposure to glucocorticoids would enhance the efficacy of ß-adrenergic agonists on the maturation of the fetal rabbit lung at 27 days gestation.

A. In vivo experiments

1. Isoxsuprine alone

The results of our in vivo experiments, in which fetal rabbits were injected with a ß-adrenergic agonist alone, indicate that this treatment does not significantly alter the rate of synthesis or secretion of surfactant in the 27th gestational day fetal rabbit. This is in agreement with a previous report which found no significant effect on surfactant synthesis or secretion with the administration of the ß-adrenergic agonist ritodrine to fetal rabbits (Hallman et al, 1985). In contrast, others have reported that ß-adrenergic agonists increase surfactant secretion in fetal rabbits of comparable age (Wyszogrodski et al., 1974; Enhorning et al., 1979). These conflicting results may be attributable to differences in treatment protocols (i.e. length of time the fetus was exposed to the agonist and age of the fetus at the time of treatment). For example, in the experiments performed by Wyszogrodski et al. (1974) the fetuses were directly injected with isoxsuprine and were delivered within three hours of treatment with the agonist. Kanajanapone et al., (1980) delivered fetal rabbits on the 26th, 28th and 30th gestational day after treating the doe 24 hours prior to delivery with isoxsuprine. In contrast, in our studies delivery took place on the 27th gestational day and isoxsuprine was administered to the fetus on the 24th or 26th gestational day. In addition, it should be noted that in the

earlier in vitro study of Kanajanapone and co-workers (1980) which showed that isoxsuprine produced an increase in choline incorporation into DPPC in fetal rabbit lung slices, the conclusions were based on changes in total lung disaturated PC levels. Our laboratory has shown that these levels are an inaccurate measure of surfactant because phosphatidylcholine is found in membranes other than those comprising the surfactant complex (Oulton and Dolphin., 1988). Using our in vivo rabbit model in which we are able to more accurately measure the actual changes in the distinct surfactant pools, we were unable to substantiate these findings.

It has been reported previously that the injection of saline causes a significant increase in the intracellular surfactant pools above the untreated fetuses (Yoon, 1985). One of the most interesting and perplexing findings of the present study was that the administration of isoxsuprine to the fetus on the 24th gestational day eliminated the saline-induced response. This suggests that isoxsuprine treatment at an early stage of gestation, i.e. during cytodifferentiation of the alveolar type II cells (Kikkawa et al., 1971), inhibits the subsequent endogenous hormonal stimulation of phospholipid synthesis (Yoon, 1985; Oulton et al., 1989).

2. Isoxsuprine and cortisol

Our studies of cortisol and isoxsuprine administered in combination to the fetal rabbit demonstrated an increase in both the

intracellular and extracellular surfactant pools. However, this effect was not greater than that observed with cortisol alone and indicates that the efficacy of glucocorticoids on fetal lung maturation was not increased with the administration of the ß-adrenergic agonist, isoxsuprine. In conflict with our results, Ekelund and Enhorning (1985) observed an increase in fetal lung compliance after the combined administration of glucocorticoids and a ß-adrenergic agonist in 27 day gestational fetal rabbits. Similarly in fetal lamb lung, Warburton et al. (1988) observed a significant increase in the saturated PC content of whole fetal lung after the combined treatment of cortisol and ritodrine compared to the untreated controls. As mentioned previously, measurement of total SPC levels in whole lung tissue does not provide a very accurate index of the actual lamellar body pool size and this may account for the conflicting results. We therefore speculate that although we did not observe any increase in surfactant pool size due to the combined treatment of these agents, glucocorticoids may actually induce components of the ß-adrenergic signal transduction system (see Section B.) if the ß-adrenergic system is mature enough to respond to such stimulation (Ekelund and Enhorning 1985). Thus, the time of treatment of these agents appears to be critical in determining their effect on fetal lung maturation.

3. Isoxsuprine - effects on fetal body and lung weight.

The results of our isoxsuprine studies are at odds with some previous observations but are in agreement with others. The work of Enhorning and co-workers (1977; 1979) as well as that from other laboratories (Saumon et al., 1989; Perks et al., 1990; Chapman et al., 1991) have shown that ß-adrenergic agonists reduce pulmonary fluid causing dehydration within the lung tissue. We observed only minor changes in fetal lung weight after isoxsuprine treatment, an effect which could be interpreted as lung fluid loss. Similar findings were observed by Wyszogrodsk et al. (1974) who administered isoxsuprine IM to the fetus three hours prior to delivery on the 28th gestational day. Other experiments exploring the effects of the ß-adrenergic agonist terbutaline on fetal lung maturation in 28th gestational day fetuses showed no significant effects on fetal body and lung weight (Ekelund and Enhorning, 1977). McDonald and co workers (1986) have also found that pulmonary fluid levels were not altered in fetuses treated directly with bromacetylal-preonolomenthane, an irreversible ß-blocker.

Taken together, previously published reports and results in the present work indicate that the ß-adrenergic agent, isoxsuprine, administered in combination with glucocorticoids, has no significant effect on fetal body and lung weight above the glucocorticoid inhibition of mitotic division during cytodifferentiation of the alveolar type II cells (on the 24th gestational day). However the

growth retardation effects of glucocorticoids alone appear to be abolished when the glucocorticoid treatment is followed by the ß-adrenergic agent later in gestation (on the 26th gestational day). Our findings differ from those of Ekelund and Enhorning (1985) who observed a potentiation of the growth retardation effects of glucocorticoids when administered with the ß-agonist, terbutaline. Again, differences in the gestational age of the fetus at the time of treatment as well as the ß-agonist used in these experiments may be the reason for the conflicting results. Experiments have also shown that the effects of ß-adrenergic agonists on lung liquid reabsorption are gestation-dependent with a greater effect noted toward term as the concentration of ß-receptors increases (McDonald et al., 1986; Barker et al., 1988; Perks et al., 1990; Walters et al., 1990; Chapman et al., 1991). Thus, isoxsuprine appears to have no significant effect on fetal body or lung weight when administered alone but is capable of either abolishing or enhancing the growth retardation properties of glucocorticoids depending on the exposure time between these two agents and the gestational age of the fetus at the time of treatment.

4. Isoxsuprine - Short term effects of isoxsuprine in the fetus versus the neonate.

Breathing has been shown to stimulate the release of surfactant from the alveolar type II cells into the alveolus (Faridy, 1976; Oyarzun and Clements, 1977; Brown and Longmore, 1981). The

stimulus for this release is thought to be the deformation of the lateral or basilar membranes. This deformation causes an increase in the Ca^{+2} permeability which in turn may initiate a cAMP-stimulated release of surfactant from the alveolar type II cells (Brown and Longmore, 1981). Our studies indicate that breathing does indeed augment the airway surfactant pool size in untreated animals on both the 28th and 30th gestational day. No corresponding pattern was observed in the size of the intracellular surfactant pool.

The effect of isoxsuprine in the fetus versus the neonate appeared to be dependent on both the gestational stage and breathing status of the animal. On the 28th gestational day, this ß-agonist augmented airway phospholipid, but once the animal was allowed to breathe, the ß-adrenergic agonist inhibited the breathing-induced increase in the surfactant pool size. The reason for this apparent inhibition in the level of extracellular surfactant in these fetuses and newborns is not readily apparent. However, the observation that inflation-induced surfactant release is mediated by a different mechanism and may not be directly under ß-adrenergic agonist control suggests that several mechanisms may be involved at different levels in the regulation of surfactant secretion. In contrast to the ß-adrenergic effect observed on the 28th day, in 30 day gestation fetuses isoxsuprine again depressed the airway pool sizes, but only in those animals that had not breathed. Furthermore, this depression of the airway surfactant correlated with high PI and low PG levels in the intracellular pool. Since anionic phospholipid synthesis switches

during the prenatal period from PI to PG and the latter phospholipid has been found to be a useful clinical predictor of fetal lung maturity, these results suggest that ß-adrenergic administration without concomitant breathing delays lung maturation. Breathing appeared to overcome this inhibition.

A possible explanation for this apparent inhibition in the 28-day gestational fetus allowed to breathe and the 30-day gestational fetus left in utero for 30 minutes might be a lack of development in any of the steps involved in a receptor-mediated response. Several lines of evidence indicate that during fetal development some tissues may not demonstrate adrenergic responses until maturation of the regulatory components of the adrenergic cascade which begins with the ß-adrenergic receptor (Maier et al, 1989; Lee et al., 1989). Most previously published reports suggest that this maturation occurs late in gestation (Corbet et al., 1985). Lung noradrenaline levels, possibly reflecting sympathetic innervation, increase gradually in the rabbit from 27 days gestation to 8 days post term (Padbury et al., 1981). Concurrently, there is a significant and progressive increase in the concentration of ß-adrenergic receptors in fetal rat, rabbit and human lung (Cheng et al., 1980; Giannopoulos, 1980; Padbury et al., 1981; Whitsett et al., 1981; 1982; Duffey et al., 1992). These receptors are first detected at 22 days gestation in the rabbit (Giannopoulos, 1980), but the concentration does not change until 26 days gestation with a threefold increase occurring by 29 days gestation. The concentration continues to increase until at least 35

days postterm in the rabbit. Therefore, although receptors can be detected at 30 days gestation, it is possible that the concentration is still too low to produce full ß-adrenergic sensitivity.

ß-adrenergic receptors stimulate the enzyme adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine 3',5'-monophosphate (cAMP). In the lung, the physiologic effects of ß-adrenergic agonists have been linked to increases in intracellular production of cAMP by activation of adenylate cyclase in alveolar type II cells and lung fibroblasts (McDonald et al., 1986; Davis et al., 1987; Skinner et al., 1989; Chapman et al., 1991). Although both ß-adrenergic receptors and adenylate cyclase are present in fetal rabbit lung early in gestation, their mere presence is not sufficient to produce a full ß-adrenergic response. There are important developmental alterations in the interaction of these components. For example, a necessary step is the formation of a high-affinity agonist-receptor complex which then acts together with other maturational events to promote increased ß-adrenergic sensitivity (Chapman et al., 1991; Griese et al., 1991b; 1992). Therefore the lack of fully mature ß-receptor interactions may account for the observed variability in response to isoxsuprine at various gestational ages and under different experimental conditions.

Another receptor mechanism which might explain our observed changes is the short term redistribution of receptors as part of the desensitization process. Prolonged or repetitive stimulation of ß-

adrenergic receptors by an agonist results in a subsequent reduction in the sensitivity of the tissue, a phenomenon known as desensitization, refractoriness, or tolerance (Deighton et al., 1988; Scarpace, 1988; Taki et al., 1988). This involves a sequestration or internalization of the receptors away from the plasma membrane. Falkay et al. (1988) has demonstrated using the ß-agonist fenoterol in pregnant rabbits that this agent promotes a "down-regulation" or a decrease in density of fetal lung ß-adrenoreceptors and concluded that desensitization of fetal pulmonary adrenoreceptors may result in diminished lung function after long term treatment with these agonists. Thus, isoxsuprine could be causing a desensitization of the ß-receptors depending on the gestation of the fetus at the time of treatment and its breathing status.

Any change in surfactant measurement must be interpreted with the understanding that alterations in synthesis, secretion, recycling and clearance or degradation all contribute in a complicated manner to the resulting alveolar pool (Steven et al., 1989; Wright and Clements, 1987; Griese et al., 1991a). Although the ratio of extracellular surfactant to intracellular surfactant appears to be decreased following isoxsuprine, one must also speculate whether this change is a result of decreased secretion of surfactant or an actual increase in the re-uptake of surfactant by the alveolar type II cells which exceeds surfactant secretion (Stevens et al., 1989). That is, in the 28th gestational day fetus that had been allowed to breathe for 30 minutes as well as the 30th gestational fetus left <u>in utero</u> for

30 minutes isoxsuprine may stimulate the process of re-uptake so that it exceeds secretion.

B. Isoxsuprine - In Vitro experiments.

Scott and co-workers (1983) have developed a method for preparing a homogenous population of undifferentiated cells from the fetal rabbit lung. These alveolar type II cells are capable of replicating in vitro in a hormone-stripped serum supplemented medium but do not differentiate until exposed to glucocorticoid hormones. Since the type II cells used in the present study were isolated prior to either the in vivo intracellular increase in total lung cell disaturated phospholipid (Scott et al., 1987) or morphological maturation of the type II cells (Kikkawa et al., 1967), we felt that a differentiation-inducing stimulus would be required before the potential for any secretory response could be examined. Presumably this stimulus would elevate DSPC synthesis. Indeed we have shown that conditioned medium which contains FPF (Scott et al., 1985), does exactly this.

The results of the present in vitro study provide support for the concept that the state of lung maturation is critical in determining the response to ß-adrenergic agents. In a single preliminary study, simply treating the cells with isoxsuprine did not seem to influence secretion of labeled DSPC, although DSPC was still present in the medium suggesting perhaps a basal secretory level. Rather, the cells

isolated on the 24th gestational day required prior exposure to the conditioned medium in order to respond to the ß-adrenergic agonist. Therefore, it would appear that, at least in vitro, induction of maturation is a prerequisite for ß-adrenergic mediated stimulation of secretion. Whether or not the medium from rabbit fetal lung fibroblasts contains an FPF-like factor which can induce elevated ß-adrenergic receptor levels in the alveolar type II cells is not known. Further examination is clearly required to establish the interrelationship between maturity and secretory potential of the type II alveolar cells.

Following isoxsuprine treatment of the cells after 24 hours in culture, the level of DSPC in the cells decreased in a dose-dependent fashion while labeled DSPC in the culture medium increased in a complementary manner. However, 48 hours after initial exposure to the ß-agonist, the cells exhibited a phase of renewed synthesis. The rate of this process appeared to be greatest in those cells with the most marked depletion of their intracellular DSPC in response to the ß-adrenergic agonist. This apparent phasic activity of the cells in regard to DSPC synthesis is interesting in light of recent observations by Ekelund et al.(1983) that a single dose of the ß-adrenergic terbutaline to fetal rabbits initially (at 3 hours) induced an increase in the phospholipid content and an improvement in lung pressure-volume loops. However, 24 hours after treatment, both of these measures were reduced compared to controls. Furthermore, the low level of phospholipid which occurred following the initial elevation

was not entirely rectified even by 48 hours after treatment. This rebound phenomenon (Ekelund et al., 1983) following stimulation may explain our findings in the differentiating type II alveolar cells. It is unlikely that our observed effects are due to isoxsuprine remaining in the culture medium over such an extended period, since its biological half-life is probably in the range of only a few hours. Thus, the lack of effect or inhibition we observed in vitro could be due to a biphasic secretory process or a stimulation of secretion followed by a period of inhibition of secretion. Gilfillan and Hollingsworth (1980) suggested that the release of PC's from the intracellular stores into the extracellular pool is normally biphasic in nature.

Summary

In summary, the effects of ß-adrenergic agents on surfactant synthesis and secretion appear to be phasic in nature. Furthermore, these agents appear to have a stimulatory or inhibitory effect which may depend on the gestational age of the fetus. It is known that ß-adrenergic receptors, Gs and catalytic adenylate cyclase are present in the fetal rabbit lung early in gestation but this ß-receptor adenylate cyclase complex must act together with other maturational events at the time of treatment to increase the maturational process of surfactant synthesis and secretion in the fetal lung.

IV. THYROID HORMONE AND GLUCOCORTICOID REGULATION OF FETAL LUNG MATURATION.

Glucocorticoid therapy is successful in the prevention of RDS in the preterm infant only under ideal conditions and therefore combined hormonal therapies have been introduced as a possible means of achieving an optimal response. Thyroid hormones have been investigated by several researchers as possible regulators of surfactant production in the fetal as well as the adult lung and shown to be important in the normal pulmonary maturation process (Douglas et al., 1970; Redding et al., 1971; Wu et al., 1971; Ballard et al., 1984b). Additive or synergistic effects of thyroid hormone plus glucocorticoids have also been investigated and shown to lead to an enhancement of biochemical, morphological and structural changes during fetal lung maturation (Rooney et al., 1979; Devaskar et al., 1987b; Ikegami et al., 1987; Liggins et al., 1988). Such combinations include cortisol plus triiodothyronine (T_3) and prolactin, cortisol plus thyrotropin releasing hormone (TRH) (which stimulates both T_3 and prolactin), and cortisol plus TRH plus either prolactin or a ß-agonist. However, despite these studies, there is still some uncertainty as to the effectiveness of this combined treatment for the prevention of RDS in the preterm infant (Ballard et al., 1990; Morales et al., 1989; Athabe et al., 1991; Tabor et al., 1990) indicating the necessity for further investigation into the effects of combined hormonal therapies on the lung maturation process.

In our studies we chose to use a combination of glucocorticoids and TRH and to administer TRH to the doe. This method of thyroid stimulation allowed us to avoid the risk associated with intraamniotic injections of thyroid hormones and limited maternal - fetal transport across the placenta (refer to Introduction, page 49). In addition, our method was preferable to direct injection of thyroid hormones to the fetus because it did not require a second maternal laparotomy. TRH has been shown to pass readily from the maternal to the fetal circulation in most species including the rabbit and cause thyroid axis stimulation (Kajihara et al., 1972; Doran et al., 1981; Rooney et al., 1986; Liggins et al., 1986) resulting in increased levels of TSH, T_3 and T_4 in the fetal circulation (Jacobson et al., 1977; Roti et al., 1981; Moya et al., 1986). Although TRH is partially degraded by the placenta, the fetal TSH response to maternal TRH administration is several times greater than the maternal TSH response (Azukizawa et al., 1976; Roti et al., 1981) and may indicate that the fetal pituitary is hyper-responsive to TRH (Roti et al., 1981).

Our data show that, unlike glucocorticoids, the effect of TRH on the intracellular surfactant pool size is not dependent on the gestational age of the fetal rabbit at the time of treatment. However, we have found that the effects of TRH on fetal lung maturation can be greatly enhanced by prior exposure to glucocorticoids. Similar observations have been reported by Gonzales et al. (1986) in human fetal lung explants in which they demonstrated a synergism between glucocorticoids and thyroid hormones that was dependent on prior

exposure to glucocorticoids. In the fetal lamb, direct infusion of T_3 (Schellenberg et al., 1988) or TRH (Liggins et al., 1988) along with cortisol resulted in a greater increase in PC synthesis and led to significant clinical response at a gestational age earlier than that at which cortisol alone has an effect. Maternal TRH administration in rabbits augments the beneficial effects of prenatal corticosteroids and exogenous surfactant on neonatal ventilatory function (Ikegami et al., 1987). In addition, an effect of T_3 in mixed fetal rat monolayer cultures was observed only when serum containing glucocorticoids was present (Smith and Torday, 1974). Although the mechanism of action of this additive effect is not clear, it is known that both actions are receptor-mediated and that the hormones probably act at different sites (Smith and Sabry, 1983). Our results agree with and also extend this theory by indicating that the two hormones act at different levels or stages of the maturational process. Considering that surfactant production begins on the 24th and 25th gestational day and continues towards term, glucocorticoids would seem to play a regulatory role in the initiation of this process but their effect is less important once this process is started. In contrast, TRH may have a reduced or negligible role in the initiation of this process but may serve as a modulator of on-going maturation.

In our study, it was interesting to note that while a response to TRH was observed at each gestational age examined, the magnitude of the response to a single treatment did not increase with gestational age and, in fact, only increased with multiple treatment

when the lung had been previously exposed to exogenous glucocorticoids. In conflict with our results, Tabor et al. (1990) found no dose-dependent effect on surfactant production with the administration of multiple doses of TRH in combination with glucocorticoids. However, it should be noted that they measured PC levels in total lung (lung homogenate plus alveolar wash) to assess the effects of the combined treatment. Our method of surfactant isolation and analysis permits not only direct assessment of the effect of these agents on surfactant phospholipids but also an evaluation of the difference between the effects on the intracellular and extracellular pools. Another important difference is that the glucocorticoid was administered at a later gestation (days 25 and 26) to the doe. Because the critical period of responsiveness of glucocorticoids to surfactant production is on the 24th gestational day (Yoon, 1985), the glucocorticoids may have been administered too late to produce an effect in these studies. This latter concept may also serve to explain why two other groups (Ikegami et al., 1987; Devaskar et al., 1987a) failed to demonstrate an additive response to a combination of glucocorticoids and TRH in their studies using fetal rabbits. These studies emphasize the importance of the early and prior treatment with glucocorticoids for maximizing the response of these hormones in combination with TRH.

Several possibilities exist for the mode of action of these hormones and the role of prior treatment with glucocorticoids in the expression of the TRH effect. Receptors specific for the thyroid

hormones, have been identified in the nucleus of the alveolar type II cells of rats (Morishige and Guernsey, 1978; Gonzales and Ballard, 1982), rabbits (Lindenburg et al., 1978) and human fetuses (Gonzales and Ballard, 1981). T3 receptors have been observed to increase in number and binding affinity as gestation increases from the 21st to the 28th gestational day in the fetal rabbit (Gonzales and Ballard, 1982) suggesting the possibility of an increased response to these hormones during the latter part of gestation. It is possible that glucocorticoids may enhance the responsiveness of the fetal lung to TRH by influencing this receptor maturation or occupancy in the alveolar type II cells or possibly by increasing the number of pituitary TRH receptors in the fetus (Tashjian et al., 1977; Tabor et al., 1990).

An alternative or additional mechanism might be via an effect of both hormones on the ß-adrenergic system. It has been shown that glucocorticoids (Man et al., 1979; Cheng et al., 1980; Barnes et al., 1984) and thyroid hormones (Whitsett et al., 1982; Das et al., 1984) up-regulate ß-adrenergic receptors in the lung. Therefore, the effect of prior treatment with glucocorticoids on receptor density may be synergistic with or greater than the effect of TRH alone. Catecholamines may than act through elevated levels of ß-adrenergic receptors to enhance surfactant production. In support of this hypothesis, recent studies have shown that cortisol and TRH increase the capacity of the fetal lamb to respond to ß-adrenergic stimulation (Warburton et al., 1988).

Glucocorticoids may also potentiate the effects of TRH in its role as an endogenous neurotransmitter. TRH is now known to be present in extra-hypothalamic locations where it appears to play a role in neurotransmission and stimulate autonomic nervous system activity (Jackson, 1982; Griffith, 1985). By acting as a neurotransmitter, TRH may act independently of the fetal pituitary-thyroid axis. This theory is supported by studies which have shown TRH - induced lung maturation in the absence of increased thyroid hormone concentrations after maternal TRH administration (Devaskar et al., 1987b; 1991; Ikegami et al., 1987; Morales et al., 1989).

Prior exposure to glucocorticoids may also be responsible for some unidentified non-receptor function which may increase the efficacy of thyroid hormones on fetal lung maturation. For example, glucocorticoids stimulate fibroblasts to release FPF (fibroblast pneumocycte factor) which subsequently binds to alveolar type II cells and increases intracellular levels of cAMP. T_3 seems to act directly on type II cells to enhance the responsiveness of these cells to FPF thereby potentiating the effects of glucocorticoids on surfactant production (Smith and Sabry, 1983; Viscardi et al., 1989).

Maternal TRH administration might also affect surfactant production through its ability to induce fetal pituitary prolactin secretion rather than thyroid gland stimulation alone. Clinical (Quirk et al., 1982; Parker et al., 1989) and animal (Rooney, 1985) studies have suggested that prolactin plays a role in surfactant production and fetal lung maturation. In fetal sheep, prolactin appears to be

permissive for glucocorticoid stimulation of surfactant synthesis but has no effect when administered alone (Ballard et al., 1978; Schellenberg et al., 1988). Other studies have shown both permissive and independent effects of prolactin on choline incorporation in cultured human lung (Mendelson et al., 1984) and in fetal rabbits in vivo (Hamosh and Hamosh, 1977). However, recent work by Devaskar et al. (1991), using a novel TRH analogue which enhances lung maturation while inhibiting prolactin, indicates that prolactin does not play a role in this accelerated pulmonary development. At the present time, the physiological role of prolactin in lung development is unclear.

TRH appears to be a relatively safe drug to administer prenatally to the fetus. In agreement with others (Ikegami et al., 1987) who used a similar dosage, we observed no signs of thyrotoxicosis in the fetus and no fetal deaths after the intravenous injection of TRH to the doe at the various gestational ages examined. This is in contrast to the high incidence of fetal death which was noted by Tabor et al. (1990) in rabbits receiving multiple doses of TRH. In Tabor's study, however, the high incidence of fetal death was probably due to the fact that the glucocorticoid was administered to the doe rather than the fetus (Yoon, 1985; Oulton et al., 1989).

TRH, unlike glucocorticoids, had no significant effect on fetal body or lung weight when compared to the untreated control fetuses. This is in agreement with some (Ikegami et al., 1987; Hitchcock, 1979; Elkady and Jobe, 1988) but not all (Liggins et al., 1988; Lohninger et

al., 1986) previous studies. TRH / cortisol combination treatment significantly reduced fetal body and lung weight only when administered together on the 24th gestational day. This suggests the possibility that an inhibition of mitotic division in favour of cellular differentiation may occur at this time.

In contrast to earlier studies by Rooney et al. (1979) and Liggins et al. (1988), we did not find any effect of TRH on surfactant secretion. The reason for this is not clear, but it is important to note that the TRH effect on secretion in those studies was much less than that observed on synthesis in the present studies. This suggests that the most important effect of treatment is an increase in the intracellular stores which will then be readily available for release to the alveoli after birth. The finding that a decrease in the incidence of RDS after maternal glucocorticoid treatment is not necessarily associated with an increased secretion of surfactant phospholipids into amniotic fluid (Farrell et al., 1983) is in keeping with this interpretation of our results.

Summary

The results of these studies clearly demonstrate the additive effects of glucocorticoids and TRH treatment on fetal lung development. Maximal response to either agent alone resulted in accelerated maturation of approximately one day, whereas, the combined treatment produced a two-day acceleration. This

advancement in fetal lung development appears to be very much dependent on the timing of the combined treatment.

CONCLUSION

The synthesis and secretion of surfactant in the fetal lung appears to be a very complex process under multihormonal control. Although it has been well established that glucocorticoids accelerate fetal lung maturation, there is a critical time period in which the fetal lung is most responsive to these agents. Thus, any change in the route of administration, length of time exposed to the exogenous agent, or the gestational age of the fetus at the time of treatment may result in a possible inhibition or stimulation of the surfactant system which may ultimately influence the incidence and severity of RDS in the premature infant.

When combined with other agents such as TRH, the efficacy of glucocorticoids is enhanced but again this effect appears to be gestation-dependent at least with regard to the glucocorticoid portion of the combined treatment. In the in vivo study, the efficacy of glucocorticoids was not increased when combined with a ß-adrenergic agonist. The ß-adrenergic effect on fetal lung maturation appears to be phasic in nature and therefore these agents may have beneficial effects at certain stages of development and deleterious effects at others. We must remember that although we may increase fetal lung maturation by using a combination of hormones for the prevention of RDS in the premature infant, possible deleterious effects, such as inhibition in lung growth may occur in non-surfactant aspects of fetal lung development. Further studies of the effects of

the combined treatment of glucocorticoids and other agents such as ß-adrenergic agonists and TRH on fetal lung maturation should be undertaken to fully evaluate the advantages as well as the possible risks of this treatment for the premature infant.

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