SOME BIOCHEMICAL ASPECTS OF POSTNATAL RAT LUNG GROWTH AND DEVELOPMENT

A Thesis

Presented to the Faculty of Graduate Studies,
University of Manitoba, in partial fulfillment
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

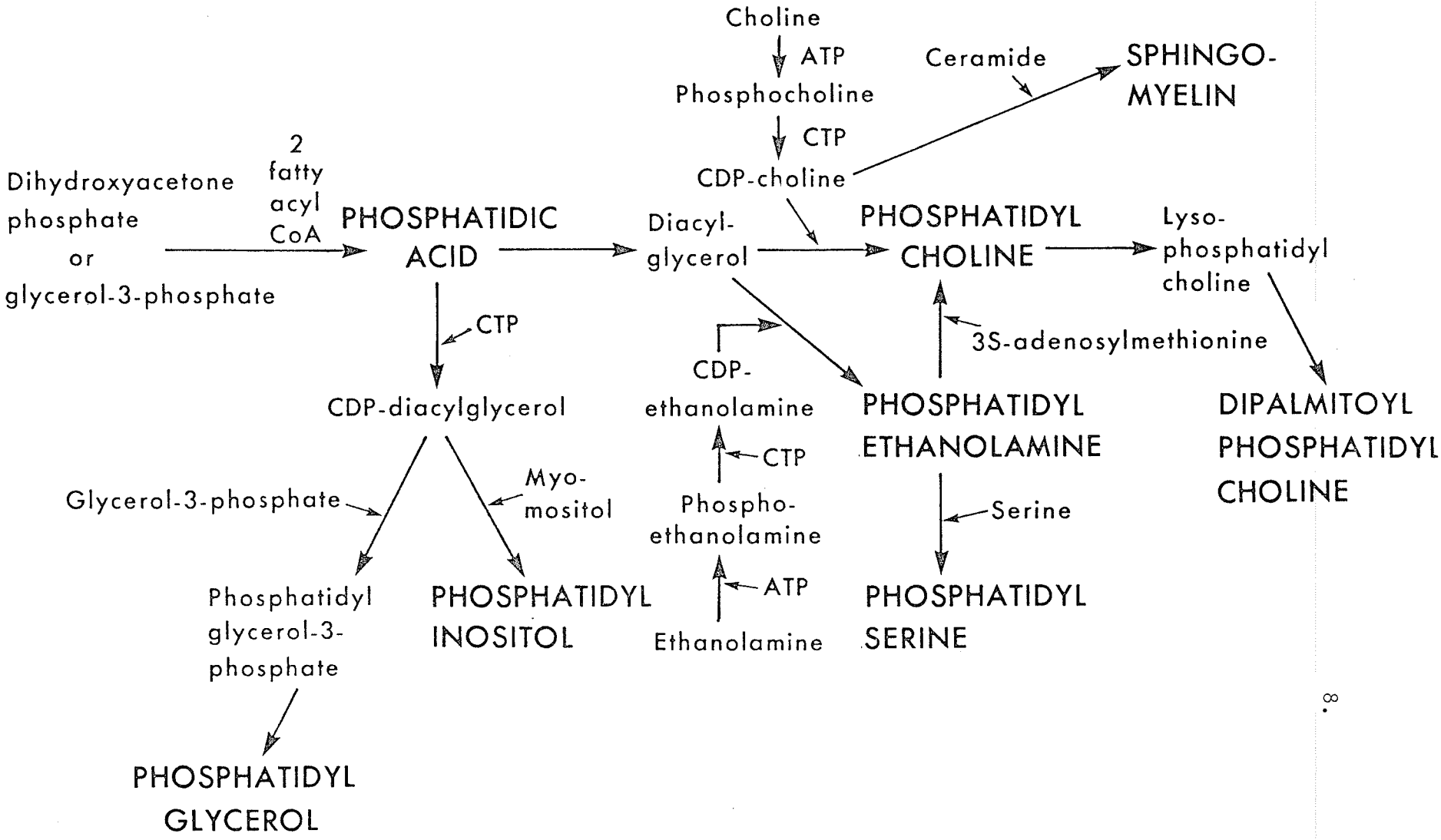


Fig. 1 PATHWAYS OF PHOSPHOLIPID SYNTHESIS

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]:

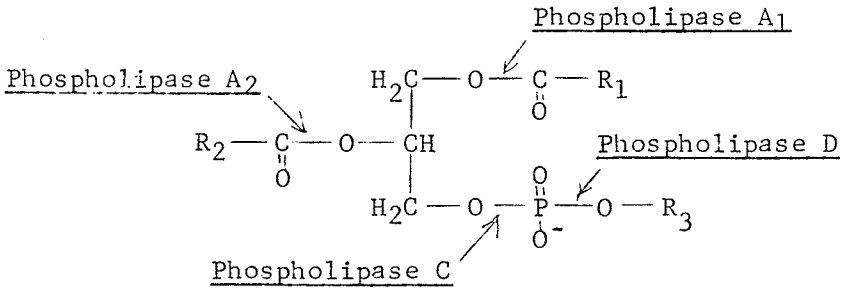


Figure 2: Action of different phospholipases on phospholipid.

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

acetate, 1.2 mM cyclic AMP, 0.1 ml of 5'-nucleotidase (0.25 U, Sigma grade III), dissolved in 10 mM Tris-HCl containing 0.5 mM magnesium acetate, pH 7.5, and 75 - 100 µg of lung homogenate protein. The tubes were pre-incubated for 3 minutes at 30°C. Reaction was initiated with the addition of cyclic AMP, continued for 30 minutes, and stopped by adding 0.1 ml of 55% ice-cold trichloroacetic acid. The denatured proteins were sedimented by centrifugation at 2000 RPM in a clinical centrifuge for 15 minutes. Aliquots of the supernatant were analyzed for inorganic phosphate. An aliquot of the clear supernatant (0.5 ml) was added to 0.5 ml of reducing agent containing 12 g sodium bisulfite, 1.2 g sodium sulfite, and 0.25 g of 1-amino, 2-napthol, 4-sulphonic acid in 100 ml of deionized water. The tubes were allowed to stand for 7 minutes at room temperature and the optical density (O.D.) was measured at 660 nm in a Pye Unicam SP8-100 spectrophotometer.

6. Estimation of protein

Proteins were measured by the colorimetric method of Lowry et al.[65]. The color reaction involved the formation of a protein-Cu^{2+} complex in alkali, and the reduction of the phosphomolybdic-phosphotungstic reagent by the copper treated protein. An aliquot of tissue fraction was dissolved in 3 ml of 1N sodium hydroxide overnight, and 0.2 ml aliquot of this solution was analyzed for protein. Water was added to make the volume to 0.4 ml and to which the color reagent containing 0.02% (w/v) sodium potassium tartrate, 0.01% (w/v) cupric sulfate, and 0.05% (w/v) of 1N sodium hydroxide in 2 ml of

2% (w/v) sodium carbonate was added. The mixture was allowed to stand for 10 minutes before 0.2 ml of Folin's reagent (1N) was added. The reaction was allowed to proceed for 30 minutes at room temperature and the absorbance was read at 750 nm. Bovine serum albumin was used as a standard.

7. Estimation of glycogen

Tissue samples were digested in 30% potassium hydroxide for 3 hours in a boiling water bath[66]. The digests were neutralized and the glucose was analyzed by the glucose oxidase method using Worthington Glucostat Reagent Kit.

8. Estimation of phospholipid content

(i) Extraction of lipid--

The extraction of lipids was carried out as described by Folch et al.[67]. Twenty five - thirty mg portions of lung slices were homogenized in 5 ml of chloroform:methanol (2:1, v/v) in a Potter-Elvehjam glass homogenizer. After extraction, 1 ml of water was added. The solution was mixed by inversion and centrifuged in an International clinical centrifuge for 5 minutes at 1000 RPM. The upper aqueous phase along with the protein interphase were discarded. The lower phase was washed twice with approximately 2.5 ml of synthetic upper phase (chloroform:methanol:water, 3:48:47, v/v). After washing, a few drops of methanol was added to prepare a single phase and portions were removed for analysis of total lipid. The remaining lipid extract

was dried under a stream of nitrogen at 37°C in a water bath. The lipid residue was redissolved in several drops of chloroform:methanol: water (75:25:2, v/v) and spotted onto thin layer chromatographic plates which have been activated at 110°C for 1 hour and cooled in a desiccator.

(ii) Separation of phospholipids by thin layer chromatography (TLC)

The glass plates were coated with 0.25 mm thick layer of silica gel H and activated for 1 hour at 110°C. Lipid samples were then applied onto the plates and chromatographed in two dimensions as described by Pumphrey[68]. The plates were first run in a solvent system which contained chloroform, methanol, 7M ammonium hydroxide (12:7:1, v/v), dried by hot air from a hair dryer, and chromatographed in the second solvent system which contained chloroform, methanol, acetic water, water (80:40:7.4:1.2, v/v).

(iii) Estimation of lipid phosphorus

After TLC separation, each plate was sprayed with 5% sulphuric acid and charred for 15-20 minutes at 160°C. Phospholipid spots were viewed under ultraviolet light, scraped, and placed into individual tubes. Perchloric acid (72%, w/v) was added to each tube and the mixture was digested at 160°C for 2 hours[69]. After cooling, 4 ml of water was added followed by 0.2 ml of 5% (w/v) ammonium molybdate and 0.2 ml of reducing agent containing 15 g sodium bisulfite, 0.5 g sodium sulfite, and 0.25 g of 1-amino-2-naphthol-4-sulfonic acid

per 100 ml of deionized water. The mixture was heated in a boiling water bath for 20 minutes, cooled, and centrifuged to sediment the silica gel. Absorbance of the clear supernatant was measured at 820 nm or 660 nm[70].

9. Studies of ^{32}P-orthophosphate ($^{32}P_i$) into phospholipids

About 25-30 mg of fresh rat lung slices was incubated in 0.5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4[71], containing 15 µCi of $^{32}P_i$ and 1 mg of glucose. Each tube was gassed with 5% carbon dioxide -95% oxygen, sealed with parafilm, and incubated at 37°C for 1 hour. Two ml of ice-cold saline was added to stop the reaction. The slices were spun down discarding the medium, and washed twice more with ice-cold saline. The slices were then homogenized in 5 ml of chloroform and methanol (2:1, v/v) in a tight fitting Potter-Elvehjam glass homogenizer to extract lipids. The lipid extract was then washed and the phospholipids separated as described earlier. After separation the developed TLC plates were subjected to autoradiography to locate radioactive lipids. Kodak no screen X-ray film was placed on the plates, exposed for 48 hour in the dark, developed, and aligned with the phospholipid spots. Silica gel containing the radioactive lipid was scraped into counting vials and mixed with 10 ml of scintillation fluid containing 4.9 g 2,5-diphenyloxazole and 0.1 g of 1,4-bis (2-(5-phenyloxazolyl)) benzene per litre of toluene. Radioactivity was measured in a Searle Mark III scintillation counter.

E. RESULTS AND DISCUSSION

Protein content of the lung increases steadily from 3 days before birth to about 2 weeks after birth (Table 1). During the same time, proliferation of lung cells is very active and new tissues are rapidly laid down. There is a net increase in the protein content per unit of lung weight, even though the amount of protein per nucleus remains rather constant[3]. After 2 weeks, lung growth enters a new phase, and the rate of cellular multiplication and formation of alveoli is markedly diminished. Lung grows mainly by cellular expansion and the amount of protein per nucleus increases rapidly although the protein content per unit of lung weight remains fairly constant.

Cyclic AMP level in lung tissues also changes with development (Table I, Figure 4). Between birth and 5 days of age, cyclic AMP level increases by 2 fold. Afterwards, it starts to decline and reaches a low level by the 15th day postpartum before it starts to rise again. The neonatal rise in cyclic AMP has been often attributed to the activation of adenylate cyclase by catecholamines which are released in response to delivery stress[1] and by glucagon whose secretion is probably enhanced by the neonatal hypoglycemia[166,167]. The postnatal rise in cyclic AMP after 2 weeks of age was apparently due to the appearance of a cytoplasmic protein modulator which activates the adenylate cyclase activity[100]. Cyclic AMP has also been ascribed

a fundamental regulatory function in the cells[162] which includes the regulation of growth and the maintainance of a differentiated state[35]. For example, cyclic AMP has been shown to inhibit cellular growth in vitro[163] and to enhance the differentiation and maturation of fibroblasts in cell cultures[164,165]. Postnatal development of rat lungs also appears to be regulated by cyclic AMP. Cellular proliferation of the rat lung is most rapid during the "phase of tissue proliferation" between 4 days and 2 weeks of age[3]. During this period cyclic AMP level starts to decline and remains at a low level. After 2 weeks, cyclic AMP level elevates when lung growth enters the "phase of equilibrated growth" which is characterized by a slow down of cellular multiplication and enhancement of maturation of the interstitium. Tissue cyclic AMP levels are dependent on the specific activities of adenylate cyclase and cyclic AMP-phosphodiesterase, which also change with age (Table I, Figure 4). Since adenylate cyclase produces cyclic AMP and cyclic AMP-phosphodiesterase hydrolyses it, a ratio of the activities of these enzymes determines the cyclic AMP level at any particular age which appears to be the case in the lung, as illustrated in Figure 6.

Glycogen content decreases rapidly from the rat lung between day 21 of gestation and the 5th day after birth (Table I). Maniscalco et al.[72] reported similar changes in the rat lung glycogen content. After the 5th postnatal day glycogen content starts to increase again and peaks around 23 days after birth before it declines. The biochemical study

of glycogen content in the rat lung in this report correlates well with the morphological studies by Curle and Adamson[73,74]. They reported a rapid depletion of glycogen from the epithelial cells around birth. After the 3rd day postpartum, glycogen starts to accumulate again, this time in the mesenchymal cells instead of the epithelial cells, and correlates to a rapid mitotic activity of the mesenchyme. About 1 month after birth, glycogen gradually disappears from the rat lungs.

In this study, the developmental profiles of cyclic AMP and glycogen appeared to be inversely related (Figure 5b,c). Glycogen was depleted rapidly between day 21 of gestation and 3 day postpartum when the cyclic AMP level was increasing. Cyclic AMP level was highest on the 5th day when the glycogen content was lowest. After 5 days postpartum glycogen content began to increase simultaneously when cyclic AMP level was declining. Glycogen content started to decrease again by the 20th day when the cyclic AMP level was rising. On the 45th postnatal day, cyclic AMP level was high and the glycogen content was low. These data suggest that glycogen content in lung tissues is regulated by the cyclic AMP level, a phenomenon well established in the liver[85]. Glycogen has been suggested to provide energy for rapid cellular multiplication[74]. Consequently, postnatal glycogen accumulated during developmental periods when there was rapid cellular multiplication and depleted when differentiation started to take place.

The lung PC content appears to increase slightly between day 21 of gestation and 1st day postpartum (Table II). This small increase in lung PC near birth has been reported on numerous occasions[6,22,72,75,76]. In contrast, other phospholipids such as PE, PS, PI, and PG all show a decrease in contents during the same period of time while the SPH content does not change very much. Among these phospholipids, the decrease in PG content is most apparent with a 60% drop in 2 days. Rooney et al.[75] also reported a significant drop in the PG content in rabbit lungs at birth; however, their report has also shown an increase in the PG content in the lung wash during the same period of time. After 5 days postpartum the contents of all phospholipids studied remain quite constant. The phospholipid composition of rat lung tissue in this report compares favorably with other reports[8]. PC is the most predominant phospholipid followed by, in the decreasing order, PE, SPH, PS, PG, and PI as the other major constituents.

The incorporation of $^{32}P_i$ into PC follows closely the pattern in the total lipid extract which increases by almost 100% between day 21 of gestation and 1 day postpartum (Table III and Figure 5a). SPH and PA also show a small increase in their $^{32}P_i$ incorporation over the same period of time, while other phospholipids show a decrease. When the $^{32}P_i$ incorporation is expressed per μg of phosphorus, there is still an increase in the incorporation into PC indicating a net increase in the metabolic activity in that particular phospholipid.

After the first day of birth, the incorporation of $^{32}P_i$ in all phospholipids decreases, an indication that they are metabolically less active. By the 16th day postpartum, $^{32}P_i$ incorporation into phospholipids has reached the lowest rate and is followed by another rise that appears to be maximum between the 23rd and 35th day declining thereafter until 51 day. The significance of this peak is not clear at present. It does not reach as high a level as obtained near birth, and it seems to appear only after lung growth has passed the stage of rapid proliferation.

Maniscalco et al.[72] have also shown a significant increase in the incorporation of (Me-^{14}C) choline into PC of rat lungs between day 21 of gestation and one day postpartum indicating an increase rate of PC synthesis. (Me-^{14}C) choline is a more specific substrate to study PC synthesis. However, $^{32}P_i$ incorporation into phospholipids has been often used as an indication of phospholipid metabolism and membrane biogenesis[76], and it is used in this report to show that the enhanced rate of phospholipid synthesis around birth involves only PC while other phospholipids show either a drop or very little change in the $^{32}P_i$ incorporation during the same period of time.

An enhanced metabolism of PC between day 21 of gestation and one day postpartum is coincident with the depletion of glycogen in the

lung tissues (Figures 5a and 5b) which has also been reported by various workers[22,39,72]. The neonatal depletion of glycogen in lungs is apparently due to an enhanced glycogen phosphorylase a activity[72,77] and appears to provide substrate for phospholipid synthesis[78]. The developmental profile and regulation of glycogen metabolism in the lung appears to be quite different from that in the liver. Liver glycogen is depleted after birth presumably to overcome neonatal hypoglycemia[31]. However, in lungs, glycogen is utilized before birth to provide substrate for the synthesis of surfactant phospholipids. Glycogen can be used for phospholipid synthesis in a number of ways[17]. It may be broken down to glucose or glucose-6-phosphate which undergo glycolysis to produce dihydroxyacetone phosphate or glyceraldehyde-3-phosphate which can be reduced to glycerol-3-phosphate and acylated to yield phospholipid. Glucose can also be broken down to acetyl CoA which is used for de novo synthesis of fatty acid and two fatty acyl CoA are combined with glycerol-3-phosphate to give diacylglycerol which is a precursor for PC synthesis. The following diagram illustrates the metabolic pathways by which glucose or glycogen may be incorporated into both the glycerol and fatty acid portions of the PC molecule[17]:

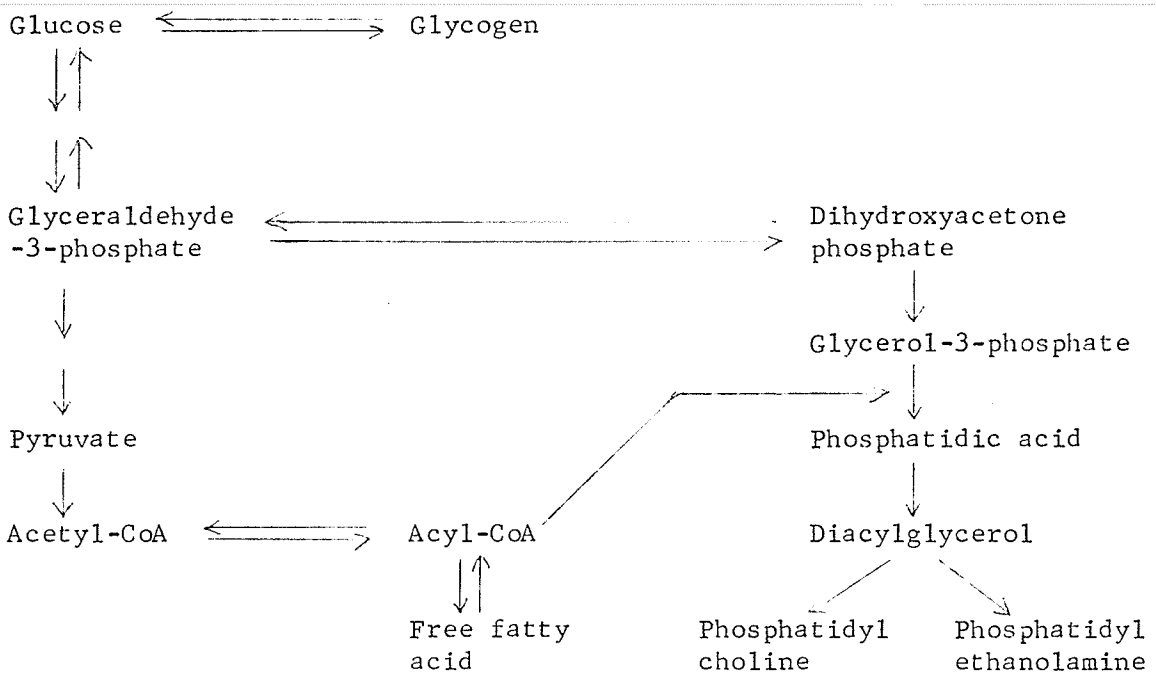


Figure 3: Scheme by which glucose can be converted into phosphatidyl-choline; single arrowhead indicates one step reactions and double arrow-heads indicate multiple step reactions[17].

There is no direct correlation between the phospholipid contents and the postnatal accumulation of glycogen in the interstitium. However, when glycogen content depletes at about 3 weeks after birth, there is a small increase in the turnover of phospholipids as indicated by an increase in the $^{32}P_i$ incorporation. Whether these two phenomena are related is not clear at present.

In summary, 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 again depleted 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 cell 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, but without much effect on the labelling of other phospholipids. This 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. Although there was a small increase in $^{32}P_i$ incorporation into phospholipids around 3 weeks of age when glycogen was depleting from mesenchymal cells, this time all phospholipids were involved. 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 level decreased between 5 day and 2 weeks of age when cellular proleferation was active. After 2 weeks cyclic AMP level elevated when maturation of the interstitium occurred.

TABLE I

Protein contents, cyclic AMP and glycogen levels, adenylate cyclase and cyclic AMP phosphodiesterase activities in rat lungs at different ages

Age	Protein Content	Cyclic AMP Level	Adenylate Cyclase Activity	Cyclic AMP Phosphodi-esterase Activity	Glycogen Level
-3	40	[a]5.1±0.2	41±11	12.7±0.7	———
-1	54±9	3.1±1.4	36±2	12.7±0.5	209±14
+1	69±7	2.9±0.4	61±2	14.3±0.6	161±22
+3	80±12	4.6±0.8	59±3	14.2±0.1	100±34
+5	91±8	7.4±0.4	85±9	15.7±0.2	95±22
+8	103±2	4.7±0.7	59±1	15.8±0.6	119±27
+15	120±8	4.2±0.3	80±2	16.5±0.1	136±16
+23	124±5	8.2±1.2	99±20	16.9±0.5	157±19
+35	123±10	10.5±2.1	95±8	13.3±0.4	100±22
+45	120±9	13.1±1.6	205±31	12.5±0.2	74±4

[a]Figures in table refer to mean ± S.E.
Results are expressed as number of days before(-) or after(+) birth (Age), mg protein/g lung wt (Protein Content), pmoles cyclic AMP/mg protein (Cyclic AMP Level), pmoles cyclic AMP/min per mg protein (Adenylate Cyclase Activity), nmoles P_i/min per mg protein (Cyclic AMP-Phosphodiesterase Activity), and ug glycogen/mg protein (Glycogen Level).

TABLE II

Phospholipid Composition of Rat Lung at Different Ages

Age	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Sphingo-myelin	Phosphatidyl-serine	Phosphatidyl-inositol	Phosphatidyl-glycerol
day	ug of phosphorus/mg protein					
-1	3.3±0.2	2.4±0.5	0.61±0.04	0.57±0.15	0.22±0.07	0.32±0.13
+1	3.6±0.3	1.3±0.3	0.58±0.06	0.49±0.03	0.12±0.01	0.14±0.04
+3	2.6±0.1	1.8±0.1	0.50±0.05	0.54±0.08	0.24±0.02	0.21±0.04
+5	3.0±0.4	2.0±0.5	0.44±0.04	0.48±0.07	0.22±0.05	0.16±0.03
+8	3.0±0.1	1.7±0.3	0.49±0.03	0.48±0.01	0.13±0.02	0.25±0.03
+15	2.8±0.1	1.7±0.2	0.47±0.02	0.42±0.02	0.10±0.02	0.15±0.04
+21	2.8±0.2	1.8±0.2	0.56±0.06	0.56±0.02	0.09±0.03	0.16±0.01
+45	2.9±0.1	1.7±0.2	0.60±0.01	0.49±0.01	0.08±0.02	0.14±0.02

Figures in table refer to mean ± S.E.

TABLE III

Incorporation of $^{32}P_i$ into Phospholipid of Rat Lung At Different Ages

Age	Total Lipid	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Sphingo-myelin	Phosphatidyl-serine	Phosphatidyl-inositol	Phosphatidyl-glycerol	Phosphatidic Acid
day	CPM/hr per mg protein							
-1	37981±5407	18889±981	981±93	685±204	307±168	3707±167	2667±556	1500±150
+1	51319±7058	33493±3855	884±145	942±261	177±44	3058±145	1870±159	1754±464
+3	33800±2413	22975±588	550±25	750±13	203±24	1625±50	1088±200	900±75
+6	22573±1656	15063±1031	250±21	292±21	118±2	896±115	698±63	438±73
+16	15074±1098	11098±803	246±16	262±74	126±68	754±49	246±8	410±90
+23	22040±589	15702±556	452±16	411±40	177±40	895±8	524±64	863±24
+30	22992±887	15556±1298	467±32	331±48	235±80	1000±25	565±40	669±24
+51	16942±2433	11067±1642	300±58	208±33	248±111	725±8	383±83	567±50

Figures in table refer to mean ± S.E.

Figure 4 A comparison of cyclic AMP level (a), adenylate cyclase activity (b), and cAMP-Phosphodiesterase activity (c) in rat lungs of different ages.

Figure 5 A comparison of $^{32}P_i$ incorporation into phospholipids (a), glycogen level (b), and cyclic AMP level (c) in rat lungs of different ages.

Figure 6 The ratio of the activities of adenylate cyclase to cyclic AMP-Phosphodiesterase in rat lungs of different ages.

Figure 4a

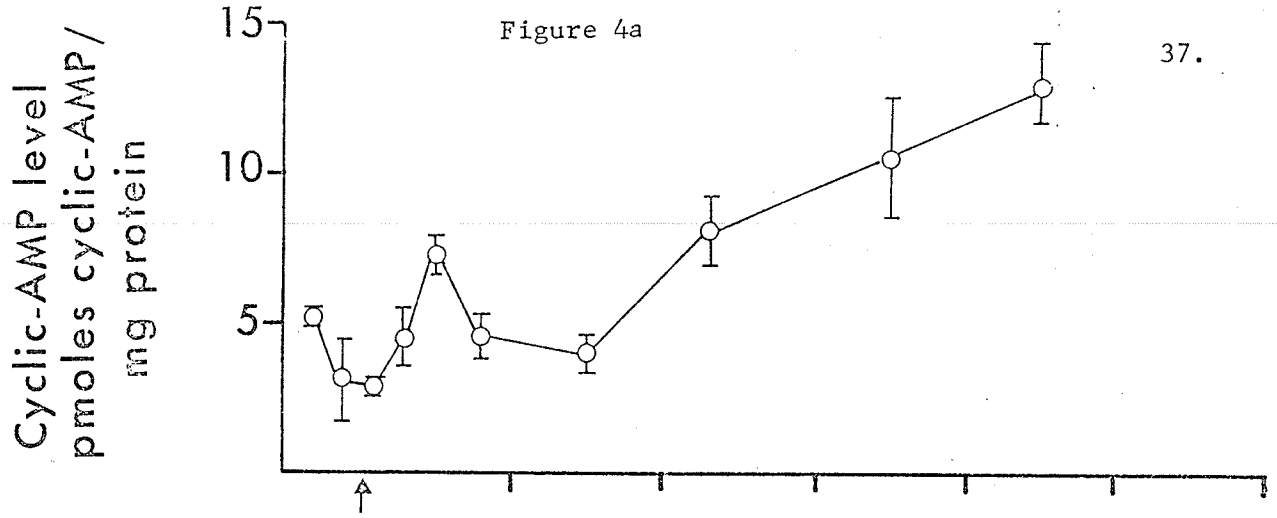


Figure 4b

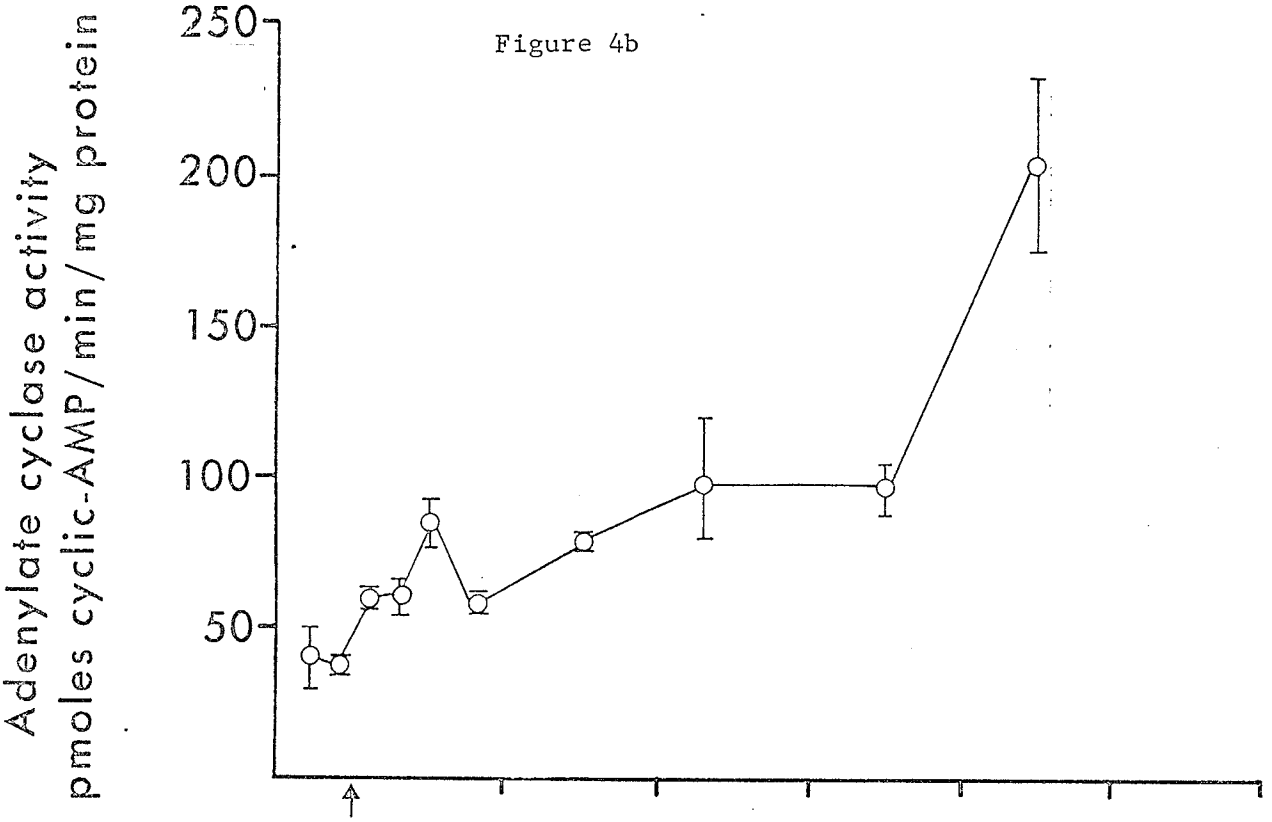


Figure 4c

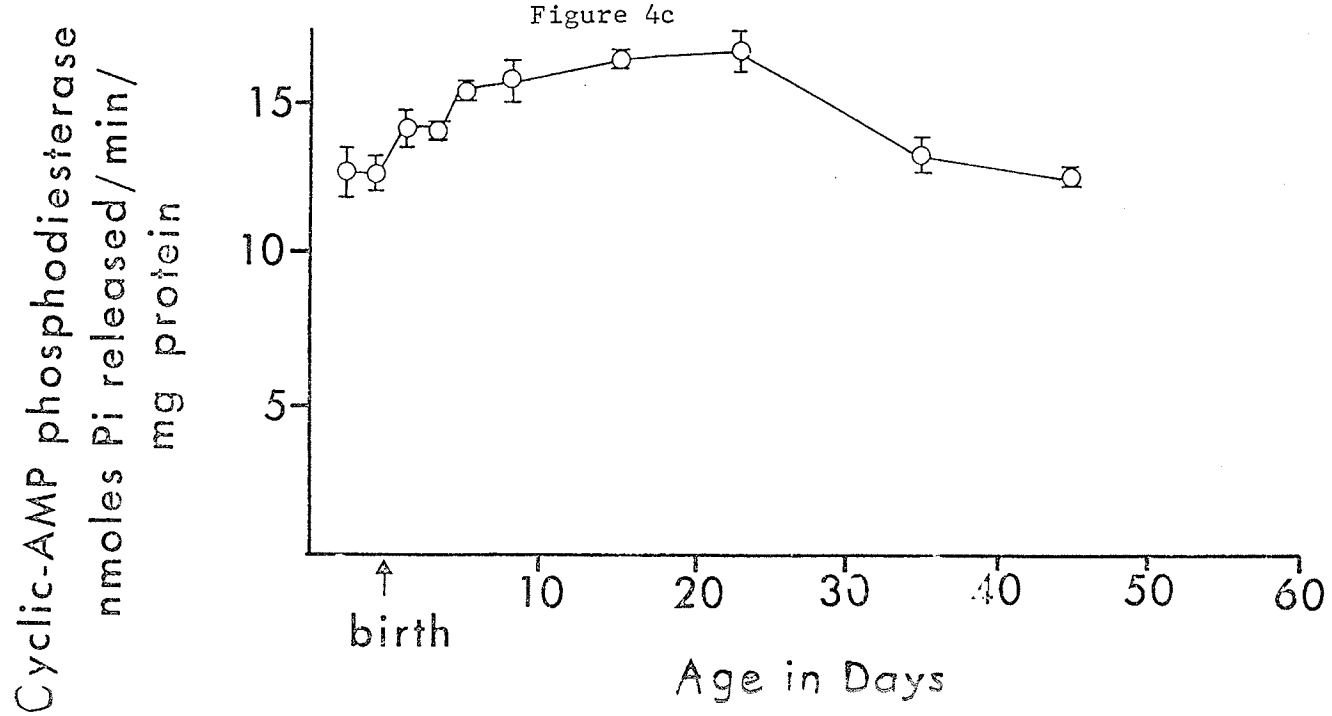


Figure 5a

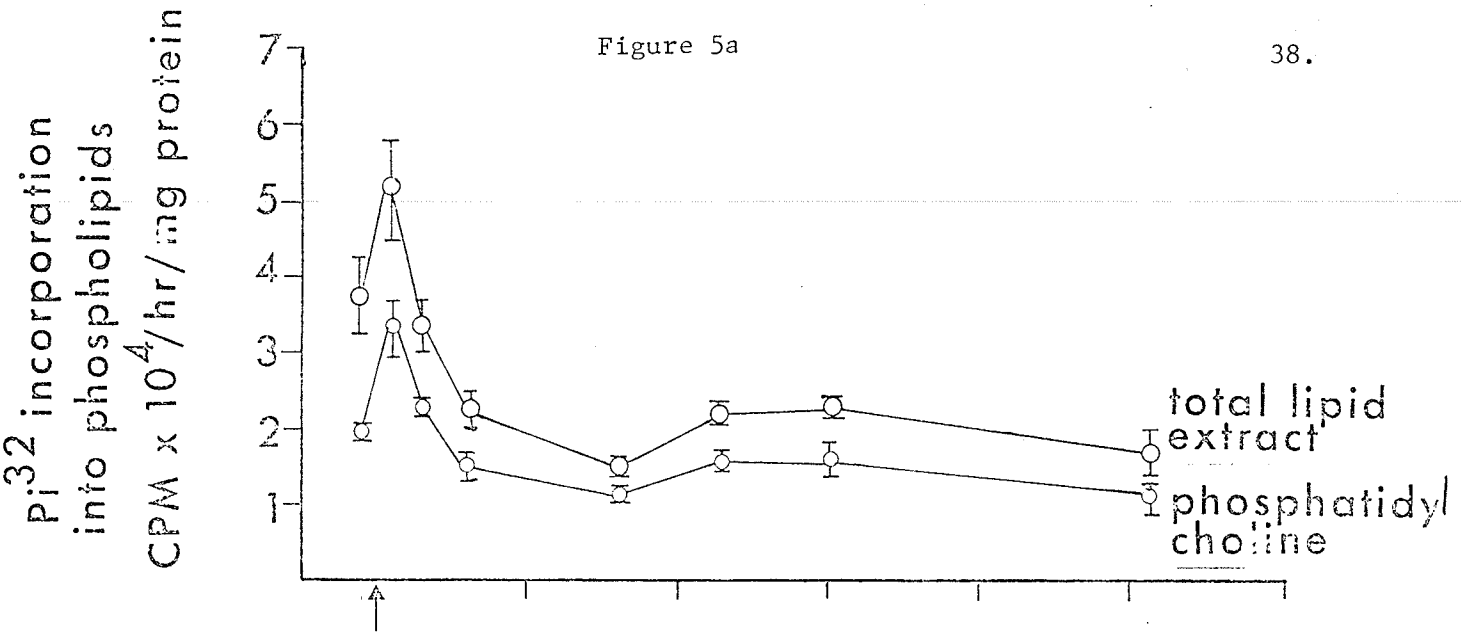


Figure 5b

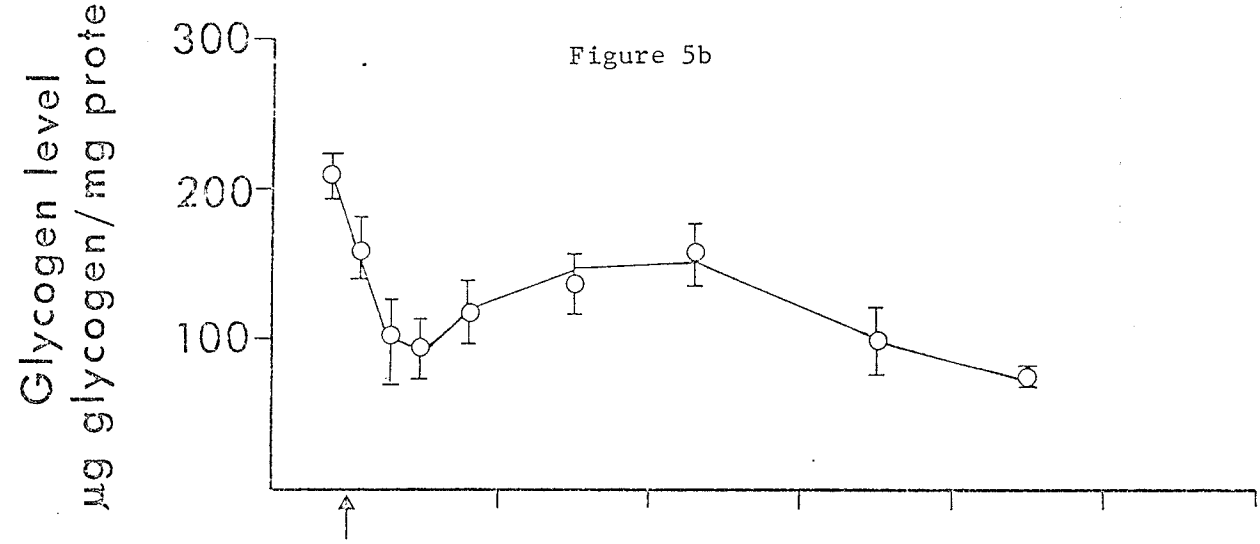


Figure 5c

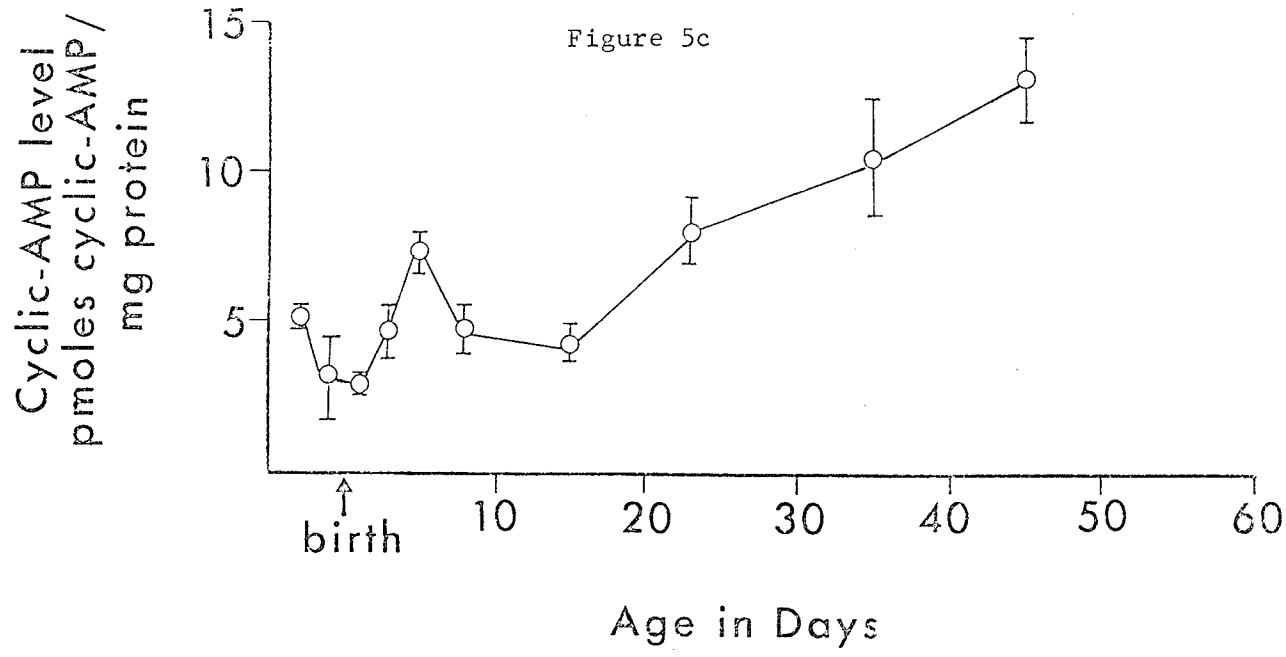
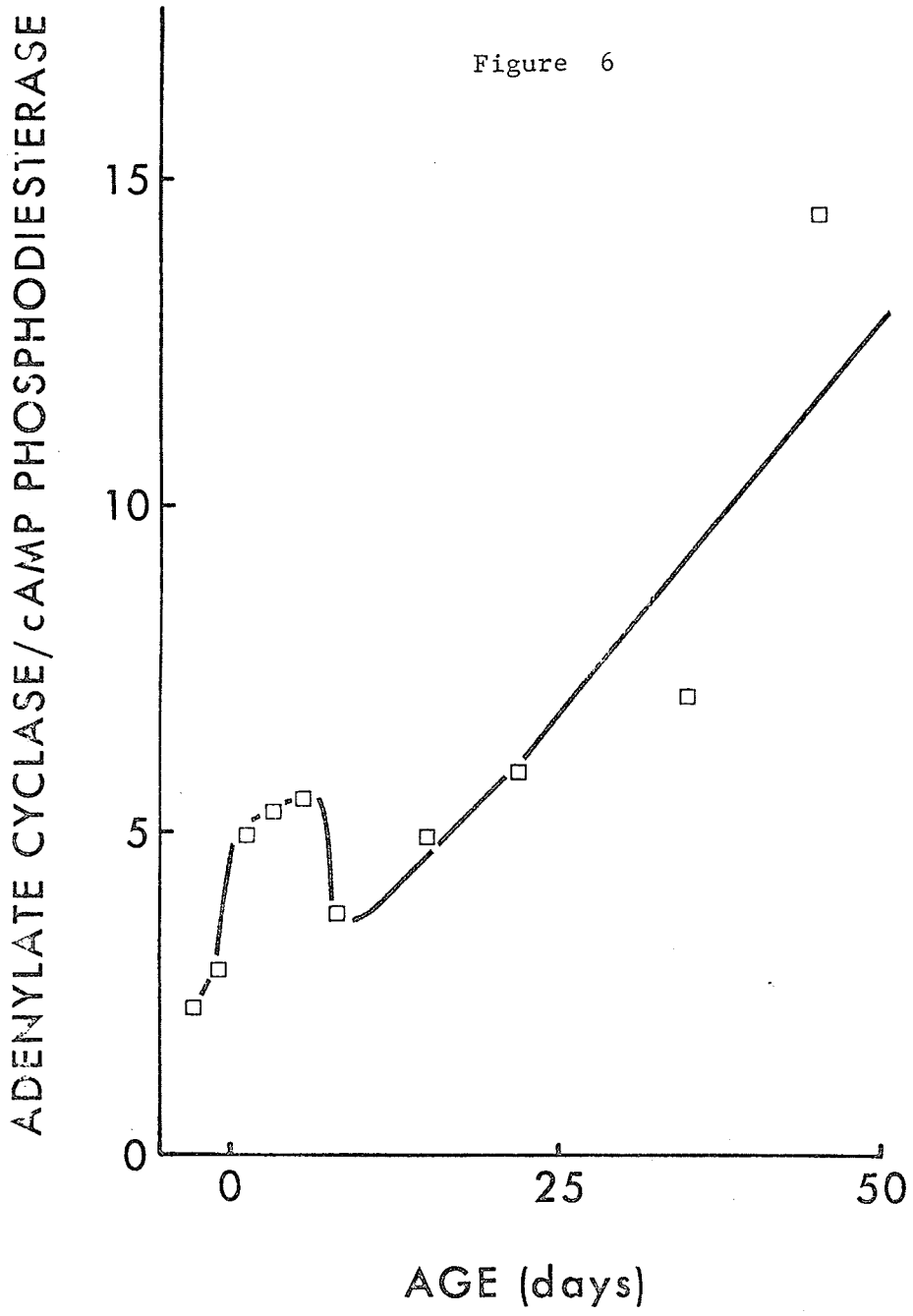


Figure 6


ADENYLATE CYCLASE/cAMP PHOSPHODIESTERASE
15
10
5
0
0
25
50
AGE (days)

II. ISOLATION OF A FRACTION RICH IN PLASMA MEMBRANES FROM LUNGS

A. INTRODUCTION

In the first part of this thesis, phasic alterations of cyclic AMP level in rat lungs have been demonstrated. Cyclic AMP level was shown to be dependent on the relative specific activities of adenylate cyclase and cyclic AMP-phosphodiesterase. These enzymes are in turn regulated by different hormones and factors. The neonatal rise in cyclic AMP has been attributed to the activation of adenylate cyclase by catecholamines and glucagon[1,166,167]. The postnatal rise in cyclic AMP after 2 weeks of age was apparently due to the appearance of a cytoplasmic protein modulator which activated the adenylate cyclase activity[100]. This protein activator may play a role in the regulation of differentiation and maturation of lung cells since its appearance coincided with the cessation of rapid cellular proliferation in rat lungs. Early studies on this protein activator were carried out in crude preparations of 100,000 xg supernatant and pellet. In future studies it was essential to obtain a reasonably pure preparation of the protein activator and plasma membranes that are rich in adenylate cyclase activity.

B. LITERATURE REVIEW

1. General Properties and Regulations of Adenylate Cyclase

In all nucleated cells thus far studied, adenylate cyclase

is predominantly found in plasma membranes of cells[86] and sometimes, in other cellular organelles[87] such as in adrenal microsomes, liver and prostate nuclei, and sarcoplasmic reticulum. In these cells adenylate cyclase is stimulated by specific hormones and by fluoride ions. Although adenylate cyclase in many types of cells such as fat cell "ghosts" is sensitive to a variety of hormones it is generally believed that only a single adenylate cyclase system is involved and is activated by the different hormones through specific receptors on the cell surface[87]. The differences in hormonal response in different cells resides in the presence of distinct receptors for specific hormones. These receptors seem to be proteic in nature, located on the external surface of the plasma membrane and linked to catalytic subunit of adenylate cyclase on the cytosolic surface by a phospholipid complex[92,93]. Divalent cations also play a crucial role in the regulation of adenylate cyclase activity which has an absolute requirement for Mg^{2+} ions[43] and the true substrate for the enzyme is Mg^{2+}-ATP while free ATP is inhibitory to the reaction[63,88]. In addition to the catalytic site there may be a second allosteric site for Mg^{2+} ions whose occupation is crucial in the expression of catalytic activity[87]. Calcium has been shown to inhibit adenylate cyclase activity although in some instances, it appears to be essential, in trace (submicromolar) quantities, for adenylate cyclase activity and its hormonal stimulation[89-91]. Besides being a substrate, ATP has also been found to augment stimulation of adenylate cyclase in liver cells by glucagon and prostaglandin but not by sodium fluoride[63,94]. This stimulatory effect of ATP

may be exerted by GTP in trace amount[95]. However, ATP shows stimulatory and inhibitory effects on adenylate cyclase response to (Arg^8)-vasopressin in the beef kidney medulla[87]. ATP stimulates the enzyme at low concentration (0.07-0.07 mM). However, it inhibits the enzyme at higher concentration (1 mM). The stimulatory effect of ATP can be mimicked by AMP and adenosine. On the other hand, GTP inhibits the (Arg^8)-vasopressin stimulated activity without affecting basal adenylate cyclase activity. These observations indicate that the hormonal response of cells is more complex than just a passive receptor dependent phenomenon and that the metabolites of target cells may play an important role in the regulation of the cellular response to the hormones. Response of adenylate cyclase to hormones also differs with age[56,57,64,77,83,96]. For example, the addition of histamine in rat lungs causes the accumulation of cyclic AMP in three distinct periods[57]. Norepinephrine, epinephrine, and sodium fluoride also elevate cyclic AMP level but their effects are most pronounced around birth and diminished with age[77]. The differential activation of adenylate cyclase by hormones during development may be due to a variable concentration of the enzyme[33,97], hormone receptors[33,35,57,97] and/or other modulators[98-100]. Recently the presence of endogenous protein modulators of adenylate cyclase has been reported in different tissues[81,98-105]. Doberska and Martin[104] and Peckor and Hanoune[101] reported the presence of a GTP-like heat stable protein factor which modulates the basal and hormonally stimulated adenylate cyclase activity in the rat liver. Sanders et al.[99] described the presence of a protein-like activator of adenylate

cyclase in the 10,000 xg supernatant of heart and other tissues. These factors have no effect on basal adenylate cyclase activity but enhance the hormonal (epinephrine and glucagon) activation of the enzyme. Katz et al.[105] reported a cytosol protein activator which restores the basal and the epinephrine, glucagon, and fluoride activation of adenylate cyclase in the particulate fraction prepared from the rat liver. Nijjar[100] reported an age dependent cytoplasmic factor from the rat lungs which enhances the adenylate cyclase activity in the particulate fraction. This activator is proteic in nature and does not resemble either GTP or calcium dependent regulator of cyclic AMP phosphodiesterase in its action on adenylate cyclase. The activator does not appear in the cytosol until around the 20th day of age. The significance of this activator in lung physiology is not clear at present but it was suggested that it may have a role in cellular differentiation as the time of appearance of the factor coincides with the time of cessation of rapid alveolar multiplication in the rat lungs. Further work on this topic awaits the isolation of the protein activator and the preparation of fraction(s) rich in adenylate cyclase activity.

2. Preparation of Plasma Membrane from Animal Tissues

Membranes are a conspicuous feature of cell structure which serve not only as barriers separating aqueous compartments with different solute composition but also as the structural base to which certain enzymes and transport systems are firmly bound[13]. The major components

of plasma membrane are proteins and lipids, particularly phospholipids, and a small amount of carbohydrates[106]. Although many models were proposed, the most satisfactory one of membrane structure appears to be the fluid-mosaic model, postulated by Singer and Nicholson[107] that the phospholipids of membranes are arranged in a bilayer to form a fluid, liquid-crystalline matrix with proteins embedded within.

Over the years a variety of procedures has been used to obtain plasma membranes from different tissues. These procedures involve gentle homogenization of the tissue in hypotonic[108,109], isotonic[110-112] and hypertonic[113] buffered medium. In some procedures, plasma membranes are obtained from pellet of low speed centrifugation of an homogenate[108,114,115] while in others, heavier material containing nuclei and cellular debris are first sedimented by low speed centrifugation and the resulting supernatant is processed for the isolation of plasma membrane[109]. Both discontinuous[108,110,111,114,115] and continuous[111] gradients are used to separate plasma membrane from other subcellular contaminants. These gradients are commonly prepared from sucrose or ficoll[112,116]. Plasma membranes prepared by different procedures vary in their relative degree of purity and hence exhibit properties characteristic of the isolation procedure. Generally, the purity of plasma membrane preparations can be increased at the expense of yield, and vice versa. The composition of the homogenizing medium appears to be a very important factor in obtaining

membrane free from other contaminants and the choice of a proper medium was dependent upon the type of tissue used. For example, it has been demonstrated that the homogenization of tumor cells in a hypotonic buffered medium led to the rupture of nuclei, mitochondria, and endoplasmic reticulum, and the plasma membrane isolated from such homogenates was contaminated with material from these subcellular organelles[114,115]. This problem was solved by supplementing the medium with either calcium chloride or magnesium chloride or both[110,111], which tend to stabilize the membranes of subcellular organelles thereby preventing their rupture. However, an isotonic homogenizing medium is often preferred over a hypotonic medium as it allows the simultaneous isolation of other subcellular organelles as well as plasma membranes[110-112]. Many times, plasma membranes are distributed almost equally between the supernatant and the pellet after low speed centrifugation[109]. The choice between these two sources of plasma membrane generally has to be made by an individual investigator and would mainly depend upon the characteristics of the tissue under investigation. In the last two decades, zonal centrifugation has become increasingly popular because it can process large amount of tissue in a single run and thus is preferred over isopycnic centrifugation when a large amount of tissue is available.

C. PRESENT STUDY

This second part is an attempt to prepare a plasma membrane

fraction from rat lungs by differential and discontinuous sucrose density gradient centrifugation. Plasma membranes along with other fractions will be characterized in terms of their purity as judged by the activities of enzyme markers for various subcellular organelles. The ability of the supernatant to activate adenylate cyclase of plasma membrane will also be tested.

D. MATERIALS AND METHODS

1. Animals

Male rate of Sprague-Dawley strain were utilized for the preparation of membrane fractions. Each preparation contained lungs from at least 3 animals of the same age. Assay of adenylate cyclase activity was carried out in fractions obtained from animals 2 days and 45 days of age. Estimation of protein, DNA, and assays of other marker enzyme activities were carried out in fractions obtained from animals between 30 and 35 days of age.

2. Preparation of plasma membrane

Animals were stunned with a blow to the head and decapitated. 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 subsequent procedures were carried out at 2-4^{o}C unless otherwise stated. The

lung slices were transferred into a Potter-Elvehjem homogenizer and hand homogenized (15 strokes) in 0.27 M sucrose - 0.01M Tris buffer, pH 7.4, with a loose fitting teflon pestle. The homogenate was filtered through 2 layers of cheese cloth to remove cellular debris. The volume of the homogenate was adjusted to yield a 20% solution (w/v), and the homogenate was then centrifuged at 900 xg in an International refrigerated centrifuge (Model B-20) for 10 minutes. The supernatant (designated S1) was carefully withdrawn with a pasteur pipette and re-centrifuged at 6000 xg for 10 minutes. The supernatant (S2) was withdrawn and centrifuged at 8000 xg for 10 minutes to give supernatant (S3). The pellets of 900x, 6000x, and 8000 xg centrifugations were designated P1, P2, and P3, respectively, and were re-suspended in about 2 ml of 0.27M sucrose - 0.01M Tris buffer (pH 7.4). Supernatant 3 was made 1mM with respect to $MgCl_2$[126] and exactly 2.3 ml was layered on top of a discontinuous sucrose gradient containing, from top to bottom, 2.3 ml of 25% sucrose (0.73 M,d 1.11), 2.3 ml of 30% sucrose (0.88M,d 1,13), 2.7 ml of 35% sucrose (1.02M,d 1.16), and 2.7 ml of 45% sucrose (1.31M,d 1.21)[134]. The gradient was centrifuged at 100,000 xg for 60 minutes in a swinging-bucket rotor in an International refrigerated centrifuge (Model B-60). After centrifugation, starting from the top of the gradient, fractions of 1.4 ml (designated F1), 1.2 ml (F2), 1.2 ml (F3), 1.7 ml (F4), 2.5 ml (F5), and 4.0 ml (F6) were withdrawn carefully and the same fractions from different tubes were pooled. Each fraction was then diluted

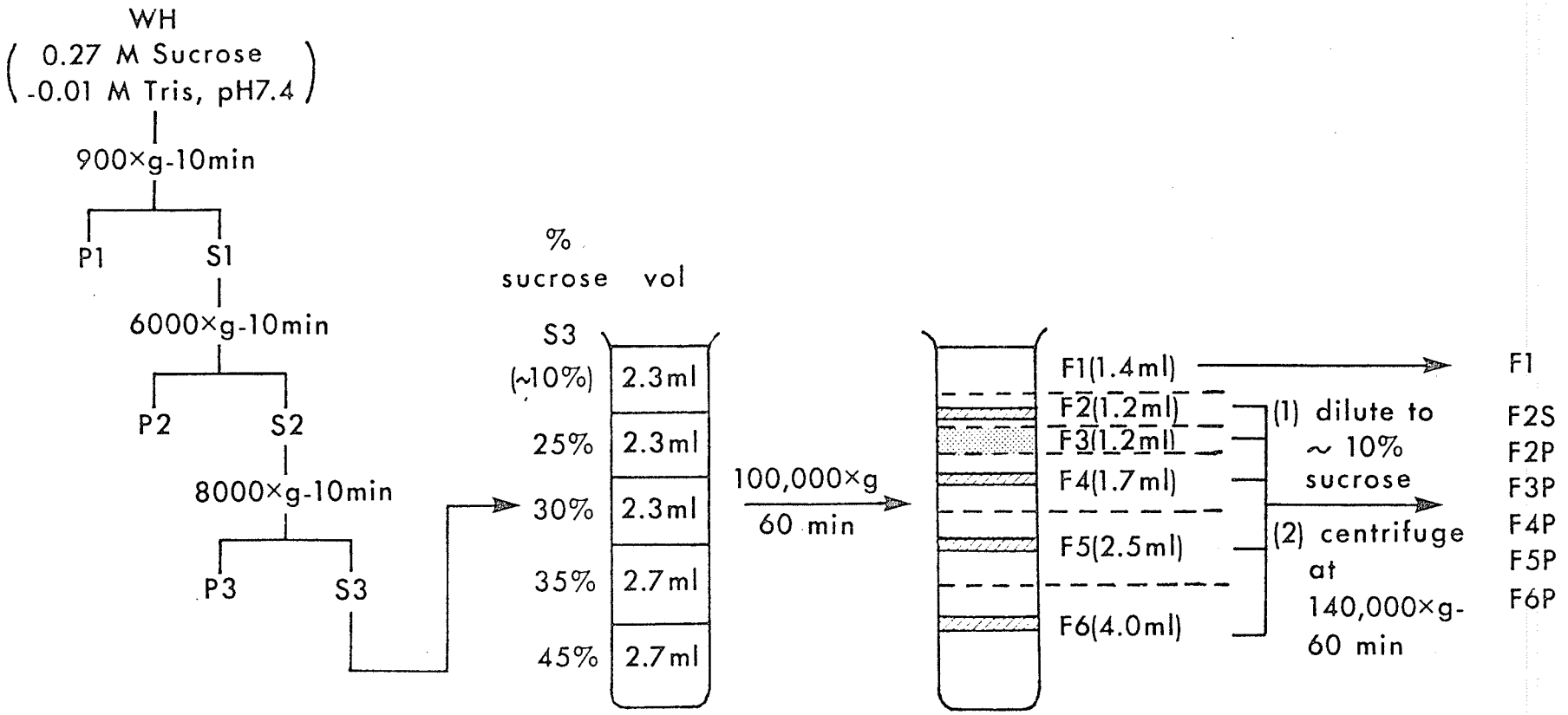


Figure 7: A flow diagram of the procedures used to isolate plasma membranes from rat lungs. F2P and F3P are the plasma membrane fractions.

with ice-cold deionized water to a sucrose concentration of about 10%, and spun at 140,000 xg for 60 minutes. The pellet from each fraction was resuspended in 1 ml of 0.27M sucrose - 0.01M Tris buffer (pH 7.4) by homogenizing in a Potter-Elvehjem homogenizer with a loose fitting teflon pestle (Figure 7). The samples were stored at -20°C until various enzyme assays were performed, usually within one week.

3. Estimation of protein content

Proteins were estimated as described previously.

4. Assay of adenylate cyclase activity

Assay of adenylate cyclase activity was described previously. In those assays when supernatant was added, the same amount of water in the incubating medium was displaced such that the volume in each assay remained constant at 100 µl.

5. Assay of 5'-nucleotidase activity

The method used was described by Michell and Hawthorne[117]. The reaction mixture in a volume of 0.5 ml contained 50 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 100 mM KCl, 10 mM sodium potassium tartrate, 2 mM 5'-AMP, and about 100 µg sample protein. The reaction was started with the addition of 5'-AMP, continued for 15 minutes at 37°C and stopped by the addition of 0.5 ml of ice-cold 10% TCA. Control tubes contained the same reagents, except 5'-AMP was added after the reaction was stopped with TCA. Denatured protein was spun

down in a clinical centrifuge at 2000 RPM. The clear supernatant was analyzed for P_i using the method described by Chen et al.[118].

6. Assay for glucose-6-phosphatase activity

The method used was described by de Duve et al.[119]. In a volume of 0.5 ml, the reaction mixture contained 70 mM histidine-10 mM EDTA buffer (pH 6.5), 40 mM glucose-6-phosphase, and about 100 µg of sample protein. Reaction was initiated with the addition of glucose-6-phosphate, continued for 20 minutes at 37°C and stopped by the addition of 0.5 ml of ice-cold 10% TCA. Control tubes were treated similarly except that glucose-6-phosphate was added after the addition of TCA. Denatured protein was removed by centrifugation and the clear supernatant was analyzed for P_i[118].

7. Assay of acid phosphatase

The method used was that described by Hubschner and West[120]. The enzyme activity was assayed in a medium of 0.5 ml containing 50 mM sodium acetate - 4 mM EDTA buffer (pH 5.4), 15 mM β-glycerophosphate and about 100 µg tissue protein. The control tubes also contained 1 mM sodium fluoride to inhibit acid phosphatase. Reaction was started with the addition of β-glycerophosphate, continued for 15 minutes at 37°C, and terminated by the addition of 0.5 ml ice-cold 10% TCA. Denatured protein was sedimented by centrifugation and the clear supernatant was analyzed for P_i[118].

8. Assay of cytochrome c oxidase activity

Enzyme activity was assayed by the method described by Wharton and Tzagoloff[121]. Into a 2 ml cuvette was added 1 ml of reagents containing 10 mM phosphate buffer (pH 7.0), 0.1% triton X-100, 0.07% reduced ferrocytochrome c, and about 100 µg of protein sample. Reaction was initiated with the addition of cytochrome c and the rate of decrease in absorbance at 500 nm was recorded in a dual beam Pye-Unicam SP8-100 Spectrophotometer. The blanks were prepared by oxidizing the cytochrome c in the cuvettes with an excess of potassium ferric cyanide.

9. Estimation of DNA content

Samples were denatured in a 20% TCA solution, and centrifuged at 5000 RPM in an International refrigerated centrifuge (Model B-20). The precipitate was washed with 15% TCA and centrifuged again. The precipitate was then suspended in 5% TCA and heated at 90°C for 20 minutes. The supernatant was analyzed for DNA content in a mixture containing 1 ml of supernatant and 2 ml of 1% diphenylamine in glacial acetic acid which contained 2.75% of sulfuric acid. The mixture was boiled for 10 minutes and the absorbance at 600 nm was recorded.

E. RESULTS AND DISCUSSION

1. Isolation of plasma membrane fraction from lungs

The current method employed to prepare plasma membrane fraction was a modification of the one described by Hallman and Gluck[10]. To prepare plasma membrane from rat lungs, Hallman and Gluck followed the method by Neville[108] as modified by Ray[122] which was designed to isolate rat liver plasma membrane from low speed pellet. This required a relatively low speed spin at 1500 xg and was followed by discontinuous sucrose gradient centrifugation to obtain a plasma membrane fraction. However, in the rat lung, this low speed pellet contained only a small portion of the plasma membrane marker enzyme activity (Table IV). In the present study, lung homogenate was subjected to 900x, 6000x, and 8000xg spins and only 15%, 8 % and 7 % of the total membrane marker 5'-nucleotidase activity were recovered in each pellet. The majority of the activity (66%) still remained in the 8000xg supernatant. Thus it was decided to prepare plasma membranes from lung tissues from the 8000xg supernatant rather than from the 1500xg pellet which was used by Hallman and Gluck. Besides, the 1500xg pellet unavoidably contained a lot of cellular debris and subcellular organelles trapped within. In the present report, lung homogenate and supernatants recovered from each spin were subjected to increasing centrifugation forces, at 900xg to remove cellular debris and nuclei, 6000xg and 8000xg to remove the majority of mitochondria and lysosomes as these speeds were employed by Hallman and Gluck to isolate these organelles

from rat lungs. For characterization purpose, these three spins were carried out. For future work when only plasma membrane is desired, only one spin at 8000xg will be adequate.

The 8000xg supernatant was placed on top of a discontinuous sucrose gradient and centrifuged at 100,000xg for 75 minutes in a swing-out bucket rotor. After centrifugation, 6 fractions were recovered. Each fraction was diluted with distilled water to give a 10% sucrose solution and then centrifuged at 140,000xg for 60 minutes. F1 was the sample applied onto the gradient and centrifugation at 140,000xg failed to yield pellet and so F1 was used as such and labelled as cytosolic supernatant. F2 contained some cytosolic supernatant also and after centrifugation at 140,000xg, the pellet was designated F2P and the supernatant F2S. The rest of the fractions F3 to F6 only the pellets were recovered and were designated F3P to F6P (Figure 7).

To characterize the distribution of plasma membrane in the fractions, different marker enzymes activities were studied. Many organelle specific enzymes have been used by various workers to characterize their preparations. These include 5'-nucleotidase, ATPases, p-nitrophenyl-phosphatase, nucleotidepyrophosphatase, leucyl-β-naphthylamidase, and adenylate cyclase which are associated with plasma membranes[123]. Several negative (degree of contamination) plasma membrane markers are

also commonly used. They were glucose-6-phosphatase and NADH-cytochrome c reductase which are microsomal enzymes; cytochrome c oxidase, succinate cytochrome c reductase, and succinate dehydrogenase which are mitochondrial enzymes; and acid phosphatase and β-glucosidase, lysosomal enzymes. The marker enzymes used in this study were 5'-nucleotidase, adenylate cyclase (plasma membranes), glucose-6-phosphatase (microsomes), acid phosphatase (lysosomes), and cytochrome c oxidase (mitochrondria). DNA content in each fraction was also studied to determine the amount of nuclear contamination.

The distribution and recovery of protein, DNA, and various marker enzymes from each step of separation were listed in Table IV. Total recovery of protein and DNA from all fractions was 80% and 90%, respectively. About 24% of the protein was recovered in the 900xg pellet which contained nuclei, cellular debris, and unbroken cells. The 6000x and 8000xg pellets together contained about 11% of the total protein. The 8000xg supernatant contained 52% of the proteins which were predominantly soluble proteins represented by a 38% recovery in the F1 and F2S fractions. The rest of the gradient fractions contained a small amount of proteins each ranging from 0.9% to 2.5% of the total. Most of the DNA (75%) came down in the 900xg pellet in the nuclear fraction P1. The 8000xg supernatant contained only 7% of the total amount of DNA. The majority (4.4%) of this was recovered in the F1 and F2S fractions and probably represented DNA that was dispersed

into the cytosol during homogenization.

Total recoveries of 5'-nucleotidase, adenylate cyclase, and glucose-6-phosphatase activities were 70%, 74%, and 64%, respectively. However, activities of these enzymes in the F2S fraction were not measured which would probably add another 5-10% to the recovery. The majority of the 5'-nucleotidase and adenylate cyclase activities remained in the 8000xg supernatant accounting for 66% and 56%, respectively, of the total whole homogenate activities. These activities were all spread out in the gradient fractions. However F2P and F3P together contained the most and accounted for 19% and 25%, respectively, of the total 5'-nucleotidase and adenylate cyclase activities. The specific activities in these two fractions were among the highest and about 6 times higher than that of the whole homogenate.

Glucose-6-phosphatase activity recovered in the 8000xg supernatant was relatively low accounting only 37% of the total activity. The distribution among the gradient fractions was quite even with about 3% in each, and the specific activity was highest in F5P with a 4 fold increase over that of the whole homogenate. The specific activity in F4P and F6P was the next highest with about 3 fold purification. The specific activity in F2P and F3P fractions was comparatively lower.

Total recoveries of acid phosphatase and cytochrome c oxidase activities were very high with values of 96% and 102%, respectively.

Most of these activities were recovered in the 6000xg pellet with a small amount in the 8000xg pellet. Consequently the gradient fractions had either none or very little activities of these enzymes.

Thus, marker enzyme studies and DNA analysis revealed that the 8000xg supernatant was a good source of plasma membranes as it was least contaminated with nuclei, mitochondria, and lysosomes. Most of the DNA was recovered in the 900xg pellet and most of the mitochondria and lysosomes recovered in the 6000xg and 8000 xg pellets. The 8000xg supernatant contained 7% of the total DNA and non-detectable acid phosphatase and cytochrome c oxidase activities while retaining 52% of the total protein, 66% of 5'-nucleotidase, and 56% of adenylate cyclase activities, the latter two being plasma membrane marker enzymes. However, the 8000xg supernatant also contained some microsomal enzyme activity (glucose-6-phosphatase) which accounted for 37% of the total. After separation on a discontinuous sucrose gradient, the specific activity of 5'-nucleotidase was highest in fractions F3P and F4P with about 6 fold purification over that of the whole homogenate. However, the recovery of total activity was highest in fractions F2P and F3P; together they contained 18.5% of the total. The specific activity of adenylate cyclase was highest among fractions F2P, F3P, and F4P with over 6 fold purification but again, recovery was highest in fractions F2P and F3P containing about 25% of the total whole homogenate activity.

Glucose-6-phosphatase activity was heavily distributed in fractions F4P, F5P, and F6P and with specific activities higher in these fractions than in F2P and F3P indicating that the majority of the microsomes was in heavier fractions. Since the activities of 5'-nucleotidase and adenylate cyclase were predominantly present in the upper fractions and the activities of glucose-6-phosphatase in the lower fractions, it was decided to choose F2P and F3P as the plasma membrane fractions for future studies. F4P contained high specific activities of 5'-nucleotidase and adenylate cyclase also but the recoveries were less and the specific activity of glucose-6-phosphatase was high, thus it was not chosen.

The plasma membrane fractions of F2P and F3P contained about 4.1% of the total protein in the whole homogenate. Contamination by DNA was minimal with about 0.5% of the total DNA present. Contamination by mitochondria was represented by about 1% of the total cytochrome c oxidase activity. There was no measurable lysosomal enzyme, acid phosphatase activity. Although there was some glucose-6-phosphatase activity (about 6.5% of the total) which indicated microsomal contamination, there have always been some glucose-6-phosphatase activity in most plasma membrane preparations and it has been suggested that some glucose-6-phosphatase is actually part of the plasma membrane[123]. Conversely it may represent endoplasmic reticulum that is in continuation with the plasma membrane and are not separable. Thus the degree of microsomal

contamination may be less than what the numbers have represented. Acid phosphatase besides being a lysosomal enzyme, is also associated to a lesser extent with microsomal and soluble fractions[123]. The failure to detect acid phosphatase activity in fractions F2P and F3P and its presence in fractions F4P and F5P may also be an indication that the plasma membrane fractions were reasonably pure with least microsomal contamination.

The activities of the various marker enzymes in the rat lung plasma membrane fractions were compared with those in plasma membranes of other tissues (Table V). In general the values are in reasonable agreement. Of particular interest was the rat lung plasma membrane prepared by Hallman and Gluck[10] who isolated the membranes from low speed pellet. They got a more pure fraction with specific activity of 5'-nucleotidase almost twice as much as that reported in this study; however, the recovery of activity in their preparation was only 5.4% of the homogenate compared with a recovery of 18.5% in membranes prepared by the current method.

The current method of preparing plasma membrane from rat lungs is simple, straight forward, and consits of only 2 major steps:

a) Spinning at 8000xg to get rid of the majority of contaminants and at the same time retain the majority of plasma membrane.

b) Applying the 8000xg supernatant onto a discontinuous sucrose gradient and separate the plasma membrane fractions from the microsomal fractions, with good recovery.

All the procedures can be finished in less than 5 hours. Thus it allows time to perform experiments that require fresh plasma membranes if freezing of the tissue overnight is not preferred.

2. Activation of plasma membrane by cytosolic supernatant F1

It has been reported that after homogenization and separation of lung crude homogenate into particulate and supernatant fractions by centrifugation at 100,000xg, the particulate adenylate cyclase enzyme lost much of its activity and addition of the supernatant led to an increase in the particulate adenylate cyclase activity while the supernatant itself did not contain any enzyme activity[100]. This loss in adenylate cyclase activity was also demonstrated in the plasma membrane fractions. Table VI compared the adenylate cyclase activity and recovery in different fractions obtained from 2 days and 45 days old rat lungs. While the 8000xg supernatant[53] in both age groups contained between 50 to 56% of the total whole homogenate activity, the recovery in the membrane fractions (F2P and F3P) from 45 day old rat lungs was only 2.9% compared to a recovery of 25.1% in similar fractions in 2 day old rat lungs. Similarly the relative specific activity in the plasma membrane fractions of 45 day old lungs was much lower (~1) than corresponding figures in the 2 day old rats which showed a 6 fold purification over the homogenate.

The ability of the cytosolic supernatant F1 to activate the lung plasma membrane adenylate cyclase was tested in another group of rats

35 days of age. Table VII showed the homogenate adenylate cyclase activity was 95 pmoles cyclic AMP/minute/mg protein which was comparable to the data reported in the first part of this thesis (Table I). The plasma membrane fraction was obtained by combining gradient fractions F2P and F3P. Instead of obtaining a 6 fold increase in adenylate cyclase activity as noted in the 2 day old lungs, the specific activity of adenylate cyclase in the plasma membrane of the 35 day old rat lungs was only 98 pmoles cyclic AMP/minute/mg protein, similar to that in the whole homogenate. While the cytosolic fraction F1 itself did not have any adenylate cyclase activity, its addition to plasma membrane activated the adenylate cyclase enzyme many fold. Activation of the enzyme by F1 was proportional to the amount of supernatant protein added up to about 160 µg of cytosolic protein to 92 µg of plasma membrane protein and has increased the specific activity of the membrane enzyme by about 800% over the whole homogenate activity (Figure 8). Thus it seems the cytosolic supernatant F1 was able to restore the lost adenylate cyclase activity in the membrane fraction. It should be emphasized here that adenylate cyclase is not a good membrane marker enzyme in older animals when the protein activator is present. In younger animals the enzyme is not modified by this activator and thus it can be used as a marker which is the case in the earlier part of this thesis when 2 day old animals were used to characterize the different fractions by

measuring the adenylate cyclase activity.

Further studies with the isolated plasma membranes and (partially) purified activator may elucidate the mechanism by which the activator modified the basal- and hormonally stimulated adenylate cyclase activity. Studies identifying the cell types that contain the activator will provide information concerning the possible significance of this activator in lung function.

Table IV Animals used were between 30 and 35 days of age except for adenylate cyclase assay where 2 day old animals were used. All enzyme activities are expressed as nmoles substrate converted /min/mg protein except for adenylate cyclase activity where the unit refers to pmoles cAMP/min/mg protein.

[a]Whole homogenate contains 94.1 mg protein/g lung wt.

[b]Whole homogenate contains 101 µg DNA/mg protein.

TABLE IV

The distribution of protein, DNA, and enzyme activities in various fractions obtained during the isolation of plasma membranes from rat lung.

Fraction	Protein % of WH	DNA % of WH	5'-nucleotidase		Adenylate Cyclase		Glucose-6-Phosphatase		Acid Phosphatase		Cytochrome c Oxidase	
			Specific Activity	% Recovery	Specific Activity	% Recovery	Specific Activity	% Recovery	Specific Activity	% Recovery	Specific Activity	% Recovery
WH	100% [a](94.1±8.9)	100% [b](101±3)	31.9±1.6	100%	31.1±2.5	100%	10.7±0.6	100%	28.7±2.1	100%	3.08±0.20	100%
P1	23.7±2.2	74.1±2.4	20.2±1.4	15.0%	18.2±1.6	13.9%	10.2±1.1	22.6%	27.1±1.7	22.4%	2.14±0.20	16.5%
P2	7.5±0.5	6.2±0.5	37.3±3.0	8.8%	38.9±4.1	9.4%	13.9±1.4	9.7%	191.0±5.8	49.9%	28.58±1.12	69.6%
P3	3.9±0.4	3.6±0.3	57.7±2.3	7.1%	87.5±5.6	11.0%	22.5±2.0	8.2%	164.2±12.4	22.3%	8.85±0.73	11.2%
S1	67.3±3.4	20.1±1.2	41.5±2.0	87.6%	39.1±3.5	84.5%	9.6±0.7	60.4%	31.0±3.2	72.6%	3.73±0.33	81.5%
S2	58.1±2.7	11.4±2.1	43.1±2.6	78.5%	38.0±7.9	71.0%	8.4±0.7	45.6%	11.9±2.9	24.2%	0.61±0.05	11.5%
S3	52.4±1.7	7.2±0.6	40.0±1.6	65.7%	33.1±2.7	55.5%	7.5±0.5	36.7%	none detected		none detected	
F1	22.5±2.1	2.4±0.2	10.9±0.5	7.7%	none detected		3.8±0.2	7.9%	none detected		none detected	
F2S	15.6±1.9	2.1±0.3	data not avail.		data not avail.		data not avail.		data not avail.		data not avail.	
F2P	2.4±0.2	0.3±0.1	108.1±7.2	8.3%	196.3±11.7	15.4%	14.7±1.4	3.4%	none detected		0.81±0.11	0.6%
F3P	1.6±0.2	0.2±0.1	201.0±6.4	10.2%	185.7±13.9	9.7%	21.4±1.7	3.3%	none detected		0.79±0.08	0.4%
F4P	1.0±0.1	0.4±0.1	222.7±9.6	6.7%	216.3±12.8	6.7%	32.4±2.5	2.9%	29.7±4.5	1.0%	2.92±0.24	0.9%
F5P	0.9±0.1	0.3±0.1	158.3±8.7	4.3%	165.3±10.0	4.6%	41.1±3.7	3.4%	23.2±2.6	0.7%	2.47±0.16	0.7%
F6P	0.9±0.1	0.5±0.1	73.9±3.1	2.0%	135.7±7.5	3.8%	33.7±2.9	2.7%	none detected		6.78±0.33	1.9%
Total	79.9%	90.0%		70.0%		74.4%		64.1%		96.3%		101.9%

TABLE V

Comparison of enzyme activities and enzymic relative specific activities ( ) of plasma membranes isolated from different tissues

Source	5'-nucleotidase Activity	Glucose-6-Phosphatase Activity	Acid Phosphatase Activity	Cytochrome c oxidase Activity	Adenylate Cyclase Activity	Reference
Rat Lung	108-201 (3.4-6.3)	14.7-21.4 (1.4-2.0)	none detected	0.79-0.81 (0.25-0.26)	186-196 (6.0-6.3)	present findings
Rat Lung	350 (13)	-	-	-	-	(10)
Rat Liver	620-1370 (16-32)	13-32 (0.2-1.1)	0-6 (not avail.)	-	17-920 (5-100)	(94, 115, 124, 128-130)
Rat Fat Cell	220 (16)	-	-	-	210 (not avail.)	(112,131)
Rat Heart	280 (5)	-	-	-	-	(132)
Rat Kidney	-	0.17 (0.006)	0.2 (0.005)	-	12 (1)	(133)
Bovine Adrenal Medulla	147 (8.4)	36 (5.4)	38 (4)	70 (1.2)	-	(126)
Rat Submandibular Salivary Glands	377-794 (18-38)	23-37 (3.5-5.5)	-	-	16-42 (not avail.)	(134)

All enzyme activities are expressed as nmoles substrate converted/min per mg protein except for adenylate cyclase activity which is expressed as pmoles cyclic AMP/min per mg protein.

TABLE VI

Comparison of adenylate cyclase activity in various fractions obtained during the isolation of plasma membranes from rat lungs of 2 days and 45 days of age

Fractions	2 days of age		45 days of age	
	Specific Activity	% Recovery	Specific Activity	% Recovery
WH	31.1±2.5	100%	220.8±20.3	100%
P1	18.2±1.6	13.9%	15.3±4.0	1.0%
P2	38.9±4.1	9.4%	41.3±5.1	2.1%
P3	87.5±5.6	11.0%	81.3±9.1	1.5%
S1	39.1±3.5	84.5%	194.8±19.7	58.3%
S2	38.0±7.9	71.0%	247.3±26.4	56.0%
S3	33.1±2.7	55.5%	235.0±16.5	50.4%
F1	none detected		none detected	
F2S	data not avail.		data not avail.	
F2P	196.3±11.7	15.4%	191.3±8.8	1.2%
F3P	185.7±13.9	9.7%	225.1±25.2	1.7%
F4P	216.3±12.8	6.7%	222.2±20.3	0.8%
F5P	165.3±10.0	4.6%	165.2±6.1	0.8%
F6P	135.7±7.5	3.8%	54.7±16.5	0.2%
Total		74.4%		9.3%

Figures are refer to mean ± S.E.

[a]Results are expressed as pmoles cyclic AMP/min per mg protein for adenylate cyclase activity.

TABLE VII

Activation of adenylate cyclase of plasma membrane fractions (F2P+F3P) by supernatant F1

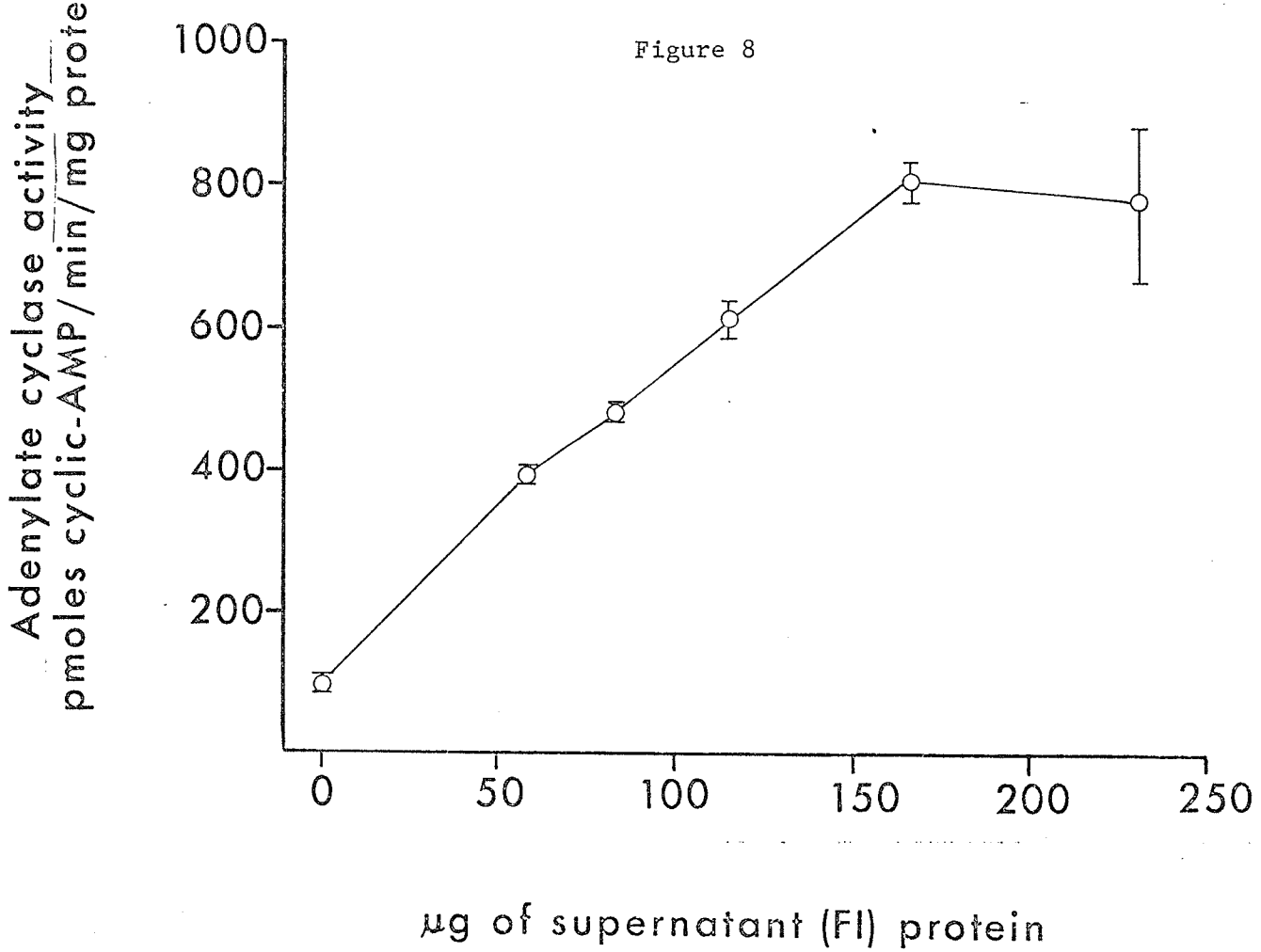
Sample	Addition of F1 (ug protein added)	Adenylate Cyclase Act. (pmoles cAMP/min per mg protein)
Whole Homogenate	0	[a] 94.5±7.5
F1	0	none detected
[b] F2P+F3P	0	98.3±3.9
F2P+F3P	58	390.1±14.2
F2P+F3P	83	481.0±14.0
F2P+F3P	115	610.0±21.2
F2P+F3P	166	808.0±22.7
F2P+F3P	230	777.1±112.2

[a] Figures refer to mean ± S.E.

[b] Each assay tube contains 92.3 ug of plasma membrane protein.

Figure 8 Activation of plasma membrane adenylate cyclase by F1 fraction obtained during isolation of plasma membranes from rat lungs.

Figure 8


Adenylate cyclase activity
pmoles cyclic-AMP/min/mg protein
1000
800
600
400
200
0
50
100
150
200
250
µg of supernatant (FI) protein

III. LUNG FUNCTION AND PHOSPHOLIPID EFFECT

A. INTRODUCTION

Neurotransmitters such as catecholamines and acetylcholine have been shown to enhance $^{32}P_i$ incorporation into acidic phospholipids (Phospholipid Effect) of a variety of tissues[136]. The enhanced phospholipid turnover by these neurotransmitters may have some role in tissue function or more likely in the process of membrane biogenesis[170]. Lungs are innervated by both sympathetic and parasympathetic nerve fibres[171] activity of which is known to influence lung functions. It was therefore expected that lungs would exhibit phospholipid effect in response to catecholamines or acetylcholine. With this objective in mind, $^{32}P_i$ incorporation into phospholipids of rat lung slices was examined in the absence or presence of neurotransmitters.

B. LITERATURE REVIEW

1. Phospholipid effect

It was originally shown by the Hokins 25 years ago that when slices of pancreatic exocrine tissues were stimulated to secrete digestive enzymes by the addition of acetylcholine there was a marked increase of ^{32}P-labelling of the acidic phospholipids, especially phosphatidylinositol (PI)[136]. By 1960, this phenomenon has been observed in several tissues in response to acetylcholine, in salivary glands in response to epinephrine, anterior pituitary exposed to corticotrophin-releasing factor, thyroid slices treated with thyroid

stimulating hormone, and pancreas treated with pancreozymin, and subsequent reviewers have been able to include an ever-increasing list of stimuli and of responsive tissues[136,137]. The interesting feature which links these stimuli is that most of them exert their effects on their target cells through interaction with receptor sites on cell surfaces. In the beginning it was not certain whether the increased labelling was due to enhanced synthesis of PI de novo or due to an increased rate of turnover of a cellular pool of PI of essentially unchanging size until in 1958 when the Hokins[138] reported that in cerebral cortex, the enhanced incorporation of $^{32}P_i$ and of labelled inositol into PI was not accompanied by increased incorporation of labelled glycerol, indicating that the phosphorylinositol portion of the molecule was being renewed in response to the stimulation but that the diacylglycerol backbone of the molecule was re-utilized. Measurements of the PI content of stimulated tissues confirmed that the effect under study did not involve an increase in de novo synthesis of PI. Review of similar studies[136] on a variety of tissues conform to the same pattern except in the case of thyroid glands where whole PI molecule was synthesized in response to stimulation by thyroid stimulating hormone. More recent studies suggested that PI hydrolysis is the key response, which has now been clearly established in several tissues. For example, Jones and Michell[139] reported that in rat parotid gland slices, 10 μM acetylcholine in the presence of 100 μM eserine caused the loss of 32% of tissue PI when incubated for 1 hour while similar loss took place in 5 minutes with 2 mM acetylcholine. Hokin-Neaverson[140] obtained similar results with

pancreas. In this case, the loss of PI was accompanied by an equivalent rise in phosphatidic acid (PA) concentration. In both cases, these effects were blocked by atropine thus indicating that muscarinic cholinergic receptors may be involved. Increases in PI labelling by radioactive precursors are thought to arise indirectly as a result of cleavage of PI into diacylglycerol and phosphorylinositol or cyclic inositol phosphate. This cycle of reactions (Figure 9) was suggested by the Hokins[141] for the avian salt gland and later by Durell et al.[142] for brain, and the four enzymes involved have been found in many tissues[137]. The enzyme thought to be responsible for this PI hydrolysis is a phosphodiesterase specific for phosphorylinositol, have an acid pH optimum, and is usually found in the cytosol but has also been reported in a plasma membrane enriched fraction from the brain[137]. It is also independent of cyclic nucleotides, calcium ions, sodium and potassium ions indicating that PI breakdown may be initiated by a direct interaction at the plasma membrane between the activated muscarinic cholinergic system and the enzyme that catalyses PI hydrolysis[143].

2. Regulation of lung function by neurotransmitters

For the last several years, physiologists are interested in the possibility that cholinergically mediated mechanisms might influence synthesis, secretion, and perhaps removal of pulmonary surfactant.

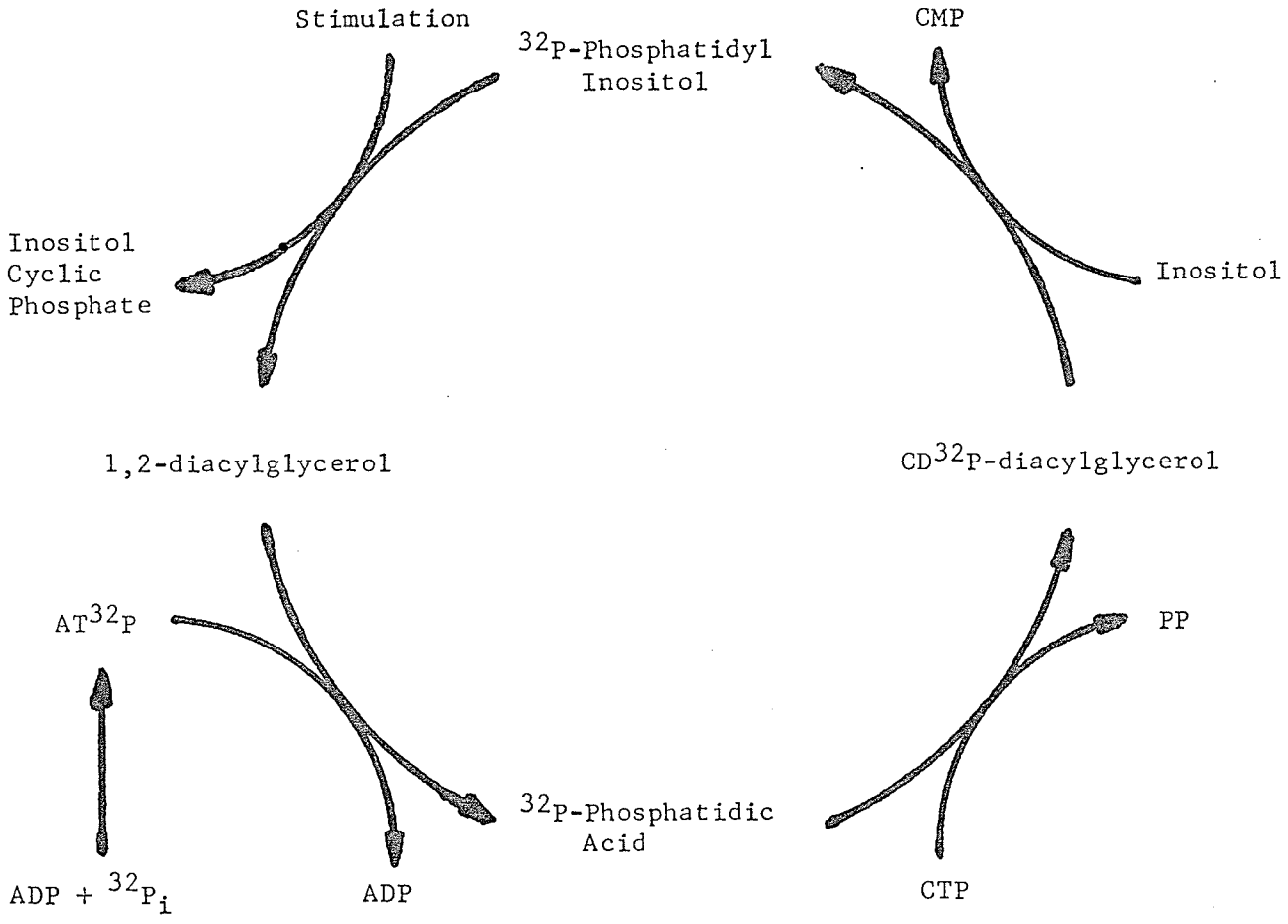


Figure 9 Phosphatidylinositol Cycle

Pulmonary surfactant is continually utilized and replaced in the normal lung, confirmed by studies of the turnover of dipalmitoyl phosphatidylcholine, the principal component of pulmonary surfactant, by tracer methods[144,145]. The possibility of cholinergic influence on surfactant flux is indicated by both biochemical and morphological observations. Bilateral vagotomy in rats caused atelectasis and a decrease in the number of secretory granules in alveolar type II cells[146] while stimulation of efferent left vagus increased the alveolar phospholipids 31% as compared to controls[147]. Administration of pilocarpine caused a rapid expulsion of secretory granules from type II cells into the alveoli and correspondingly, a decrease in the number of lamellar bodies in alveolar type II cells[148]. Administration of acetylcholine and pilocarpine also caused an increase of phospholipids in lung lavage and these effects of acetylcholine and pilocarpine were blocked by atropine[147,149]. Rats treated with pilocarpine to study the incorporation of ^{14}C-palmitate into disaturated lecithin in lung tissue and in the lavage showed an earlier appearance and higher specific activity of disaturated lecithin in the lavage fluid as compared to control animals[150]. ^{14}C-leucine labelling experiments showed that pilocarpine stimulated the release of radioactive protein into the surfactant fraction isolated from lung lavage fluid and this effect was again completely blocked by atropine[151]. Electron microscopic studies revealed innervation of alveolar walls of rat by two different types of nerve endings[152,153], one of which was in close contact with a type II cell and its appearance was consistent with a motor function although ultrastructurally the nerve ending was

not typically adrenergic or cholinergic. Mechanical ventilation of in vivo and in vitro lungs also has been shown to affect lung stability. Increasing the minute ventilation by 100% by augmenting dead space increased alveolar phospholipids by 45% and was prevented by the administration of atropine[147], suggesting that increased ventilation stimulates surfactant release through a cholinergically mediated mechanism.

Lung tissues have also been shown to respond to catecholamines such as norepinephrine and epinephrine[57,77,83] which elicit intracellular responses through both alpha and beta receptors[154]. The intracellular messenger for the beta adrenergic receptor is undoubtedly cyclic AMP which is produced by the activation of adenylate cyclase[155]. The stimulation of alpha adrenergic receptor generally does not lead to a rise in the cellular cyclic AMP concentration but instead causes an increase in cellular cyclic GMP concentration suggesting it to be an alpha adrenergic second messenger[156]. A major controlling factor in the control of cellular cyclic GMP concentration is the cytosolic calcium concentration, rather than another direct control of guanylate cyclase by interaction with a receptor[157]. There are also calcium independent events during an alpha adrenergic stimulation, one of which is the 'PI' response, a sequence of events that involves alpha adrenergically activated PI breakdown and subsequent re-synthesis[136].

It was suggested that the most probable reason for the calcium independence of stimulated PI breakdown was that it has some essential role in the coupling between the activated receptor and the increase in cell surface calcium permeability[136] as opposed to the suggestion that the two were different responses involving different mechanisms[158]. Insofar as appropriate experiments have been carried out it has been found that PI breakdown is an invariable response to the activation of receptors that initiate calcium mobilization[156] which is similar to those proposed for muscarinic cholinergic receptors that act by mobilization of calcium[136].

C. PRESENT STUDY

In view of the ability of muscarinic cholinergic and adrenergic agents to elicit a phospholipid response in a number of experimental systems, the present study has tried to determine whether this would occur in the lung. Such studies would provide information concerning the regulation of lung functions by these hormones and the mechanisms involved.

D. MATERIALS AND METHODS

Male, Sprague-Dawley rats of age 35 days were used in these experiments. Lung slices were prepared as described previously and for each experiment, slices pooled together from 3 animals were utilized.

Pineal glands were obtained from the same animals and whole glands were used as such. The tissues were incubated in 0.5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 containing 15 μCi of $^{32}P_i$ and 1 mg of glucose. Incubation was carried on for 1 or 2 hour period and in the absence or presence of norepinephrine (10 or 30 μM), epinephrine (10 or 30 μM), acetylcholine (250 μM or 1 mM) plus eserine sulphate (100 μ M), myo-inositol (0.5 mg), or norepinephrine (30 μM) and myo-inositol (0.5 mg). After incubation, tissue lipids were extracted and separated by thin layer chromatography. Two solvent systems were used. In the first two experiments where $^{32}P_i$ incorporation into phospholipids was examined for different time intervals and in the presence of different neurotransmitters, the solvent system described by Getz et al.[168] was employed. This system failed to separate PS and PI from each other and the values reported represented a total of the two phospholipids. In later experiments, the solvent system of Pumphrey[68] was employed. This system enabled separation of PS and PI from each other. After separation, the estimation of phospholipid contents and the determination of radioactivity incorporated into phospholipids were carried out as described previously.

E. RESULTS AND DISCUSSION

$^{32}P_i$ incorporation into phospholipids was measured against incubation time for 1 and 2 hour periods. Table VIII showed that the

radioactivity incorporated into the total lipid extract and into phospholipids during a 2 hour incubation period was slightly less than the double of the radioactivity incorporated in 1 hour suggesting that incubation should not be run for more than an hour in future experiments.

Table IX showed that in the presence of different neurotransmitters, there was a marginal increase in the $^{32}P_i$ incorporation into the total lipid extract and into most of the phospholipids examined except PG which did not show any difference from the control. These increases, however, were very slight and were not dose dependent in many cases. For example, the increase in $^{32}P_i$ incorporation into PC in the presence of 10 and 30 μM norepinephrine were the same, about 14%. In the presence of 10 μM norepinephrine or epinephrine, the incorporation of $^{32}P_i$ into PI and PS was actually lower than the control. When the concentration of the neurotransmitters was increased to 30 μM, the incorporation into PI and PS was then higher than the control although not by much. The incorporation of $^{32}P_i$ into phospholipids in the presence of acetylcholine followed a pattern similar to other neurotransmitters, that is, a small increase in radioactivity incorporation into all phospholipids except into PG. Since the phospholipid effect reported by other workers involved only one or two specific phospholipids, the current enhanced rate of $^{32}P_i$ incorporation seems to be, rather, a non specific effect.

To show that the incubation medium in the current study was appropriate for studying the phospholipid effect, pineal glands were incubated under identical conditions and the effect of norepinephrine on $^{32}P_i$ incorporation into phospholipids was studied. Norepinephrine has been shown to enhance $^{32}P_i$ incorporation into PI and PG by several fold in rat pineal glands[159]. Table X showed that 30 μM norepinephrine stimulated $^{32}P_i$ incorporation into pineal PI by about 6 fold, PS by 3 fold, and PG by 6 fold but has no effects on the labelling of PC and PE. However, in lungs slices, the addition of norepinephrine slightly increased $^{32}P_i$ incorporation into all the phospholipids. One may conclude that the experimental conditions used in this study were adequate to study phospholipid effect, and lungs do not appear to exhibit enhanced phospholipid turnover in response to neurotransmitters.

To show that the availability of immediate precursors was not rate limiting in the present study , myo-inositol was added to the incubation medium[169]. Results in Table X showed that norepinephrine produced similar effects on phospholipid labelling as noted previously (Table IX). Addition of myo-inositol alone to the incubation medium did not change $^{32}P_i$ incorporation into PI and SPH, but reduced the labelling of other phospholipids, particularly that into PG. Since PG and PI synthesis shared a common precursor, CDP-diacylglycerol, in the last step of the pathways (Figure 1), addition of myo-inositol undoubtedly has channelled CDP-diacylglycerol

into biosynthesis of PI and very little PG was produced. The synthesis of other phospholipids also shared a common precursor, PA, in earlier parts of their synthetic pathways, therefore these phospholipids also showed a decrease in $^{32}P_i$ incorporation. The exception was SPH which does not share a common precursor used in PI synthesis and thus was not affected. However it should be emphasized that the increase into PI was very small and probably not significant at all. Addition of myo-inositol in the presence of norepinephrine showed a combined effect. $^{32}P_i$ incorporation into most phospholipids has increased compared to the control however this increases were not as much as when norepinephrine alone was present. SPH was again the exception in here while PG showed a substantial decrease. The incorporation of $^{32}P_i$ into PI, however, was not any more than that when norepinephrine alone was present. Thus, the addition of myo-inositol did not alter the incorporation into PI and was therefore, not rate limiting. One might question if the substrates were penetrating into cells in these studies. The fact that addition of myo-inositol decreased the $^{32}P_i$ incorporation into many phospholipids and reduced the incorporation into PG to about 12% of the control suggested that penetration of substrates into the lung cells was not a problem.

Since it has been reported that the first step in a phospholipid effect was the hydrolysis of PI and several reports have shown that incubation with hormones caused a significant loss of tissue PI in a short period of time[139,140], this possibility was also investigated.

Lung slices were incubated with different neurotransmitters and afterwards the phospholipid contents were determined. The data in table XI showed that there was very little difference in the phospholipid content after incubating the lung slices in the presence of norepinephrine or acetyl choline.

At this point it seems quite clear that the phospholipid effect involving specific phospholipids in other tissues was not detected in lung slices. However, lung has many cell types[160] and not one constitutes a major portion of the total cell population. Thus the possibility exists that there was some phospholipid effect in some minority cell types but this effect was not detected when $^{32}P_i$ incorporation was measured in lung tissue as a whole. However, it is quite reasonable to say that the majority of the cell types in the lung do not elicit a phospholipid effect in the presence of these neurotransmitters. Michell in his review has listed tissues in a table which do not show any phospholipid effect[136]. Lung was not one of these tissues and the result of present study showed that it should be added to the list of non-responding tissues.

TABLE VIII

Incorporation of $^{32}P_i$ into Phospholipids for different time time intervals.

Incubation Time	Total Lipid Extract	Phosphatidylinositol + Phosphatidylserine	Phosphatidyl Choline	Phosphatidyl Ethanolamine
hour		CPM / mg tissue		
1	3180	214	2119	52
2	5677	319	3706	98

TABLE IX

Incorporation of $^{32}P_i$ into phospholipids of rat lungs in the presence of neurotransmitters.

Additions	Total Lipid Extract	Phosphatidyl Choline	Phosphatidylinositol + Phosphatidylserine	Phosphatidyl Ethanolamine	Phosphatidyl Glycerol	Phosphatidyl Acid
			CPM / hr / mg tissue			
Control	[a]2934±139	2034±118	197±9	49±4	61±4	104±4
Norepinephrine (10 uM)	3285±131	2322±10	145±3	69±9	62±5	118±2
Norepinephrine (30 uM)	3719±299	2344±93	214±11	84±11	59±8	132±15
Epinephrine (10 uM)	3227±190	2246±134	146±3	78±7	57±3	122±10
Epinephrine (30 uM)	3657±203	2376±107	233±16	70±10	63±3	122±19
[b]Acetylcholine (250 uM)	3107±99	2202±52	202±11	60±5	49±6	130±8
[b]Acetylcholine (1 mM)	3078±112	2408±97	256±13	67±7	52±4	155±11

[a]Figures refer to mean ± S.E.

[b]The incubation medium also contains 100 uM Eserine Sulphate.

TABLE X

Incorporation of $^{32}P_i$ into phospholipids of rat pineal glands and lung slices in the presence of norepinephrine and/or myo-inositol

Additions	Total Lipid	Sphingo myelin	Phosphatidyl Choline	Phosphatidyl Serine	Phosphatidyl Inositol	Phosphatidyl Ethanolamine	Phosphatidyl Glycerol	Phosphatidic Acid	Tissue
Control	[a]18941±1584	80±14	6869±628	202±19	4649±57	2876±25	322±36	790±16	Pineal Gland
NE (30uM)	[a]43532±2712	190±21	6479±317	722±31	27042±3578	2963±148	1966±235	1073±215	Pineal Gland
Control	[b]2966±133	30±1	1876±85	28±5	171±12	49±3	57±6	127±14	Lung
NE (30uM)	[b]3453±63	42±4	2327±35	29±2	239±29	55±3	62±7	158±5	Lung
Myo-Inositol	[b]2591±102	33±2	1660±25	22±4	185±11	41±2	7±2	108±7	Lung
NE (30uM) + Myo-Inositol	[b]3244±160	45±3	2250±170	27±4	230±36	53±4	7±1	144±12	Lung

[a]Results are expressed as CPM/hr per pineal gland

[b]Results are expressed as CPM/hr per mg lung tissue

TABLE XI

Phospholipid contents of rat lungs after incubation in the presence of neurotransmitters.

Additions	Sphingo myelin	Phosphatidyl Choline	Phosphatidyl Serine	Phosphatidyl Inositol	Phosphatidyl Ethanolamine	Phosphatidyl Glycerol	Phosphatidic Acid
	nmoles phosphorus / mg tissue						
Control	[a]1.27±0.06	11.80±0.30	1.20±0.14	0.64±0.15	4.24±0.38	0.59±0.19	0.15±0.12
NE (100uM)	1.25±0.02	10.20±0.78	1.05±0.06	0.50±0.04	3.78±0.09	0.56±0.01	0.14±0.01
[b]Acetylcholine (100 uM)	1.35±0.01	11.30±0.10	1.21±0.02	0.60±0.11	3.84± 0.01	0.51±0.02	0.18±0.05

[a]Figures refer to mean ± S.E.

[b]The incubation medium also contains 100 uM Eserine Sulphate.

IV. REFERENCES

1. Greengard, O. (1971) Enzymic differentiation in mammalian tissues. Essays Biochem. 7:159.

2. Sorokin, S.A. (1961) A study of development in organ cultures of mammalian lungs. Dev. Biol. 3:60.

3. Thurlbeck, W.M. (1975) Postnatal growth and development of the lung. Am. Rev. Resp. Dis. 111:803.

4. Emery, J. (1969) The Anatomy of the Developing Lung, Spastis International Medical Publications, England.

5. Burri, P.H., Dbaly, J., Weibel, E.R. (1974) The postnatal growth of the rat lung. I. Morphometry. Anat. Rec. 178:711.

6. Farrell, P.M., Avery, M.E. (1975) Hyaline membrane disease. Am. Rev. Resp. Dis. 111:657.

7. King, R.J., Clements, J.A. (1972) Surface active materials from dog lung. II. Composition and physiological correlations. Am. J. Physiol. 223:715.

8. Van Golde, L.M.G. (1976) Metabolism of phospholipids in the lung. Am. Rev. Resp. Dis. 114:977.

9. King, R.J. (1974) The surfactant system of the lung. Fed. Proc. 33:2238.

10. Hallman, M., Gluck, L. (1975) Phosphatidylglycerol in lung surfactant, II. Subcellular distribution and mechanism of biosynthesis in vitro. Biochim. Biophys. Acta 409:172.

11. Kennedy, E.P. (1961) Biosynthesis of complex lipids. Fed. Proc. 20:934.

12. Bremer, J., Greenberg, D.M. (1961) Methyl transferring enzyme system of microsomes in the biosynthesis of lecithin. Biochim. Biophys. Acta 46:205.

13. Lehninger, A.L. (1975) Biochemistry, Worth Publishers Inc., New York, 2nd ed., p. 279-308, 659-691.

14. Havel, R.J., Felts, J.M., Van Duyne, C.M. (1962) Formation and fate of endogenous triglycerides in blood plasma of rabbits. J. Lipid Res. 3:297.

15. Popjak, G., Beeckmans, M. (1950) Extrahepatic lipid synthesis. Biochem. J. 47:233.

16. Schiller, H., Bensch, K. (1971) De novo fatty acid synthesis and elongation of fatty acids by subcellular fractions of lung. J. Lipid Res. 12:248.

17. Gildes, C., Sevanian, A., Tierney, D.F., Kaplan, S.A., Barrett, C.T. (1977) Regulation of fetal lung phosphatidyl choline synthesis by cortisol: role of glycogen and glucose. Pediat. Res. 11:845.

18. Niden, A.H. (1967) Bronchiolar and large alveolar cell in pulmonary phospholipid metabolism. Science 158:1323.

19. Azzopardi, A., Thurlbeck, W.M. (1969) The histochemistry of the nonciliated bronchiolar epithelial cell. Am. Rev. Resp. Dis. 99;516.

20. Etherton, J.E., Conning, D.M., Corrin, B. (1973) Autoradiographical and morphological evidence for apocrine secretion of dipalmitoyl lecithin in the terminal bronchiole of mouse lung. Am. J. Anat. 138:11.

21. Macklin, C.C. (1954) The pulmonary alveolar mucoid film and the pneumocytes. Lancet 1:1099.

22. Kikkawa, Y., Motoyama, E.K., Gluck, L. (1968) Study of the lungs of fetal and newborn rabbits: morphologic, biochemical and surface physical development. Am. J. Path. 52:177.

23. Adamson, I.Y.R., Bowden, D.H. (1973) The intracellular site of surfactant synthesis. Autoradiographic studies on murine and avian lung explants. Exp. Mol. Path. 18:112.

24. Chevalier, G., Collet, A.J. (1972) In vivo incorporation of choline-^{3}H, leucine-^{3}H and galactose-^{3}H in alveolar type II pneumocytes in relation to surfactant synthesis: A quantitative radioautographic study in mouse by electron microscopy. Anat. Rec. 174:289.

25. Dickie, K., Massaro, D. (1974) Protein transport by lung. Proc. Soc. Exp. Bio. Med. 145:154.

26. Spitzer, H.L., Rice, J.M., MacDonald, P.C., Johnston, J.M. (1975) Phospholipid biosynthesis in lung lamellar bodies. Biochem. Biophys. Res. Commun. 66:17.

27. Rooney, S.A., Page-Roberts, B.A., Motoyama, E.K. (1975) Role of lamellar inclusions in surfactant production: Studies on phospholipid composition and biosynthesis in rat and rabbit lung subcellular fractions. J. Lipid Res. 16:418.

28. Garcia, A., Sener, S.F., Mavis, R.D. (1976) Lung lamellar bodies lack certain key enzymes of phospholipid metabolism. Lipids 11:109.

29. Engle, M.J., Sanders, R.L., Longmore, W.J. (1976) Phospholipid composition and acyltransferase activity of lamellar bodies isolated from rat lung. Arch. Biochem. Biophys. 173:586.

30. Watts, C., Gain, K.R. (1976) Glycogen metabolism in the liver of developing rat. Biochem. J. 160:263.

31. Shelley, H.J., Bassett, J.M., Milner, R.D.G. (1975) Control of carbohydrate metabolism in the fetus and newborn. Brit. Med. Bull. 31:37.

32. Novak, E., Drummond, G.I., Skala, J., Hahn, P. (1972) Developmental changes in cAMP, protein kinase, phosphorylase kinase, and phosphorylase in liver, heart, and skeletal muscle of rat. Arch. Biochem. Biophys. 150:511.

33. Hommes, F.A., and Beere, A. (1971) The development of adenyl cyclase in rat liver, kidney, brain and sketal muscle. Biochim. Biophys. Acta 237:296.

34. Greengard, O., Dewey, H.K. (1967) Initiation by glucagon of the premature development of tyrosine aminotransferase, serine dehydratase and glucose-6-phosphatase in fetal rat liver. J. Biol. Chem. 242:2986.

35. Christoffersen, T., Morland, J., Osnes, J.B, Øye, I. (1973) Development of cAMP metabolism in rat liver. A correlative study of tissue levels of cAMP. Accumulation of cAMP in slices, adenylate cyclase act. and cyclic nucleotide PDE activity. Biochim. Biophys. Acta 313;338.

36. Ballard, J., Hanson, R.W. (1967) Phosphoenolpyruvate carboxykinase and pyruvate carboxylase in developing rat liver. Biochem. J. 104: 866.

37. Shelley, H.J. (1961) Glycogen reverses and their changes at birth and in anoxia. Brit. Med. Bull. 17:137.

38. Sorokin, S. (1960) Histochemical events in developing human lungs. Acta Anat. 40:105.

39. Williams, M.C., Mason, R.J. (1977) Development of the type II cell in the fetal rat lung. Am. Rev. Resp. Dis. 115:37.

40. Mims, L.C., Mazzuckelli, L.F., Kotas, R.V. (1975) The significances of circulating glycerol as a precursor of pulmonary phosphatidylcholine in the developing mammalian lung. Pediat. Res. 9:165.

41. Felts, J.M. (1965) Carbohydrate and lipid metabolism of lung tissue in vitro. Med. Thorac. 22:89.

42. Robison, G.R., Butcher, R.W., Sutherland, E.W. (1968) Cyclic AMP. Am. Rev. Biochem. 37:149.

43. Sutherland, E.W. Roll, T.W. (1958) Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J. Biol. Chem. 232:1077.

44. Kikkawa, Y., Kaibara, M., Motoyama, E.K., Orzalesi, M.M., Cook, C.D. (1971) Morphologic development of the fetal rabbit lung and its acceleration by cortisol. Am. J. Path. 64:423.

45. Farrell, P.M. (1973) Regulation of pulmonary lecithin synthesis in respiratory distress syndrome, Villee, Villee, Zuckerman, eds. Academic Press, New York, p. 311.

46. Farrell, P.M., Zachman, R.D. (1973) Induction of choline phosphotransferase lecithin synthesis in the fetal lung by corticosteroids. Science 179:297.

47. Rooney, S.A., Gross, I., Motoyama, G.K., Warshaw, J.B. (1974) Effects of cortisol and thyroxine on fatty acid and phospholipid biosynthesis in fetal rabbit lung. Physiologist 17:323.

48. Adamson, I.Y.R., Bowden, D.H. (1975) Reaction of cultured adult and fetal lung to prednisolone and thyroxine. Arch. Path. 99:80.

49. Mukhergie, S.P., Mukhergie, C. (1973) Control of rat adipose tissue metabolism by cortisone in relation to epinephrine. Am. J. Physiol. 224:898.

50. Tombropoulos, E.G. (1973) Palmitate incorporation into lipids by lung subcellular fractions. Arch. Biochem. Biophys. 158:911.

51. Mims, L.C., Kotas, R.J. (1973) Glycerol as a PC precursor in the developing mammalian lung. Biol. Neonate 22:436.

52. Redding, R.A., Douglas, W.H.J., Stein, M. (1972) Thyroid hormone influence upon lung surfactant metabolism. Science 175:994.

53. Barrett, C.T., Sevanian, A., Larin, N., Kaplan, S.A. (1976) Role of cyclic AMP in maturation of fetal lungs. Pediat. Res. 10:621.

54. Gross, I., Rooney, S.A. (1977) Aminophylline stimulates the incorporation of choline into phospholipids in explants of fetal rat lung in organ culture. Biochim. Biophys. Acta 488:263.

55. Levey, G.S., Epstein, S.E. (1969) Myocardial adenyl cyclase: Activation by thyroid hormones and evidence for two adenyl cyclase system. J. Clin. Invest. 48:1663.

56. Klainer, M.A., Orange, R.P., Koopman, W.J., Austen, K.F., Lavaia, P.J., (1971) Cyclic AMP in human lung. Biochim. Biophys. Acta 252:160.

57. Palmer, G.C. (1972) Cyclic AMP response in the rabbit lung--adult properties and development. Biochem. Pharm. 21:2907.

58. Collins, M., Palmer, G.C., Baca, G., Scott, H.R. (1973) Stimulation of cAMP in the isolated perfused rat lung. Res. Comm. in Chem. Path. and Pharm. 6(3):805.

59. DiMarco, P.N., Oliver, I.T. (1978) cAMP in perinatal rat liver. Ontogeny and response to hormones. Eur. J. Biochem. 87:235.

60. Steiner, A.L., Ferrendelli, J.A., Kyines, D.M. (1972) Radioimmunoassay for cyclic nucleotides. J. Biol. Chem.247:1121.

61. Davis, C.W., Kuo, J.F. (1976) Ontogenic changes in levels of phosphodiesterase for cAMP and cGMP in the lung, liver, brain, and heart from guinea pigs. Biochim. Biophys. Acta 444:554.

62. Sicard, R.E., Aprille, J.R. (1977) Adenylate cyclase and cyclic nucleotide phosphodiesterase in the developing rat liver. Biochim. Biophys. Acta 500:235.

63. Drummond, G.I., Duncan, L. (1970) Adenyl cyclase in cardiac tissue. J. Biol. Chem. 245:977.

64. Butcher, R.W., Sutherland, E.W. (1962) Adenosine 3',5'-phosphate in biological materials. J. Biol. Chem. 237:1244.

65. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

66. Morris, D.L. (1948) Quantitative determination of carbohydrates with dreywood's anthrone reagent. Science 107:254.

67. Folch, J., Lees, M., Sloane-Stanley, G.H. (1957: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497.

68. Pumphrey, A.M. (1969) Incorporation of ^{32}P orthophosphate into brain slice phospholipids and their precursors. Effect of electrical stimulation. Biochem. J. 112:61.

69. Yagihara, Y., Bleasdale, J.E., Hawthorne, J.N. (1973) Effects of acetylcholine on the incorporation of ^{32}orthophosphate in vitro into the phospholipids of subsynaptosomal membranes from guinea pig brain. J. Neurochem. 21:173.

70. Fisk, C.H. and Subbarow, Y. (1925) The colorimetric determination of phosphorous. J. Biol. Chem. 66:375.

71. Umbreit, W.W., Burris, R.H., Stauffer, J.F. (1964) Monometric techniques. Burgess Publishing Co., 4th ed.

72. Maniscalco, W.M., Wilson, C.M., Gross, I., Gobran, L., Rooney, S.A. Warshaw, J.B. (1978) Development of glycogen and phospholipid metabolism in fetal and newborn rat lung. Biochim. Biophys. Acta 530:333.

73. Curle, D.C., and Adamson, I.Y.R. (1978) Retarded development of neonatal rat lung by maternal malnutrition. J. Histochem. Cytochem 26(5):401.

74. Curle, D.C. (1977) M.Sc. Thesis, University of Manitoba. Histo- and cytochemistry of developing lungs in normal and malnourished rats.

75. Rooney, S.A., Lee, T.S.W., Gobran, L,, Motoyama, E.K. (1976) Phospholipid content, composition and biosynthesis during fetal lung development in the rabbit. Biochim. Biophys. Acta 431:447.

76. Weinhold, P.A., Vilee C.A. (1965) Phospholipid metabolism in the liver and lung of rats during development. Biochim. Biophys. Acta 106:540.

77. Nijjar, M.S. (1979) Role of cAMP and related enzymes in rat lung growth and development. Biochim. Biophys. Acta (in press).

78. Gross, I. (1977) Nutritional and hormonal influences on lung phospholipid metabolism. Fed. Proc. 36:2665.

79. Wang, N.S., Kotas, R.V., Avery, M.E., Thurlbeck, W.M. (1971) Accelerated appearance of osmiophilic bodies in fetal lungs following steroid injection. J. Appl. Physiol. 30:362.

80. Lizzins, G.C., Howie, R.N. (1972) A controlled trial of ante- partum glucocorticoid treatment for the prevention of respiratory distress syndrome in premature infants. Pediat. 50:515.

81. Anderson, W.B., Pastan, I. (1975) Altered adenylate cyclase activity: Its role in growth regulation and malignant transoformation of fibroblasts. In: advances in cyclic nucleotides research, Vol. 5, Drummond, G.I., Greengard, P, Robison, G.A. eds. Raven Press, Publishers, New York. p. 681.

82. Wu, B., Kikkawa, Y., Orzalesi, M.M., Motoyama, E.K., Kaibara, M., Zigas, C.J., Cook, C.D. (1973) The effect of thyroxine on the maturation of fetal rabbit lungs. Biol. Neonate 22:161.

83. Barrett, C.T., Sevanian, A., Kaplan, S.A. (1974) Adenyl cyclase in immature rabbit lungs. Pediat. Res. 8:244.

84. Stahlman, M.T., Gray, M.E., Lieu, S., Chytil, F. (1974) The role of cAMP in lamellar body synthesis and secretion. Pediat. Res. 8:470. (Abstract)

85. Robison, G.A., Butcher, R.W., Sutherland, E.W. (1971) Cyclic AMP. Academic Press, New York.

86. Davoren, P.R., Sutherland, E.W. (1963) The cellular location of adenyl cyclase in the pigeon erythrocyte. J. Biol. Chem. 238:3016.

87. Birnbaumer, L. (1973) Hormone sensitive adenylyl cyclases. Useful models for studying hormone receptor functions in cell free systems. Biochim. Biophys. Acta 300:129.

88. Birnbaumer, L., Pohl, S.L., Rodbell, M. (1969) Adenyl cyclase in fat cells. I. Properties & effects of adrenocorticotropin and fluoride. J. Biol. Chem. 244:3468.

89. Campbell, B.J., Woodward, G., Borberg, V. (1972) Calcium mediated interactions between the antidiuretic hormone and renal plasma membranes. J. Biol. Chem. 247:6167.

90. Bar, H.P. and Hechter, O. (1969) Adenyl cyclase and hormone action III. Ca^{2+} requirement for ACTH stimulation of adenyl cyclase. Biochim. Biophys. Res. Comm. 35:681.

91. Lelkovitz, R.J., Roth, J., Pastan, I. (1970) Effects of Ca^{2+} on ACTH stimulation of the adrenal stimulation of hormone binding from adenylate cyclase activation. Nature 228:864.

92. Levey, G.S. (1971) Restoration of Norepi-responsiveness of solubilized myocardial adenylate cycase by PI. J. Biol. Chem. 246:7405.

93. Rethy, A., Tomasi, V., Trevisain, A. (1971) The role of lipids in the activity of adenylate cyclase of rat liver plasma membrane. Arch. Biochem. Biophys. 147:36.

94. Pohl, S.L., Birnbaumer, L., Rodbell, M. (1971) The glucagon-sensitive adenyl cyclase system in plasma membrane of rat liver. I. Properties. J. Biol. Chem. 246:1849.

95. Rodbell, M., Birnbaumer, L., Pohl, S.L., Krans, H.M.J.(1971) The glucagon sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. J. Biol. Chem. 246:1877.

96. Palmer, G.C. (1971) Characteristics of the hormonal induced cAMP response in the rat and guinea pig lung in vitro. Biochim. Biphys. Acta 252:561.

97. Vinicor, F., Higden, G., Clark, J.F., Clark, CM.Jr. (1976) Development of glucagon sensitivity in neonatal rat liver. J. Clin. Invest. 58:571.

98. Rodbell, M. (1975) On the mechanism of activation of fat cell adenylate cyclase by guanine nucleotides. J. Biol. Chem. 250:5826.

99. Sanders, R.B., Thompson, W.J., Robison, G.A. (1977) Epi and glucagon stimulated cardiac adenylyl cyclase act. Regulation by endogenous factors. Biochim. Biophys. Acta. 498:10.

100. Nijjar, M.S. (1979) Regulation of rat lung adenylate cyclase by cytoplasmic factors during development. Biochim. Biophys. Acta. (In press)

101. Pecker, F. and Hanoune, J. (1977) Activation of epi sensitive adenylate cyclase in rat liver by cytosolic protein nucleotide complex. J. Biol. Chem. 252:2784.

102. Pecker, F. and Hanoune, J. (1977) Uncoupled B-adrenergic receptors and adenylate cyclase can be recoupled by a GTP-dependent cytosolic factor. F.E.B.S. Lett. 83:93.

103. Londos, C., Salomen, Y, Lin, M.C., Harwood, J.P., Schramin, M., Wolff, J., Rodbell, M. (1974) 5'-guanylylimidodiphosphate, A potent activator of adenylate cyclase systems in erkcryotic cells. Proc. Natl. Acad. Sci. (USA) 71:3087.

104. Doberska, C.A., Martin, B.R. (1977) Activation of rat liver plasma membrane adenylate cyclase by a cytoplasmic protein factor. FEBS Lett. 82:273.

105. Katz, M.S., Kelly, T.M., Pineyro, M.A., Gregerman, R.I. (1978) Activation of epi and glucagon sensitive adenylate cyclases of rat liver by cytosol protein factors. Role in loss of enzyme activities during preparation of particulate fractions, quantitation and partial characterization. J. Cyclic Nucleotide Res. 5: 389.

106. Benedetti, E.L., Emmelot, P. (1968) Structure and function of plasma membranes isolated from liver in The Membranes, Dalton, A.J., Hagnenan, F., eds. 4:33. Academic Press, New York.

107. Singer, S.J., Nicolson, G.L. (1972) The fluid mosaic model of the structure of membranes. Science 175:720.

108. Neville, D.M. Jr. (1960) The isolation of a cell membrane fraction from rat liver. J. Biophys. Biochem. Cytol 8:413.

109. Bosmann, H.B., Hagopian, A., Eylar, E.H. (1968) Cellular membranes. The isolation and characterization of the plasma and smooth membranes of Hela cells. Arch. Biochem. Biophy. 128:51.

110. Coleman, R., Michell, R.H., Finean, J.B. and Hawthorne, JN. (1967) A purified plasma membrane fraction isolated from rat liver under isotonic conditions. Biocheim. Biophys. Acta 135:573.

111. Berman, H.M., Gram, W., Spirtes, M.A. (1969) An improved, reproducible method of preparing rat liver plasma membranes in buffered isotonic sucrose. Biochim. Biophys. Acta 183:10.

112. McKeel, D.W., Jarrett, L. (1970) Preparation and characterization of a plasma membrane fraction from isolated fat cells. J. Cell Biol. 44:417.

113. Kono, T., Colowick, S.P. (1961) Isolation of skeletal muscle cell membrane and sone of its properties. Arch. Biochem. Biophys. 93:520.

114. Emmelot, P., Bos, C.J. (1962) Adenosine triphosphatase in the cell membrane fraction from rat liver. Biochim. Biophys. Acta. 58:374.

115. Emmelot, P., Bos, C.J., Bennedetti, E.L., Rumka, P. (1964) Studies on plasma membranes. I. Chemical composition and enzymic content of plasma membranes isolated from rat liver. Biochim. Biophys. Acta 90:126.

116. Kamat, V.B., Wallach, D.F.H. (1965) Separation and partial purification of plasma membrane fragments from Ehrlich Ascites Carcinoma microsomes. Science 148:1343.

117. Michell, R.H., Hawthorne, J.N. (1965) The site of diphosphoinositide synthesis in rat liver. Biochem. Biophys. Res. Comm. 21:333.

118. Chen, P.S., Toribara, T.Y., Warner, H. (1956) Microdetermination of phosphorus. Anal. Chem. 28:1756.

119. DeDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., Applemans, F. (1955) Tissue fractionation studies, 6. Intracellular distribution patterns of enzymes in rat liver tissue. Biochem. J. 60:604.

120. Hubscher, G., West, G.R. (1965) Specific assays of some phosphatases in subcellular fractions of small intestinal mucosa. Nature 205:799.

121. Wharton, D.C., Tzagoloff, A. (1967) Methods in enzymology: Estabrook, R.W., Pulmann, M.E., eds. 10:245, Academic Press, New York.

122. Ray, T.K., Skipski, V.P., Barclay, M., Essner, E.E., Archibald, F.M. (1969) Lipid composition of rat liver plasma membranes. J. Biol. Chem. 224:5528.

123. Solyom, A., Trams, E.G. (1972) Enzyme markers in characterization of isolated plasma membranes. Enzyme 13:329.

124. Gavard, D., DeLamirande, G., Karasaki, S. (1974) Highly purified plasma membranes from rat heptocytes following rate isopycnic zonal centrifugation. Biochim. Biophys. Acta 332:145.

125. Brumley, G.W., Chernick, V., Hodson, W.A., Normand, C., Fenner, A., Avery, M.E. (1967) Correlations of mechanical stability, morphology, pulmonary surfactant, and phospholipid content in the developing lamb lung. J. Clin. Invest. 46:863.

126. Nijjar, M.S., Hawthorne, J.N. (1974) A plasma membrane fraction from bovine adrenal medulla: Preparation, marker enzyme studies and phospholipid composition. Biochim. Biophys. Acta 367:190.

127. Buckingham, S., Avery, M.E. (1962) Time of appearance of surfactant in the fetal mouse. Nature (London)1930:688.

128. Lanter, C.J., Solyam, A., Trams, E.G. (1972) Comparative studies on enzyme markers of liver plasma membranes. Biochim. Biophys. Acta 266:511.

129. Emmelot, P., Bos, C.J. (1971) Studies in plasma membranes. XIV. Adenyl cyclase in plasma membrane isolated from rat and mouse livers, and hepatomas, and its hormone sensitivity. Biochim. Biophys. Acta 249:285.

130. Marinetti, G.V., Ray, T.K., Tomasi, (1969) Glucagon and epinephrine stimulation of adenyl cyclase in isolated rat liver plasma membranes. Biochem. Biophys. Res. Com. 36:185.

131. Avruch, J., Wallach, D.F. (1971) Preparation and properties of Plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. Biochim. Biophys. Acta 233:334.

132. Kidwai, A.M., Radcliffe, M.A., Duchon, G., Daniel, E.Z. (1971) Isolation of plasma membrane from cardiac muscle. Biochem. Biophys. Res. Comm. 45:901.

133. Wilfong, F.R., Neville, D.M. Jr. (1970) The isolation of a brush border membrane fraction from rat kidney. J. Biol. Chem. 245:6106.

134. Nijjar, M.S., Pritchard, E.T. (1972) Characterization of plasma membranes isolated from rat submandibular salivary glands. Arch. Oral. Biol. 17:1679.

135. Hokin, M.R., Hokin, L.E. (1953) Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. J. Biol. Chem. 203: 967.

136. Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. Biochim. Biphys. Acta 415:81.

137. Hawthorne, J.N., White, D.A. (1975) Myo-inositol lipids in vitamins and hormones. Munson, P.L., Diszfalusy, E., Glover, J., Olson, R.E. eds. 33:529. Academic Press, New York.

138. Hokin, L.E., Hokin, M.R. (1958) Acetylcholine and the exchange of phosphate into phosphatidic acid in brain microsomes. J. Biol. Chem. 233:822.

139. Jones, L.M., and Michell, R.H. (1974) Breakdown of PI provoked by muscarinic cholinergic stimulation of rat parotid gland fragments. Biochem. J. 142:583.

140. Hokin-Neaverson, M.O. (1974) Acetylcholine causes a net decrease in PI and a net increase in PA in mouse pancreas. Biochem. Biophys. Res. Com. 58:763.

141. Hokin, M.R., Hokin, L.E. (1964) Metabolism and physiological significance of lipids. Dawson, R.M.C., Rhodes, D.N. Eds. John Wiley, London, p. 423.

142. Durell, J., Garland, J.T., Friedel, R.O. (1969) Acetylcholine action: biochemical aspects--two major approaches to understanding the mechanism of action of acetylcholine are examined. Science 165:862.

143. Jones, L.M., Michell, R.H. (1976) Cholinergically stimulated PI breakdown in parotid-gland fragments is independent of the ionic environment. Biochem. J. 158:505.

144. Naimark, A. (1973) Cellular dynamics and lipid metabolism in the lung. Fed. Proc. 32:1967.

145. Tierney, D.F., Clements, J.A., Trahan, H.J. (1967) Rates of replacement of lecithins and alveolar instability in rat lungs Am. J. Physiol. 213:671.

146. Goldenberg, V.E., Buckinaham, S., Sommers, S.C. (1967) Pulmonary alveolar lesions in vagotomized rats. Lab. Invest. 16:693.

147. Oijarzun, M.J., Clements, J.A. (1977) Ventilatory and cholinergic control of pulmonary surfactant in the rabbit. J. Appl. Physiol. 43:39.

148. Goldenberg, V.E., Buckingham, S., Sommers, S.C. (1969) Pilocarpine stimulation of granular pneumocyte secretion. Lab. Invest. 20:147

149. Olsen, D.B. (1972) Neurohumoral hormonal secretory stimulation of pulmonary surfactant in the rat. Physiol. 15:230.

150. Morgan, T.E., Morgan, B.C. (1973) Surfactant synthesis, storage and release by alveolar cells. In: Respiratory Distress Syndrome, Villee, C.A., Vilee, D.B. , Zuckerman, J. eds. Academic Press, New York, p. 117.

151. Massaro, D.C. (1975) In vivo protein secretion by lung. Evidence for active secretion and interspecies differences. J. Clin. Invest. 56:263.

152. Meyrick, B., Reid, L. (1971) Nerves in rat intra-acinar alveoli: an electron microscopic study. Resp. Physiol. 11:367.

153. Hung, K.S., Hertweek, M.S., Hardy, J.D., Loosli, C.G. (1972) Innervation of pulmonary alveoli of the mouse lung: an electron microscopic study. Am. J. Anat. 135:477.

154. Ahlquist, R.P. (1948) A study of the adrenotropic receptors. Am. J. Physiol. 153:586.

155. Lefkowitz, R.J. (1975) Catecholamine stimulated myocardial adenylate cyclase: Effect of phospholipase digestion and the role of membrane lipids. J. Molec. Cell. Card. 7:27.

156. Jones, L.M., Michell, R.H. (1978) Stimulus-response coupling at alpha adrenergic receptors. Biochem. Soc. Trans. 6(3):673.

157. Goldberg, N.D., Haddox, M.C. (1977) cGMP metabolism and involvement in biological regulation. Ann. Rev. Biochem. 46:823.

158. Oron, Y., Lowe, M., Selinger, Z. (1975) Incorporation of inorganic ^{32}P phosphate into rat parotid phosphatidyl inositol. Molec. Pharm. 11:79.

159. Hauser, G., Nijjar, M.S., Smith, T.L., Erchberg, J.C. (1978) Nature of the receptors which mediate enhanced phosphatidylinositol turnover in rat pineal gland. In: Cyclitols and phosphoinositides. Wells, W.W., Eisenberg, F.Jr. eds. Academic Press, New York, p. 167.

160. Sorokin, S.P. (1970) The cells of the lungs. In Morphology of Experimental Carcinogenesis. Nehesheim, P., Hanna, M.G. Jr., Deatherage, J.W. Jr. eds. Oak Ridge, Tennessee, U.S. Atomic Energy Commission, p. 3.

161. Carson, S.H., Taeusch, W., Avery, M.E. (1973) Inhibition of lung cell division after hydrocortisone injection into fetal rabbits. J. Appl. Physiol. 34:660.

162. Sutherland, E.W. (1972) Studies on the mechanism of hormone action. Science 177:401.

163. Ryan, W.L., Heidrick, M.L. (1968) Inhibition of cell growth in vitro by cyclic AMP. Science 162:1484.

164. Hsie, A.W., Puck, T.T. (1971) Morphological transformation of Chinese hamster cells by dibutyryl adenosine cyclic 3':5'-monophosphate and testosterone. Proc. Natl. Acad. Sci. USA 68:358.

165. Johnson, G.S., Friedman, R.M., Pastan, I. (1971) Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine-3':5'-cyclic monophosphate and its derivatives. Proc. Natl. Acad. Sci. USA 68:425.

166. Girard, J.R., Guiendet, G.S., Marliss, E.B., Kerorum, A., Rilutort, M., Assan, R. (1973) Fuels, hormones and liver metabolism at term and during the early postnatal period in the rat. J. Clin. Invest. 52: 3190.

167. Blazquez, E., Sugase, T., Blazquez, M., Foa, P.P. (1974) Neonatal changes in the concentration of rat liver cyclic AMP and of serum glucose, free fatty acids, insulin, pancreatic and total glucagon in man and in rat. J. Lab. Clin. Med. 83:957.

168. Getz, G.S., Jakoucic, S, Heywood, J., Frank, J., Rabinowitz, M. (1970) A two dimensional thin layer chromatographic system for phospholipid separation. The analysis of yeast phospholipids. Biochim. Biophys. Acta 218:441.

169. Freinkel, N., El Younsi, C., Dawson, R.M.C. (1975) Inter-relations between the phospholipids of rat pancreatic islets during glucose stimulation and their response to medium inositol and tetracaine. Eur. J. Biochem. 59:245.

170. Toback, F.G., Havener, L.J., Dodd, R.C., Spargo, B.H. (1977) Phospholipid metabolism during renal regeneration after acute tubular necrosis. Am. J. Physiol. 232:E216.

171. Greep, R.O., Weiss, L. (1973) The respiratory system in: Histology, 3rd ed., p. 706, Blakiston Publication, Toronto.