SOME BIOCHEMICAL ASPECTS OF POSTNATAL RAT LUNG GROWTH AND DEVELOPMENT

A Thesis

Presented to the Faculty of Graduate Studies,
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of the requirements for the degree of
Master of Science.

by

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To my parents

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ABSTRACT

Some biochemical aspects of postnatal lung growth and development in rats have been investigated. Protein content in lung increased steadily from 3 days before birth to about 2 weeks after birth but remained constant thereafter and correlated to the cessation of rapid tissue proliferation. Glycogen disappeared rapidly around birth, began to accumulate 5 days after birth but was depleted again after 1 month. Depletion of glycogen around birth occurred in epithelial type II cells presumably to provide precursors for surfactant(phospholipid) synthesis. The postnatal changes in lung glycogen occurred mainly in mesenchymal cells during the lung growth phase of rapid tissue proliferation, and may provide energy for rapid lung growth. Phospholipid composition changed between birth and 5 days postpartum but remained quite constant thereafter. Incorporation of $^{32}P_i$ into PC molecules was enhanced greatly around birth, without much effect on the labelling of other phospholipids. Enhancement of $^{32}P_i$ incorporation into PC molecules around birth correlated to the rapid depletion of glycogen in type II cells. This relationship between phospholipid and glycogen metabolism was less clear during postnatal development. The developmental changes in glycogen content appeared to be regulated by the cyclic AMP level which was dependent on the relative specific activities of adenylate cyclase and cyclic AMP-phosphodiesterase. Changes in cyclic AMP level also coincided with different phases of postnatal development of the lung. Cyclic AMP levels decreased between 5 days and 2 weeks of age when

cellular proliferation was active. After 2 weeks cyclic AMP level elevated when maturation of the interstitium occurred.

The postnatal rise in cyclic AMP level after 2 weeks of age was apparently due to the presence of a cytoplasmic protein modulator which enhanced basal adenylate cyclase activity. To elucidate the mechanism of this protein modulator action on adenylate cyclase, it was essential to isolate the protein modulator and a plasma membrane fraction rich in adenylate cyclase activity. A simple procedure to isolate a membrane fraction from rat lungs was described. The preparation was relatively free of nuclear, mitochondrial, lysosomal, and microsomal contaminations as determined by marker enzyme studies and DNA analysis. Adenylate cyclase activity in plasma membrane fractions obtained from older animals showed a loss in the enzyme activity when compared to similar fractions from young rats. This loss of activity was restored when cytosolic supernatant was added to the membrane fractions. It is concluded that adenylate cyclase is not a good marker for plasma membrane from lungs of older animals because of the presence of the cytosolic factor(s).

Neurotransmitters such as acetylcholine and catecholamines influence numerous lung functions including the secretion of surfactant. These neurotransmitters have been shown to enhance $^{32}P_i$ incorporation into acidic phospholipids (Phospholipid Effect) in a variety of other tissues. However, these neurotransmitters failed to elicit a phospholipid

effect in rat lungs in vitro, under the condition of these experiments. This failure of response was not due to the limiting concentration of myo-inositol and/or other cofactors.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. DEVELOPMENTAL STUDIES OF GLYCOGEN, PHOSPHOLIPIDS, AND CYCLIC AMP METABOLISMS

A. INTRODUCTION

Each organ or tissue of an animal has its own characteristic pattern of biochemical activities which is associated with its physiological functions and morphological features[1]. These biochemical activities of an organ alter with age as the morphology and physiology are being modified with growth and development[2]. Studies examining these biochemical alterations should provide fundamental information concerning the function of that organ and any characteristics that may be different from other tissues. The lung performs a vital function allowing gaseous exchange between circulating blood and inhaled air. Previous interest of biochemical events in the lung has mainly focused on the fetal and neonatal stages when the lung first experiences the mechanics of breathing and relatively few studies have been done on the postnatal development. Postnatal lung does not grow by simple expansion as some have believed in the past and it appears likely that most, if not all, of the lung units (alveoli) develop after birth, in many species[3]. It was therefore decided to examine the biochemical changes during postnatal rat lung growth and development particularly those in cyclic AMP which mediates the effects of many hormones.

B. LITERATURE REVIEW

1. Growth and development of the lung

The whole of the epithelial structure of the lung arises from the foregut. A groove forms in the anterior portion of the foregut early in development. The caudal end of the groove forms a small pouch. Immediately following the formation of this pouch, the foregut anterior to this elongates rapidly, separating the future stomach from the primary lung bud. The foregut anterior to the primary lung bud forms the primitive esophago-trachea which divides by the fusion of the lateral ridges. While the esophagus and trachea are separating, the lung bud divides into left and right secondary buds. These outgrowings of the endodermal cavity carry with them a mass of surrounding mesenchymal tissue, together termed the "lung buds" which divide to form the main bronchial pattern. The proliferating lung buds grow into the pleuro-peritoneal space and growth at this stage is extremely rapid. The cartilage, muscle, elastic tissue and lymphatic tissue of the lung are derived from these mesenchymal elements surrounding the

lung bud while the bronchi, alveolar membrane, and mucous glands are derived from the endoderm[4].

The intrauterine growth of the lung is usually divided into four stages[3,4]: (a) the embryonic period includes the earliest phase of lung development, (b) the pseudoglandular phase with the completion of bronchial development which gives the lung a distinctly glandular appearance with airways lined by columnar epithelium and separated from each other by poorly differentiated mesenchyme, (c) the canalicular phase is characterized by the proliferation of mesenchyme and the development of a rich blood supply within it, together with flattening of the epithelium that lines the airways, and (d) the terminal sac period when there is progressive thinning of epithelium and protrusion of capillaries leading to close approximation of capillary lumen to airway surface. Alveoli are not yet present in the walls of these terminal sacs although respiration can now be maintained. In the human, this phase commences at approximately the twenty-fourth to twenty-sixth week of pregnancy. In some species such as the rat and the mouse, birth occurs during this period.

Using rat as an example, the postnatal development of the lung can be divided into three phases[3,5]: (a) phase of lung expansion takes place from days one to four. During this period lung growth occurs by simple expansion and little lung tissue is added. At birth the

peripheral respiratory units are rather simple structures which are larger and different in shape from the adult lung alveoli. These are called the primary saccules and are lined by type I and type II alveolar epithelial cells; (b) phase of tissue proliferation lasts from the fourth postnatal day to approximately day thirteen. This phase is characterized by the subdivision of the primary saccule by secondary crests and by the formation of definitive alveoli. The rate of lung growth is relatively faster than that of the previous phase and there is a rapid increase in alveolar surface area; (c) phase of equilibrated growth begins at approximately two weeks of age and is characterized by a slowing in the increase of lung volume. New alveoli are added continuously although at a slower rate. The general features are of maturation of the interstitium. The rate of cellular multiplication is markedly diminished and the lung grows mainly by cellular enlargement, dilatation, and modification of pre-existing structures.

2. Pulmonary surfactant system

(i) Introduction--

The major functions of the lung are to provide the living organism with oxygen from the air and to remove excess carbon dioxide from the bloodstream. To perform these functions the lung contains a continuous layer of extremely flattened and distended squamous cells covering its alveolar surfaces with a very rich capillary bed underneath. These epithelial type I cells, together

with the capillary endothelium and the reticulin basement membranes form the blood-air barrier where effective gas exchange takes place. Between the squamous superficial cells at sporadic intervals are cuboidal shaped type II cells which contain lamellar bodies in their cytoplasm. These lamellar bodies are rich in surfactant, a surface active material which is mainly composed of phospholipids and small amounts of proteins and carbohydrates. Surfactant production is turned on in the fetus only in the later stage of prenatal development. For example, in the developing lamb lungs, the phospholipid concentration and the degree of saturation of phosphatidylcholine (main constituent of surfactant) were rather constant from day 60 to day 120 of gestation. Thereafter, significant increases of both parameters were noticed and these were accompanied by an increase in the lamellar bodies in the type II cells[125]. In mice, the appearance of lamellar bodies and surfactant occur around the 17th or 18th day of gestation[127]. Surfactant lowers the surface tension of alveoli, stabilizes the air spaces, and enables the lung to retain air at low inflational pressures, thus preventing alveolar collapse during expiration and greatly reducing the inspirational force required to expand the lungs[6]. Alveolar collapse occurs at birth in children with inadequate synthesis and secretion of surfactant, the syndrome known as hyaline membrane disease or respiratory distress syndrome which is the leading cause of neonatal death in developed countries.

As mentioned, the principle components of surfactant are phospholipids. They are associated with certain apoproteins and it has been postulated that the apoproteins may accelerate the extracellular transport of pulmonary surfactant to the alveolar interface and to insure that adequate amounts of pulmonary surfactant are available to the alveolar surface to prevent the collapse of alveoli during expiration[7].

(ii) Phospholipid composition, whole lung tissue versus surfactant--

The phospholipid composition of whole lung tissue has been examined in a variety of mammalian species. There appears to be little species variation in lung phospholipid patterns. In general, phosphatidylcholine is the most predominant phospholipid followed by, in the decreasing order, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol as the other constituents[8]. In most tissues, the majority of the molecular species within a phospholipid class show an asymmetric distribution of their fatty acyl constituents between the 1- and 2- positions of the glycerol moiety. The 1- position is normally occupied by a saturated fatty acid, whereas unsaturated fatty acids are found predominantly at the 2- position. However, the lung phosphatidylcholine molecules contain large amounts of palmitic acid at both the 1- and 2- positions. Considerable amounts of mono- and dienoic phosphatidylcholine molecules, particularly those containing palmitic acid at the 1- position are also found in the lung tissue[8].

The lipid composition of the surfactant differs from that of whole lung tissue. In surfactant, phospholipids may comprise as much as 87% by weight of the total lipid as compared to about 75% in whole lung tissue. Major phospholipid is phosphatidylcholine, and in surfactant it may account for as much as 58% by weight of the total lipid as compared to 16% in the whole lung tissue[9]. Phosphatidylglycerol content is also higher in the surfactant containing about 11% of phospholipids compared with about 3% in the whole lung tissue[10]. Other phospholipids such as phosphatidylethanolamine, sphingomyelin, phosphatidylserine, and phosphatidylinositol are present in much lower concentrations in surfactant.

(iii) Synthesis of pulmonary phospholipids--

The pathways of phospholipid synthesis are summarized in Figure 1. Briefly,

Phosphatidylcholine (PC) Based on the present knowledge, de novo biosynthesis of PC in the lung involves two pathways: (a) Cytidine diphosphate (CDP) choline pathway established by Kennedy in liver[11] which begins with the phosphorylation of choline by the action of choline kinase to yield phosphocholine which then reacts with cytidine triphosphate (CTP) to yield cytidine diphosphocholine. In the last step the CMP part of CDP-choline is cleaved and the phosphocholine portion is transferred to diacylglycerol to yield PC. (b) The conversion of PE into PC by three sequential N-methylation steps catalyzed by phosphatidylethanolamine methyltransferase[12]. These enzymes involved

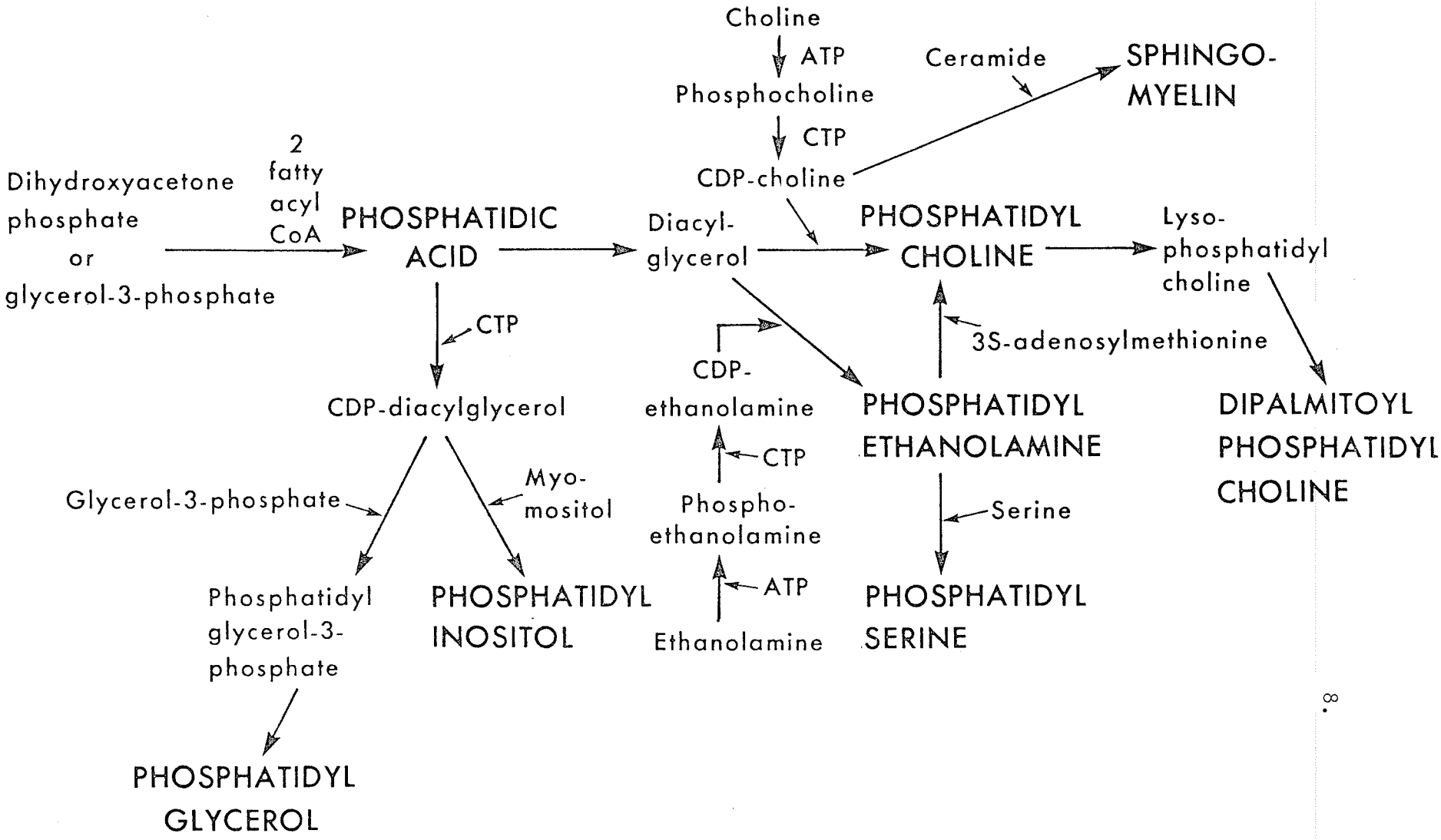

Fig. 1 PATHWAYS OF PHOSPHOLIPID SYNTHESIS

in both pathways are present in the lung in either the 105,000xg supernatant or in the microsomal fraction. In the lung, the CDP-choline pathway is the major route involved in the de novo formation of PC. However, this pathway probably produces predominantly unsaturated PC molecules. Two auxilliary mechanisms are involved in the transformation of them into dipalmitoyl-PC. These mechanisms include deacylation-reacylation process and deacylation-transacylation process with 1-palmitoyl lyso-PC as an intermediate metabolite[8]. It is important to stress that these auxilliary mechanisms cannot lead to a net increase of the total PC pool but can only accomplish a shift in the molecular composition of the total PC fraction.

Phosphatidylethanolamine (PE) In contrast to PC, lung PE does not contain significant amounts of disaturated molecules. Following the incorporation of labeled glycerol into the various molecular species, it was found that de novo synthesis of PE occurred mainly through the CDP-ethanolamine pathway[8].

Phosphatidylglycerol (PG) The synthesis of PG in the lung is a two step reaction involving CDP-diacylglycerol and glycerol-3-phosphate to form phosphatidylglycerol-3-phosphate which is then dephosphorylated to form PG[8].

Phosphatidic acid (PA) The synthesis of PA in the lung first involves the acylation of glycerol-3-phosphate with a fatty acyl CoA to form acylglycerol-3-phosphate (lyso-phosphatidic acid) or with dihydroxyacetone phosphate which is then reduced to acylglycerol-3-phosphate. This is followed by another acylation reaction to yield PA[8].

Phosphatidylinositol (PI) Lung microsomes were shown to have the capacity to catalyze the synthesis of PI from CDP-diacylglycerol and myo-inositol[8].

Biosynthesis of other phospholipids: The metabolism of other pulmonary phospholipids has not been established. However, their ways of metabolism were examined in other animal tissues. Briefly, phosphatidylserine (PS) is formed by the enzymatic exchange of the "head" alcohol of PE, namely, ethanolamine, with another alcohol group, that of L-serine. Sphingomyelin (SPH) is formed by transferring phosphocholine from CDP-choline to a ceramide group[13].

Origin of fatty acids for pulmonary phospholipid synthesis: Under normal conditions the uptake of free fatty acid from the circulation is probably a major source of fatty acid for the lung. The uptake of palmitic acid from the blood stream by the lung was shown many years ago. A second external source of fatty acid is represented by circulating lipoproteins, either very low density lipoproteins, synthesized predominantly in the liver or chylomicrons, originating from the intestine[14]. However, lung also has a great potential for fatty acid synthesis. After intravenous injection of ^{14}C-acetate into rabbits, the specific activity of the fatty acyl constituents of lung phospholipids exceeded that of liver phospholipids by a factor of thirty[15]. The two enzyme systems, acetyl-CoA carboxylase and fatty acid synthetase,

involved in the de novo synthesis of fatty acids are both present in the lung cytosol[16]. The major product of lipogenesis in the cytosol appears to be palmitic acid while the mitochondria possesses the capacity to elongate fatty acids already formed.

In addition to fatty acid, glycerol-3-phosphate or dihydroxyacetone phosphate are required as precursors in the biosynthesis of phospholipids. Under normal conditions, glycerol-3-phosphate arises from glucose via dihydroxyacetone phosphate, an intermediate of the glycolytic pathway, or from glycerol[17].

(iv) Degradation of pulmonary phospholipids--

The degradation of pulmonary phospholipids probably follows the same routes as in other tissues as the presence of phospholipase A, lipases, and lysophospholipase in the lung has been demonstrated[8]. Different phospholipases act at different sites of the phospholipid molecule, as illustrated below in Figure 2[13]:

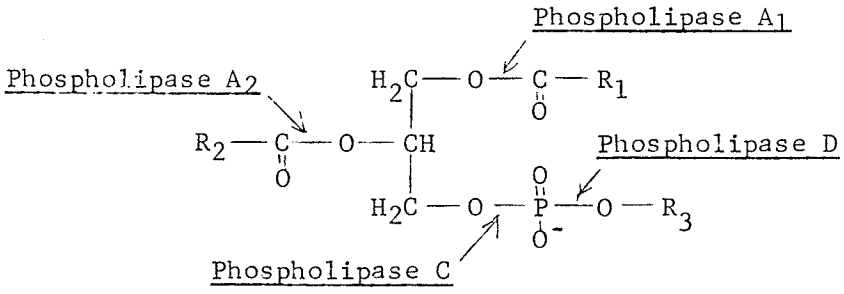

Figure 2: Action of different phospholipases on phospholipid.

The removal of surfactant phospholipids may follow one or more of the following possibilities[8]: (a) degradation by alveolar macrophages and phospholipases A_1, A_2, and lysophospholipases were found in alveolar macrophages; (b) passage up the respiratory tree and swallowed; (c) reabsorption into the alveolar epithelium; and (d) in situ enzymatic degradation, and phospholipase A was found in fetal pulmonary fluid or at the surface of alveolar epithelial cells.

(v) Cellular source of surfactant production--

There was some controversy about the involvement of Clara cells in the secretion of surfactant[18-20]. However, it is quite certain now that pulmonary alveolar type II cells are the sites of surfactant production[21-23]. Autoradiographic and electron microscopic studies following the incorporation of choline and leucine into various subcellular fractions of type II cells suggested that endoplasmic reticulum is the locus of PC synthesis. Subsequently the radioactivity was found to be rapidly transferred via the golgi complex to the lamellar bodies for storage, in the form of lipoprotein[24,25]. The role of lamellar bodies in the biosynthesis of surfactant is not clear at this moment; there are evidence both for[23,26,27] and against[10,28] it. It does seem that lamellar bodies have the capacity to transform unsaturated PC into saturated PC by a deacylation-reacylation process[29].

3. Role of glycogen in lung maturation

(i) Glycogen metabolism and its role in phospholipid synthesis

It has been shown that during development tissue glycogen

levels vary considerably[30]. In fetal animals, glycogen is accumulated in a variety of tissues during mid-gestation. After birth, glycogen stores are mobilized to produce glucose as the maternal supply ceases abruptly[31]. Activities of enzymes concerned with glycogenolysis, for example, protein kinase[32,33], phosphorylase kinase[32], phosphorylase[32], and glucose-6-phosphatase[34] also change with age. Gluconeogenesis[1,35] along with the activities of phosphoenolpyruvate kinase[36] and tyrosine aminotransferase[1] are also enhanced. However, in all mammalian species examined, the developmental profile of glycogen in lungs appears to be distinct from that of most other organs[37-39]. Pulmonary glycogen content increases throughout most of gestation but peaks prior to term and falls rapidly to low levels by birth. Accumulation of glycogen in rats is abundant in the undifferentiated cuboidal cells from day 17 to day 20 of gestation. Its presence correlates with active mitosis of the epithelium and may provide energy for cellular division[74]. The rapid prenatal fall in fetal lung glycogen content is coincident with differentiation of epithelial cells, an increase in pulmonary phospholipid content, and the appearance of lamellar bodies in the type II cells[22,39]. Thus, it has been suggested that lung glycogen may play a role in phospholipid synthesis[78]. Glucose can be utilized for PC synthesis in a number of ways. It may be broken down to acetyl-CoA which is the starting point for de novo synthesis of fatty acid or it may be converted into glycoerol-3-phosphate, the

backbone of the phospholipid[40]. The importance of glucose in lung PC synthesis is well illustrated by the observation that in the absence of glucose in the incubation medium, lung slices oxidize acetate to carbon dioxide, whereas when glucose is present, acetate tends to be used for phospholipid synthesis[41].

(ii) Regulation of glycogen metabolism by different hormones and by cyclic AMP

Cyclic AMP mediates the action of many hormones in a variety of tissues[42]. For example, glycogen degradation is mediated by cyclic AMP and this correlates to a rapid depletion of glycogen content of the liver cell. However, there are only few reports on the role of cyclic AMP in the regulation of glycogen metabolism in the lungs. Around birth, glycogen may provide precursors for surfactant synthesis in the type II cells. Administration of glucocorticoid and other hormones such as thyroxin results in a decrease in the glycogen content of the type II cells and an increase in the rate of PC synthesis[44-48] presumably due to the increase in choline phosphotransferase activity[46], the rate limiting enzyme in PC synthesis[45]. Glucocorticoids also promote lipolysis in adipose tissue releasing glycerol and free fatty acid[49],

both of which are precursors for PC synthesis[50,51]. Cortisol has also been shown to inhibit lung cell division and consequently may stimulate cellular differentiation[161]. Increasing fetal serum concentrations of cortisol near term and increased amniotic fluid cortisol, associated with advancing gestation and maturation of fetal lungs, indicate that increased output of cortisol by fetal adrenal glands may be one mechanism by which normal maturation of lungs occurs[6]. Likewise, thyroid hormone may also be involved[52] although its participation has been challenged by some workers[48].

Glucocorticoids have been shown to enhance cyclic AMP concentrations by inhibiting cyclic AMP phosphodiesterase[53,54]. The administration of hydrocortisone phosphate or aminophylline (an inhibitor of cyclic AMP phosphodiesterase) into fetal lungs increases the cellular cyclic AMP level and at the same time, increases lung PC content and ^{14}C-choline incorporation into PC molecules. Thyroid hormones have also been shown to elevate concentrations of cyclic AMP in target tissue through stimulation of adenylate cyclase[55]. During late gestational and early neonatal ages, lungs contain adenylate cyclases that are very responsive to different hormones such as catecholamines, histamine, prostaglandin E[56-58] all of which augment cyclic AMP concentration. Based on this evidence, it has been suggested that cyclic AMP is a mediator involved in the maturation of the lung near birth by promoting

glycogen degradation and surfactant phospholipid synthesis[53,54].

4. Developmental changes in cyclic AMP, adenylate cyclase, and cyclic AMP phosphodiesterase

During development, phasic elevations in cyclic AMP have been reported in liver[35,59], brain[60], heart[32] and other tissues[32,57]. In most tissues studied, there is a marked rise in cyclic AMP immediately after birth which decreases with age. In the rabbit lung, basal cyclic AMP level is low around birth, peaks at eight days after birth and decreases thereafter to adult level[57].

Hommes and Beere described the development of adenylate cyclase in various tissues of rats[33]. Adenylate cyclase activity in brain and kidney is low prenatally, remains low until ten to fifteen days postpartum, and increases markedly thereafter until adult life. Adenylate cyclase activity in muscle, however, begins to increase immediately after birth, reaching a maximum level on the twentieth day and declines a little thereafter to an adult level. In contrast, the development of adenylate cyclase in liver mostly occurs during fetal life, the enzyme activity increases prenatally to a maximum level by birth, and remains elevated thereafter.

Developmental studies of cyclic AMP phosphodiesterase in guinea pigs showed that the highest activity of the enzyme is in the fetal lungs Throughout late gestation cyclic AMP phosphodiesterase activity remains

higher than that found in postnatal life. After birth the enzyme activity remains quite constant at adult level[61]. In the rat liver, cyclic AMP phosphodiesterase activity is high during the perinatal period and then decreases gradually to adult level from four days after birth[62].

These studies of the developmental changes concerning cyclic AMP and related enzymes in lung tissues are few in number. However, a study of the developmental pattern of cyclic AMP metabolism is important for a number of reasons. Cyclic AMP has been ascribed a fundamental regulatory function in the cells[85]. Of special relevance to development are the indications that cyclic AMP, in at least some tissues, is involved in regulation of growth and is of central importance in maintaining the differentiated state[35].

C. PRESENT STUDY

The first part of this thesis will study the developmental profiles of glycogen and phospholipid metabolism in the rat lung, the changes in cyclic AMP and related enzymes' activities, and the possible role of cyclic AMP in the regulation of the glycogen content. Previous studies on similar subjects have focused on the changes in fetal and neonatal lungs. However, this study will examine the biochemical changes from 3 days before birth to about 45 days after birth and correlates them with lung growth and function.

D. MATERIALS AND METHODS

1. Animals

Sprague-Dawley strain rats of varying ages were obtained from Faculty of Dentistry, University of Manitoba. Fetal rats were obtained by Caesarean section and both males and females were utilized. Only male rats were used in the postnatal studies. All the animals were divided into three groups, one for the studies of protein content, cyclic AMP and glycogen levels, and adenylate cyclase and cyclic AMP-phosphodiesterase activities, another group was utilized for the study of phospholipid composition, while the third group was used for the study of $^{32}P_i$ incorporation studies. In these experiments, lungs from a minimum of 3 animals were used, at each age interval.

2. Preparation of lung tissues

The animals were stunned, decapitated, and the lungs were quickly excised and placed in a chilled petri dish. Extraneous tissues including trachea were removed as much as possible and the lungs were sliced with a sharp razor blade. All operations were carried out at 2-4^{o}C unless otherwise stated.

3. Estimation of cyclic AMP level

Lung slices were homogenized with a glass homogenizer in 6% ice-cold trichloroacetic acid, and then centrifuged at 4000 RPM in an International clinical centrifuge for fifteen minutes. The supernatant was mixed with 5 ml of ethylether saturated with water, phases allowed to separate, and the upper ether phase discarded. The lower aqueous phase was washed twice more with ethylether and then evaporated to dryness under a stream of nitrogen in a water bath at 60 - 70° C. The residue was dissolved in one ml of 0.05M sodium acetate buffer, pH 6.2. An appropriate volume was assayed for cyclic AMP by the procedure described in the Schwarz/Mann cyclic AMP radio-immunoassay kit. In this assay, succinyl cyclic AMP tyrosine methyl ester (^{125}I) is allowed to react with cyclic AMP antisera for 20 h at 2-4°C in the absence or presence of increasing amounts of cyclic AMP and unknown samples. A semi-logarithmic plot between the binding of radioiodinated cyclic AMP to cyclic AMP antisera and cyclic AMP concentration was obtained and the amount of cyclic AMP in unknown samples was determined.

4. Assay of adenylate cyclase activity

The method of Drummond and Duncan was followed using (8-^{14}C)-ATP as a substrate[63]. In a total volume of 100 µl, the assay medium contained 40 mM Tris-HCl buffer (pH 7.5), 8 mM caffeine,

2 mM cyclic AMP, 5.5 mM KCl, 15 mM $MgCl_2$, 20 mM phosphoenolpyruvate, 130 µg/ml of pyruvate kinase, 0.4 mM ^{14}C-ATP (10 µCi/µmol ATP), and about 100 µg tissue protein. The reactants were pre-incubated at 37°C for 3 minutes in a metabolic incubator. Reaction was initiated with the addition of ^{14}C-ATP, continued for 15 minutes and terminated by placing the tubes in a boiling water bath for 3 minutes. Denatured proteins were removed by centrifugation at 2000 RPM for 15 minutes in a clinical centrifuge in the cold room. The controls were treated similarly except that they were boiled before the addition of ^{14}C-ATP. An aliquot (75 µl) of the clear supernatant was applied to Whatmann no. 3MM filter paper. Cyclic AMP standard was always run on each sheet of paper to identify the cyclic AMP spot under ultraviolet light. Descending chromatography was performed for 18 hours at room temperature in a solvent containing 1 M ammonium acetate - 95% ethanol (3:7, v/v). The papers were air dried and the area containing cyclic AMP was cut out and placed in 18 ml of scintillation fluid containing 4.9 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(2-(5-phenyloxazolyl)) benzene per liter of toluene, and the radioactivity was measured in a Searle Mark III counter. The amount of cyclic AMP was calculated from the specific activity of the ^{14}C-ATP used as substrate. Counts were corrected for radioactivity in the cyclic AMP spot of each boiled sample.

5. Assay of cyclic AMP phosphodiesterase activity

The enzyme activity was measured by the method of Butcher and Sutherland[64]. In a total volume of 0.9 ml, the assay medium contained 40 mM Tris - 40 mMImidazol buffer, pH 7.5, 3 mM magnesium