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The Effect of Maternal Administration of Pilocarpine on the Maturation of the Rabbit Fetal Lung

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THE EFFECT OF MATERNAL ADMINISTRATION OF
PILOCARPINE ON THE MATURATION OF
THE RABBIT FETAL LUNG

by

Dennis M. Smith

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
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VITA

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PUBLICATIONS


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INTRODUCTION

It is well established that immaturity of the lung is the primary etiologic factor predisposing to the development of the respiratory distress syndrome of the newborn, the major cause of death in premature infants. The overall maturity of the fetal lung has been found to depend to a great extent on the maturity of the type II epithelial cells of the alveolus. These cells are the recognized source of lung surfactant, a surface tension lowering substance, rich in dipalmitoylphosphatidylcholine, which lines the alveolar space and prevents the collapse of the lung at expiration. The type II cells contain characteristic lamellar inclusion bodies, the intracellular storage form of surfactant, as well as other secretory elements including rough endoplasmic reticulum, Golgi apparatus and multivesicular bodies, the presumed precursors of lamellar inclusion bodies.

Previous studies have indicated that glucocorticoids, and perhaps other pharmacologic agents, promote fetal lung maturation by accelerating the structural, functional and biochemical development of the alveolar type II cells. Pilocarpine, a parasympathomimetic alkaloid, has been shown by previous studies to stimulate the secretion of surfactant by type II cells in the adult. There is little information available concerning the effects of pilocarpine on the surfactant system of fetal animals. There is, however, some evidence that it is capable of causing increased secretion of surfactant in the later
stages of gestation.

This study was designed to investigate the reactions of the developing fetal rabbit lung to multiple maternal doses of pilocarpine on successive days at a point in gestation when the surfactant system is just beginning to mature. These reactions include morphologically demonstrable changes in surfactant synthesis and secretion as well as changes in the maturation process of the fetal lung, such as changes in cell numbers or cell division.

Ultrastructural studies constituted the main emphasis of this project and employed morphometric and stereologic analyses. These data are correlated with light microscopic morphometric analysis, a biochemical study and a new application of the rapid technique of scintillation autoradiography.
General Concepts of the Pulmonary Surfactant System

A Swiss physician, Von Neergard ('29), was the first to suggest that the surface tension of fluid in the alveoli of the lung might be lower than that of other body fluids, and that this might be due to the accumulation of surface active material. Studying pressure-volume relationships in excised degassed pig and dog lungs filled with either fluid or air, he found that the transpulmonary pressures were lower for a given volume of fluid than air and concluded that 2/3 to 3/4 of the retractive pressure of the lung was due to surface forces. The theoretical significance of surface forces in the initial expansion of the lungs at birth was appreciated by Wilson and Farber ('33) and Gruenwald ('47). Surface properties of the lung were discussed by Radford et al. ('54) in relation to the maintenance of alveolar stability and the determination of respiratory surface area.

A major contribution was made by Pattle ('55) who demonstrated that bubbles expressed from the lungs were very stable in air and had a high resistance to anti-foam agents. He concluded that these bubbles had a very low surface tension, less than 0.06 dynes/cm. Furthermore, low surface tension was found to be a unique property of these pulmonary bubbles since bubbles made of fluid from other sources, such as blood, showed no such stability and decreased rapidly in size when ex-
posed to air. Further studies supported his original conclusions that
the surface tension of the lining film of the lungs was very close to
zero (Pattle, '60, '65). In addition, he theorized that in the absence
of extremely low surface tension, the alveolar "bubble of air" would
collapse upon itself due to the law of Laplace \( P = \frac{2\gamma}{r} \), where \( P \) = internal pressure, \( \gamma \) = surface tension and \( r \) = the radius of the alveolus) giving rise to massive atelectasis.

Clements et al. ('57, '58) measured the surface tension lowering
properties of lung extracts obtained by rinsing lungs with 0.09% sa-
line, mincing whole lungs in saline and squeezing material from the
cut surfaces of lungs into saline. They found that, in vitro, as a
film of this lung material was compressed, the surface tension dropped
and, furthermore, that the changes in surface tension during compres-
sion and expansion of the film did not coincide. About the same time
Mead et al. ('57) described static pressure-volume characteristics of
saline and air filled dog lungs. They found that in excised lungs the
surface forces were minimized by saline filling and consequently hyst-
eresis, or the difference between the curves on inflation and defla-
tion, was small. In air-filled lungs, however, hysteresis was marked
and much like that observed by Clements and co-workers in films of lung
extracts.

It is now well established that the major function of pulmonary
surfactant is the maintenance of alveolar stability. As the radius of
the alveolus decreases, pulmonary surfactant reduces the surface ten-
sion at the air-liquid interface. If pulmonary surfactant were not
present, upon expiration there would be massive alveolar collapse, or
avtelectasis. Due to the action of surfactant in lowering the surface
tension, a residual volume of air always remains in the lung (Avery and
Said, '65).

Other demonstrated or theorized physiologic functions of surfac-
tant, as reviewed by many authors including Avery and Mead ('59),
Scarpelli ('68), Morgan ('71) and Read ('71), may be summarized as
follows. **Alveolar liquid balance.** It is thought that in the absence
of surfactant the increased surface tension in the alveoli would aug-
ment capillary hydrostatic pressure and result in transudation of fluid
into the alveoli. This may occur in some pathologic conditions of the
lung, resulting in massive pulmonary edema. **Pulmonary capillary flow.**
The surface tension in the alveoli does rise during inflation of the
lung, as shown in a typical hysteresis loop. This controlled rise in
surface tension exerts a limited amount of force which does augment
capillary pressure, as described above. This regulated force aids in
overcoming gravity and "pulling" blood up to the apices of the lungs.
**Clearance of the alveolar space.** Although this task is accomplished
primarily by the alveolar macrophages, pulmonary surfactant does appear
to play a role. Surfactant is cleared from the alveolar spaces, in
part, by a central flow through the bronchiolar tree and trachea to the
pharynx, along with the mucous sleeve of the airways. In so doing,
debris which was trapped in the surfactant layer is carried along and
thus cleared from the alveolar space. **Reaction to infection.** The al-
veolar surface layer has also been implicated as part of the body's de-
fense mechanism. This is thought to involve an antigen-antibody reaction which localizes bacteria at the alveolar surface (Shifrine and Gourlay, '65).

The presence of a fluid lining layer in the alveoli of mammalian lungs was first described morphologically by Terry ('26) and subsequently elaborated upon by him (Terry, '45). The significance of this lining layer was, for the most part, overlooked until the 1950's when the physiology of the surfactant system was being described. Macklin ('54, '55) provided the first strong morphologic evidence of an extracellular lining layer in the alveoli. He observed that the alveoli were lined by an "exterior alveolar mucoid fluid" which had the staining characteristics of mucopolysaccharides. By phase contrast microscopy, he observed "myelin figures" forming the layer, which he postulated as consisting of either lecithin or cholesterol. His studies were followed by several reports dealing with the staining affinities of the lining layer, all using light microscopy (Groniowsky and Biczyskowa, '69; Bernstein et al., '69; Kikkawa et al., '70).

Convincing electron microscopic demonstration of this alveolar lining layer was for a time lacking, due to the difficulty in preserving it during fixation. Weibel and Gil ('68) were the first to clearly show the ultrastructure of this layer. Their observations were confirmed by Kikkawa ('70), Kuhn ('72) and others. It is now well established that the alveolar lining layer consists of two portions, the first being a mucinous coat which is an integral part of the alveolar epithelial cell membranes (Balis et al., '71). This is similar to
other such coats which exist on the surfaces of many cells and is distinct from the second part of the alveolar lining layer, the surfactant layer. This surfactant layer, in turn, consists of two portions, a base layer, or hypophase, which contains proteins and mucopolysaccharides and a 4 nm thick superficial lamellar layer. This lamellar layer is thought to be a monolayer of highly polar lipids which forms naturally, since it is a physical property of the lipids which comprise it to form monolayers in water (Luzzati and Husson, '62). Weibel's electron microscopic description agreed well with what was postulated as the physical composition of the surfactant layer by earlier authors (Pattle and Thomas, '61; Klaus et al., '61; Clements, '67) based on biochemical data.

The hypophase of the surfactant layer appears, for the most part, homogenous due to protein and mucopolysaccharide components. However, there are also surfactant lipids present in the hypophase which appear as characteristic "myelin figures" or lattice-like tubular myelin, due to the same physical forces which cause them to form monolayers (Klaus et al., '62; Balis and Conen, '64). These lipids are 80% phospholipids (King, '74) of which the major proportion is phosphatidylcholine and phosphatidylglycerol, with some phosphatidylethanolamine and sphingomyelin (King and Clements, '72; Hallman and Gluck, '75; Van Golde, '76). The molecular species of phosphatidylcholine are also quite distinct, being mostly disaturated molecules and, in fact, predominantly dipalmitoylphosphatidylcholine (DPC) which comprises about 50% of the total surfactant lipids (Toshima and Akino, '72; Van Golde, '76).
DPC owes its great surface activity largely to its bipolar molecular configuration - a hydrophilic choline group and two hydrophobic saturated fatty acid side chains. This bipolar configuration causes it to form packed layers, and the presence of saturated side chains, as opposed to unsaturated, allows the molecules to be very tightly packed (Read, '71; Balis and Shelley, '72). Radioisotope studies have indicated that the major pathway for the de novo synthesis of surfactant phosphatidylcholine is the CDP-choline pathway (Van Golde, '76).

Some of the confusion concerning the surfactant layer of the alveolus was due to a long standing controversy as to whether the alveolar epithelium was a continuous epithelium, covering the capillary endothelial cells, or discontinuous, leaving the capillary cells exposed directly to the air in the alveolus. In the early 1950's Low and co-workers published electron microscopic studies of mammalian lungs, which convincingly demonstrated for the first time that the epithelium of the alveolus was indeed continuous (Low and Daniels, '52; Low, '53, '54; Low and Sampaio, '57). This work was substantiated by the similar findings of Karrer ('56) and Kisch ('60). It is now universally accepted that the alveoli of the mammalian lung are lined by a continuous layer of endodermally derived cells. The epithelial cells can be characterized as two distinct types with particular, specialized functions. The type I cells cover the great majority of the alveolar surface and are essential for gaseous exchange in the lung. They are characterized by a central nucleus and wide, attenuated cell extensions which form the epithelial constituent of the three layered blood-air
barrier, the other two portions being a fused basal lamina and the pulmonary capillary endothelium. The attenuated cytoplasm is approximately 0.1 to 0.2 μm thick and thus may only be visualized using electron microscopy. The nuclear region is approximately 0.7 μm thick and here are found the majority of the cellular organelles which include mitochondria, Golgi complex and rough endoplasmic reticulum. The attenuated cytoplasm contains few organelles but numerous pinocytotic vesicles which play a role in transepithelial transport. Contacts between type I cells, or between type I and type II cells, are typical "tight" junctions with close apposition of cell membranes. Synonyms for the type I alveolar epithelial cell include type-B cell, small alveolar cell, surface epithelial cell and membranous pneumocyte (Kilburn, '74).

Interspersed among the type I cells are type II alveolar epithelial cells which have also been called type-A cells, corner cells, vacuolated and non-vacuolated alveolar cells, septal cells and granular pneumocytes (Kilburn, '74). The morphometric relationships of alveolar epithelial cells have been documented by Weibel ('76). In the lung of the adult rat he found the type II cells to cover far less surface area along the alveolar lumen than type I cells (69 μm²/cells vs. 4003 μm²/cells). He also observed that type II cells are smaller than type I cells (336 μm²/cell vs. 812 μm²/cell) but more numerous (14.1% vs. 8.3% of lung parenchymal cells).

The type II cells were first noted by Reinhardt in 1847 and so were resolvable by light microscopy. In paraffin sections they are easily confused, however, with alveolar macrophages, but are readily
studied in 1 to 2 \( \mu m \) plastic sections. They may be "tucked" away in a niche in the alveolar wall, or bulging out into the lumen, being exposed to air on the lateral surfaces of the cell. They may occur alone or in groups of two or three and tend to occur more often at points where several adjacent alveoli meet.

The ultrastructure of the type II alveolar epithelial cells has been described by many authors including Macklin ('54), Karrer ('56), Balis and Conen ('64), Sorokin ('67), Kikkawa ('70) and others. The cells rest on a continuous basal lamina, have a diameter of from 7 to 14 \( \mu m \), a spherical vesicular nucleus, which is frequently eccentric, and a nucleolus which is usually visible. They are roughly cuboidal but do exhibit a great amount of pleomorphism. The cells also exhibit some degree of polarity in that the apical surface is covered with short stubby microvilli which have fine axial filaments similar to those found in microvilli on the cells of other organs (Kuhn, '68). Mitochondria are fairly numerous within the cytoplasm and are seen as "plump" or sometimes "rod-like" structures with parallel cristae. These cristae often fail to reach the center of the mitochondrion and are less numerous than those in mitochondria of other cell types, such as striated muscle (Sorokin, '67). Peroxisomes have been identified in type II cells of some species and are seen as enlarged bodies with a single limiting membrane (Schneeberger, '72). Lysosomes have also been observed by most authors and contain acid phosphatase and other hydro- lases. There are usually two centrioles located apically in the cell, although they are seldom both seen in the same ultrathin section. One of these may, during cytodifferentiation, send out a short cilium into
the alveolar space; however, this never persists into the mature stage of the cell.

The secretory nature of the cell is suggested by an abundant rough endoplasmic reticulum which is found throughout the cytoplasm of the cell, taking up much space between other organelles. The cisternae are loosely packed, differing in this respect from those of other secretory cells, such as pancreatic acinar cells. Some authors have described a flocculent precipitate within the cisternae, particularly when they are dilated (Sorokin, '67). Ribosomes are abundant on the cisternae as well as free within the cytoplasm, forming arrays. The Golgi apparatus appears in section to consist of both vesicles and stacks of lamellae in paranuclear, often apical, locations. In some cases there appear to be multiple vesicles of Golgi apparatus scattered widely throughout the cytoplasm. Multi-vesicular bodies, although known to exist in other cells, such as the ovum (Sotello and Porter, '59), are particularly prominent in type II pneumocytes. These membrane bound structures may contain a few, or many, smooth surfaced vesicles and have been observed to incorporate Golgi vesicles (Sorokin, '67).

The most unique structure to be found within the type II cell is what are now termed lamellar inclusion bodies. These are, or have been, also known as cytosomes, multilamellar bodies or alveolar inclusion bodies. Macklin ('54), using light microscopy, observed osmiophilic granules in the cuboidal alveolar epithelial cells and thus originated the term granular pneumocyte in reference to these
cells. In paraffin embedded tissue sections processed routinely these granules appear as empty holes or vacuoles. When lung tissue is processed with minimal use of organic solvents, however, their contents stain intensely with osmium, periodic acid-Schiff, sudan black and toluidine blue. They are completely extracted by chloroform-methanol treatment. These and other histochemical reactions lead to the conclusion that the inclusion bodies contain lipids. By electron microscopy, lamellar inclusion bodies appear as roughly spherical or elongate, membrane bound and varying greatly in size. They consist of intensely osmiophilic lamellae, or membranes, which may be concentrically arranged or present in parallel arrays. Some problems were encountered in the study of the ultrastructure of the lamellar inclusion bodies in past years due to the partial or total extraction of their lipid content by polar dehydrants. Because of this Scarpelli ('68) suggested the adjective "lamellar" be omitted from their descriptive title since the morphology of these inclusion bodies was undetermined and variable from one report to the next. Studies utilizing the freeze-fracture technique (Lauweryns and Gombeer-Desmecht, '72; Smith et al., '72) established that the lamellar inclusion bodies were indeed packed with lipid membranes. The periodicity of the membranes, however, remains controversial. Some (Pattle et al., '72; Lauweryns and Gombeer-Desmecht, '72) have reported a 40 nm periodicity, while others (Smith et al., '72) felt a periodicity of 80 nm to be more accurate. Recently, the use of special lipid retention procedures, such as tannic acid fixation, has added considerably to the knowledge of the ultrastructure of the lamellar inclusion bodies. Stratton ('76), using these techniques
and high voltage electron microscopy, found a periodicity of 66 nm to be the most probable in vivo.

In interpreting ultrastructural observations of the lamellar inclusion bodies, the problem of interspecies variation must be considered. The studies which have dealt with the periodicity of the lamellar membranes have employed primarily rat lung, where the lamellae are highly regular and densely packed. Kikkawa and Spitzer ('69) demonstrated that a great deal of variation exists in the morphology of lamellar inclusion bodies among mammals. For example, the inclusion bodies of adult rabbits are somewhat vacuolated with far fewer membranes than those of the rat, while guinea pig inclusion bodies are intermediate in terms of lamellar appearance.

Some investigators have claimed that the type II cell is merely a phagocytic cell (Niden, '67). Others (Shulz, '59; Klaus et al., '62) suggested that the lamellar inclusion bodies are transformed mitochondria. However, it is now well established that the type II cells are the source of pulmonary surfactant and that the lamellar inclusion bodies are the intracellular storage form of surfactant. Using a method developed in 1938 (Sjostrand and Sjostrand), Macklin ('54) was the first to suggest that the "mucoid" film of the alveolar space was secreted by the granular pneumocytes. Ten years later Balis and Conen ('64) and Bensch ('64) reported a secretory function for the type II cells, based on the electron microscopic observation of lamellar inclusion bodies being secreted into the alveolar space. Similar observations have since been made by many investigators in the field (O'Hare
and Sheridan, '70; Smith et al., '79). Some have also claimed that this appearance might just as easily represent phagocytosis of surfactant material by type II cells. Detailed ultrastructural studies of the type II cell surface, however, have yielded evidence that the lamellar bodies are indeed being secreted from, not ingested by, the type II cell (Askin and Kuhn, '71). Moreover, it is thought that normal type II cells possess no phagocytic properties (Karrer, '60).

Buckingham and Avery ('62) correlated the appearance of lamellar bodies in the fetal mouse with the first appearance of normal surface tension properties. This temporal relationship between the appearance of surface activity, the alveolar lining layer and lamellar inclusion bodies adds support to the concept of the lamellar bodies as intracellular surfactant and has since been described in the rat (Buckingham et al., '64), rabbit (Kikkawa et al., '68), lamb (Kikkawa et al., '65) and human (Campiche et al., '63). Histochemical studies at the ultrastructural level have also indicated that the lamellar inclusion bodies consist of phospholipids very similar in all respects to those found in the surfactant layer of the alveolus (Campiche, '60; Sorokin, '66; Dermer, '70), thereby linking the two.

More recently, strong evidence in support of the relationship between lamellar inclusion bodies and surfactant synthesis has come from studies employing radiolabelled surfactant precursors. Askin and Kuhn ('71) administered \(^{3}H\)-palmitate intravenously to rats and found that it was incorporated primarily into the surfactant lipid lecithin. At all time periods studied they found heavy labelling of type II
alveolar cells and specifically the lamellar inclusion bodies. Chevalier and Collet ('72) studied the incorporation of several pre-
cursors into lung epithelial cells, using electron microscopic auto-
radiography. They observed that $^3$H-choline, a specific precursor of
surfactant phosphatidylcholine, selectively labelled type II pneumocytes.
In addition, they provided evidence that the lamellar inclusion bodies
are formed through a process similar to that found in other secretory
cells. After initial incorporation in the rough endoplasmic reticulum,
the labelling was found progressively over the Golgi apparatus, the
lamellar bodies, and then finally in the alveolar space over the
myelin figures, which are thought to represent secreted surfactant.
These findings have been substantiated by Massaro and Massaro ('72)
in an autoradiographic study of intracellular surfactant protein transfer
in type II cells. Although autoradiographic evidence has appeared which
favors the view of the Clara cell of the terminal bronchiole as the
source of surfactant (Niden, '67; Etherton et al., '73), other reports
have convincingly demonstrated that the secretion of the Clara cells is,
in major part, proteinaceous in nature (Roth and Meyer, '72; Ebert et al.,
'76). In addition, comparative autoradiographic studies have also
added support to the concept of the type II cell as the source of
surfactant (Adamson and Bowden, '73; Frasca, '77).

Since the lung consists of over 40 individual cell types (Sorokin,
'70), detailed biochemical or functional studies of type II cells alone
have been lacking. Recently, however, some investigators have been
able to isolate relatively pure populations of viable type II cells.
Kikkawa and Yoneda ('74) were the first to accomplish this in rat lung,
and with co-workers ('75) published a valuable study of the composition and synthetic activity of type II cells. Others (Fisher and Furia, '77; Mason et al., '77; Pfleger, '77) have since accomplished similar isolations and have contributed information concerning the role of the type II alveolar epithelial cell in surfactant synthesis, storage and release.

Through the various lines of evidence discussed above, and innumerable studies, the following scheme of lamellar body synthesis and surfactant secretion has been well established. The lipids and proteins are synthesized in the rough endoplasmic reticulum and passed on to the Golgi apparatus, where vesicles are "pinched" off and packaged as multivesicular bodies. These then undergo a lamellar transformation to mature inclusion bodies while approaching the apex of the cell. Upon reaching the apex the limiting membrane of the lamellar body fuses with the cell membrane, and the membranous contents of the inclusion body are discharged into the hypophase of the existing alveolar lining layer. In the alveolar space the molecules of surfactant may take part in forming the 4 nm thick surface active film, or remain in reserve in the hypophase, forming myelin figures or tubular myelin arrays. The process of the lamellar body membranes unfolding and rearranging to form tubular myelin arrays in the hypophase has recently been convincingly demonstrated by the use of high voltage electron microscopy (Williams, '77; Sanderson and Vatter, '77). To complete the story, "used" surfactant is thought to leave the alveolar space in several ways. It may flow up the respiratory tree, be phagocytized by the alveolar macrophages present in the hypophase,
be absorbed through the alveolar epithelium or enzymatically degraded **in situ** (Goerke, '74).

**Fetal Lung Development**

The lungs of all mammals, including man, which have been studied during the course of their development have been found to share a common pattern of origin, development and maturation. The epithelium of the respiratory tract originates as a diverticulum from the endoderm of the caudal pharynx (Moore, '73; Yokoh, '77). This diverticulum elongates as the laryngotracheal tube, developing lung buds which grow and divide by dichotomous branching to form primitive respiratory passages. The mesenchyme surrounding the endodermal outgrowth is destined to become the smooth muscle, cartilage and blood vessels of the lung. It has been shown that specific interaction between the endoderm and mesoderm is essential for normal lung growth and cellular differentiation (Taderara, '67; Masters, '76).

Further development of the lung may be divided into three major periods. The glandular (pseudoglandular) period is the earliest and is so called because the microscopic appearance of the lung at this stage resembles glandular tissue. The air conducting system develops during this period and the potential airways are lined by a low columnar to high cuboidal epithelium. Also during this period, the mesenchymal mass around the airways is condensing. The canalicular period is characterized by enlarging respiratory passages which are lined by cuboidal epithelium, by a vascularization of the surrounding mesenchyme and by the appearance of primitive respiratory bronchioles
and what may be termed alveolar septa (Conen and Balis, '69). The alveolar period is the final stage of development and is characterized by the massive proliferation of alveoli and the thinning of the alveolar septa. Concurrent with this is the development of the great majority of the blood-air barriers which will be required for gaseous exchange. Since only 1/8 to 1/6 of the adult number of alveoli are present in the lungs at birth, in the human this stage is said to continue up till the age of ten years (Moore, '73).

Although it was generally accepted that the epithelium of the air conducting system was an endodermal derivative, the germ layer origin of the alveolar epithelium was, for a time, in question. Policard et al. ('57) felt that both the type I and type II alveolar epithelial cells were of mesodermal origin. Many investigators, influenced by its reported phagocytic characteristics, believed that the type II cell was of mesodermal origin, as is the alveolar macrophage (Divertie and Brown, '64; Bertalanffy, '64). Others (Low and Sampaio, '57; Campiche et al., '63; Suzuki, '66) thought that a common endodermal origin was more likely, judging by the junctional complexes between type I and type II cells and the presence of a common basal lamina. Morphologic (Kikkawa et al., '68; O'Hare and Sheridan, '70) and autoradiographic (Hitchcock, '68) studies have established the concept of a common endodermal derivation for all the respiratory passages, including both types of alveolar epithelial cells.

The mature type I and type II epithelial cells which are found in the alveolus of the term fetus both stem from a common immature
cuboidal cell which is already present at the canalicular phase of development. The internal organization of this undifferentiated cell is relatively simple, in that a great deal of the cytoplasm is filled with fairly densely packed glycogen and varying, usually small, amounts of rough endoplasmic reticulum. A few mitochondria are also present, but inconspicuous, and the Golgi is normally lacking. Lamellar inclusion bodies or multivesicular bodies are not present. If a given immature cuboidal cell is destined to become a type I cell, extensions begin to grow out from its lateral borders while a gradual thinning of the entire cell takes place (Balis et al., '66; Adamson and Bowden, '75). The internal organization of the cell remains at about the same simple level of the undifferentiated cell, although occasionally a poorly formed lamellar inclusion may be observed (Mercurio and Rhodin, '76). This, however, is occasionally true of type I cells in the adult lung as well (Kikkawa and Kaibara, '72). The major intracytoplasmic change which occurs during the differentiation of type I cells is the loss of glycogen as the extensions thin to form blood-air barriers. As these blood-air barriers mature, they become thinner by the attenuation of the type I epithelium and, often, by the repositioning of the capillary cell nucleus. At earlier stages these nuclei lie directly under the epithelial component of the blood-air barrier but, as maturation proceeds, they come to lie at the opposite pole of the capillary, toward the alveolar septa.

The same immature cuboidal cells may also undergo major changes during the differentiation to mature type II cells. These changes all correspond with the cell developing the cytoplasmic specializations
required for the manufacture and secretion of pulmonary surfactant. A cuboidal cell which lines the fetal alveolus may be termed a type II cell, as such, at the first appearance of lamellar inclusion bodies. This occurs rather late in gestation and, as mentioned, is one criterion which helped link the lamellar inclusion bodies to the synthesis of surfactant. Of course the secretory apparatus which manufactures the surfactant is also becoming highly developed during this same time.

One of the hallmarks of the differentiation of type II cells from their immature cuboidal precursors is the loss of the glycogen content which had been built up by the immature form (Brandstrup and Kretchmer, '65; Kikkawa, '68). It is thought that this decrease in glycogen content occurs because of the increase in surfactant synthesis. Glucose has been shown to be an important precursor for phosphatidylcholine synthesis in the adult rabbit lung (Felts, '64), and there is indirect evidence that glycogen is a precursor for, or is somehow necessary for, phosphatidylcholine synthesis in fetal rabbit lung (Gilden et al., '77). Finally, as the cell matures there occurs an increase in the number of lamellar inclusion bodies and their precursor forms, multivesicular bodies.

This basic scheme of differentiation and maturation in the alveolar epithelium has been studied in many mammals including the human (Balis and Conen, '64; Campiche, '63; McDougall and Smith, '75), lamb (Kikkawa et al., '65), rat (O'Hare and Sheridan, '70; Williams, '77), mouse (Woodside and Dalton, '58), pig (Baskerville, '76) and
hamster (Banks and Epling, '71). Kikkawa and co-workers ('68, '71) and Motoyama et al. ('71), working with Kikkawa, have contributed greatly to the understanding of the morphologic development of the alveolar epithelium in the rabbit. Their findings may be summarized as follows: On the 21st gestational day a columnar epithelium containing a moderate amount of glycogen lined the respiratory passages. These cells also contained quite a few profiles of rough endoplasmic reticulum which, in some cases, were distended. The Golgi apparatus was often present, but inconspicuous. The alveolar epithelium of the 24th gestational day was characterized by the presence of large amounts of glycogen packed densely into the cells. The cells also contained a few mitochondria and rough endoplasmic reticulum similar to that observed at the 21st gestational day. Various oval shaped, electron dense bodies were found in the vicinity of a well defined Golgi. In 26 day fetal rabbit lungs they observed some differentiation of the alveolar epithelium into type I and type II cells. The type I cells at this point contained no glycogen and were already fairly well attenuated. The type II cells contained large amounts of glycogen, some lamellar inclusion bodies and an occasional multivesicular body. Fetal lung examined at 27 days showed type I and type II cells which were fairly well differentiated, and a further decrease in the glycogen content of the type II cells. At 28 days of gestation Kikkawa concluded that the fetal rabbit lung, including the type II epithelial cells, was completely mature. He based this primarily on the lack of glycogen, the presence of blood-air barriers and the observation of relatively numerous lamellar inclusion bodies.
Kikkawa has also described a maturation process for the lamellar inclusion bodies within the fetal rabbit type II cells ('69). The most immature of the inclusion bodies are uniformly electron dense with perhaps one or two lamellae within them and were termed "dense bodies" by Kikkawa. "Transitional forms" were the next stage of the maturation process and were characterized as possessing a number of well formed lamellae with varying amounts of amorphous, electron dense material in the center. Finally, "fetal inclusions" were the most mature forms described by Kikkawa and consisted of many well formed lamellae. The mature lamellar inclusion body of the fetal rabbit lung possesses many more lamellae than that of an adult rabbit, although the reason for this difference has not become clear. Recently, this high degree of lamellar development in fetal rabbit inclusion bodies has been confirmed by the application of freeze-fracture techniques (Kikkawa and Manabe, '78).

Any study dealing with the maturation of the fetal lung has had to take into account the fact that different portions of the lung mature at different rates. Howatt et al. ('65) observed that the upper lobes of premature newborn lambs were more compliant and that surfactant appeared earlier here than in the lower lobes. Kikkawa et al. ('71), using morphologic criteria, reported that the apex of the right upper lobe of the fetal rabbit lung was advanced 1.5 gestational days beyond the maturational stage of the base of either lower lobe.

Cellular proliferation in the fetal lung is also of interest in
describing maturational changes occurring prenatally. Sorokin ('61) demonstrated that the mitotic index of lung parenchymal cells decreased with advancing gestational age. In an extensive autoradiographic study of cell proliferation in fetal rat lung, O'Hare and Townes ('70) reported that the labelled cell indices of alveolar epithelial cells and lung interstitial cells was constant in the midportion of gestation, but abruptly decreased in late gestation. They administered tritiated thymidine to pregnant rats and, in others, injected colchicine in order to study mitotic indices. The results of the two studies showed comparable trends and thus provide evidence that the \(^3\text{H}\)-thymidine indices are accurate reflections of mitotic activity in fetal lung cells. They also concluded that the decreasing mitotic and labelling indices paralleled the reduction of glycogen levels in pulmonary epithelial cells. Essentially the same results were reported by Kauffman ('75) in a similar autoradiographic study of cell proliferation in fetal mouse lung. She concluded that the decrease in cell proliferation in late gestation was due to the fact that the cells undergoing differentiation were removed from the proliferative "pool" of earlier gestational ages due to a significant increase in cell cycle time.

Scintillation Autoradiography

Although the foregoing studies utilized autoradiography, they illustrate a drawback which is common to all autoradiographic studies, the length of time necessary to obtain adequate exposure of the emulsion. The technique of scintillation autoradiography was pioneered by
Panayi and Neill ('72) based on a somewhat similar technique which had been previously described for use in paper chromatography (Wilson, ('58) and thin layer chromatography (Luthi and Waser, '65). By dipping emulsion coated slides in a scintillation mixture, Panayi and Neill were able to reduce the exposure time to one day in a study of cell division in lymphocyte cultures. Durie and Salmon ('75) also studied blood cell division using this technique and were able to shorten the exposure time to six hours by the use of tritium with a high specific activity. Platkowska ('76) employed stripping film autoradiography and a scintillation mixture in a study which employed 6 μm paraffin tissue sections as well as lymphocyte smears. This technique has also been applied to the study of chromosomal DNA synthesis in the lily plant (Sen et al., '77) and drosophila (Mukherjee and Chatterjee, '77). In the only study to date using plastic embedding, Fischer et al. ('71) used a related technique of mixing scintillator with Epon resin and obtained electron microscopic autoradiographic results after 14 days of exposure.

Respiratory Distress Syndrome of the Newborn

Interest in the type II epithelium, its maturation and the secretion of surfactant in the fetus was stimulated by the observation of Avery and Mead ('59) that the lungs of infants who had died of respiratory distress syndrome of the newborn (RDS) lacked sufficient amounts of pulmonary surfactant. RDS afflicts approximately 1,000 premature infants per week in the United States alone (Gluck, '73) and although therapeutic measures designed to date have increased the
likelihood of survival, it remains a major disease with a high mortality rate. Those infants who do survive often develop neurologic and intellectual deficits due to the significant post-natal anoxia associated with RDS.

The clinical disease entity as such has been the subject of excellent reviews (Villee et al., '73; Farrell and Avery, '75) and therefore only morphologic features relevant to this project will be discussed briefly. It has been clearly demonstrated that the lungs of most infants who develop RDS are at an immature stage of maturation at birth, in that the alveoli are predominantly lined by characteristically immature type II epithelial cells and possess relatively few blood-air barriers (Balis et al., '66). This anatomic immaturity of the lung may lead to a deficiency of surfactant secretion, difficulty in inflating the lung at birth and consequent expiratory atelectasis. Anoxia and other factors lead to necrosis of type I epithelium and increased transudation of plasma proteins. This results in formation of the characteristic hyaline membranes found at autopsy in the lungs of infants who succumb to RDS. Lungs of infants who survive following therapeutic management show a regenerated alveolar epithelium, composed of immature type II cells, which progressively matures. It should be emphasized that the precise structural, functional and biochemical characteristics of the infant lung at birth, with or without RDS, have not been well documented and many in utero factors may influence these characteristics.

The normal infant, possessing a mature lung whose alveoli
contain sufficient surfactant at birth, retains 50% of the air which it inhales with its first breath (Karlberg et al., '62). This functional residual volume is the hallmark of pulmonary maturity and what is decidedly lacking in the premature infant with insufficient surfactant at birth.

Drug Induced Alterations of Fetal Lung Maturation

The findings of Avery and Mead ('59) have been substantiated by many investigators. This established relationship between RDS, lung surfactant and the maturity of the type II alveolar epithelium led to investigations concerned with factors which could possibly influence the maturation of the type II epithelial cells and the production of surfactant in the fetus. Buckingham et al. ('68) were the first to propose that glucocorticoids might accelerate lung cell differentiation. They theorized that since glucocorticoids were known to have a stimulatory effect on cell differentiation in the epithelium of the developing digestive tract (Moog and Richardson, '55) they might also have this effect on the epithelium of the fetal alveolus since it too is an endodermal derivative.

Liggins ('69) administered glucocorticoids to pregnant sheep and found that when he delivered the lambs prematurely, they survived significantly more often than control lambs delivered at the same gestational age. Direct injection of cortisol into rabbit fetuses in utero has been morphologically demonstrated to accelerate the maturation of type II alveolar epithelial cells (Kikkawa et al., '71;
Motoyama et al., '71). This conclusion was based on an earlier decrease in glycogen content associated with an increase in the number of lamellar inclusion bodies present within the type II pneumocytes. In addition, type I cells began the attenuation process sooner, resulting in the appearance of blood-air barriers at an earlier stage of gestation than in controls. Related findings indicating increased maturation in the lungs of glucocorticoid-treated rabbit fetuses have also been reported by Wang et al. ('71) and Kotas and Avery ('72).

In order to rule out the interference of other hormones which may have been stimulated through pituitary feed-back loops, Blackburn et al. ('72) using rats and Chisweck et al. ('73) using rabbits, decapitated fetuses in utero and studied type II cell maturation. The results of both studies indicated a retardation of type II cell differentiation with decreased numbers of lamellar inclusion bodies present within the cells. However, in the study of Chisweck, when a synthetic analog of ACTH was administered to the fetal rabbits, these changes were abolished, indicating a possible hypophyseal controlling mechanism for lung maturation in vivo.

Since the first experiments of Liggins, there has appeared a wealth of literature documenting the acceleration of maturation of the fetal lung following glucocorticoid treatments (for review see Farrell and Avery, '75). In addition, controlled clinical trials have been implemented which have suggested that the administration of steroids to pregnant mothers 1-2 days prior to delivery may decrease the incidence of RDS in high risk pregnancies, but is only effective if delivery occurs before the thirty-second week of gestation (Liggins and
As promising as the foregoing studies appear to be, however, glucocorticoid administration has not gained universal acceptance as the clinical answer to RDS. Although there have been reports of decreased cortisol levels in infants afflicted with RDS (Murphy, '74), others have indicated no such decrease and, indeed, found elevated cortisol levels in infants similarly afflicted (Reynolds, '73). In addition to the problem of these conflicting clinical reports, some studies have revealed possible deleterious effects of steroid treatment of the fetus and/or newborn. Kotas et al. ('74) and Carson et al. ('73) have documented an inhibition of total lung cell number after hydrocortisone therapy in fetal rabbits. Picken et al. ('74) found that the administration of corticosteroids, on a longer term basis, resulted in a significant increase in the elastic recoil of the lungs in treated animals and the appearance of "giant" lamellar bodies within the type II cells. They concluded that the long-term administration of glucocorticoids resulted in a decided impairment of surfactant secretion. In addition to these possible deleterious effects in the lung, other, far reaching, problems have been reported following corticosteroid administration. Impaired cellular development of the cerebrum and cerebellum was observed in rats treated post-natally with steroids (Cotterrell et al., '72) and neurologic deficits including impaired motor co-ordination and balance were reported in mice following similar treatment (Howard and Granoff, '68). Older reports of side effects in the glucocorticoid therapy of human infants
also hold significance. Treating premature infants with ACTH for retrolental fibroplasia, Silverman et al. ('51) observed a retardation of growth which was reversible following the cessation of treatment. In addition, neurologic deficits were described by deLemos and Haggerty ('69) following prednisolone therapy of infants suffering from bacterial meningitis. More recently, several reports have appeared dealing with previously unknown side effects of the clinical trials of steroid therapy for RDS which were attempted. Taeusch et al. ('73) as well as Ewerbeck and Helwig ('72) have reported extensive intraventricular hemorrhages following steroid treatment. In addition, various neurologic abnormalities have also been reported as long-term sequelae of such clinical steroid intervention for RDS (Fitzhardinge et al., '74).

Because of the conflicting reports of the usefulness and possible deleterious side effects of corticosteroid treatment for the prevention and/or treatment of RDS, investigations have begun into the possibility of influencing fetal lung maturation through other means. Other agents which are thought to be capable of affecting the maturation of the fetal lung are discussed in reviews by Cowan ('74) and Avery ('75). The list of such agents now includes thyroxine (Wu et al., '73), aminophyllin (Karotkin et al., '76), prolactin (Hamosh and Hamosh, '77), plasminogen (Ambrus et al., '77) and heroin (Taeusch et al., '73). In addition, there has been an increase in studies designed to ascertain the mechanisms at work in vivo which control the maturation of the lung and the initiation of surfactant synthesis and
secretion in the fetus.

**Autonomic Manipulation of the Surfactant System**

The first indication that the parasympathetic nervous system could be involved in the maintenance of the type II alveolar epithelium and the secretion of surfactant came from studies of vagotomized animals. Early investigators in the surfactant field had noted abnormal alveolar surface tension (Klaus et al., '62) as well as morphologic changes in type II alveolar cells (Bolande and Klaus, '64) in the lungs of vagotomized animals. In addition, Miller et al. ('51) observed hyaline membranes similar to those found in the lungs of infants who had succumbed to RDS. Goldenberg et al. ('67) performed an in depth study of the ultrastructural changes in alveolar type II cells of rats following bi-lateral cervical vagotomy. They described a severe loss of osmiophilia and membrane structure within the lamellar inclusion bodies as quickly as one hour post-vagotomy. They also observed a loss of multivesicular bodies and changes in the secretory apparatus of the type II cells. They concluded that intact vagal connections were a necessity for normal surfactant production and secretion. Based on these findings, they reasoned that a parasympathomimetic should have a stimulatory effect on surfactant secretion. They chose to try pilocarpine (Goldenberg et al., '69) a cholinomimetic (parasympathomimetic) alkaloid which is used by ophthalmologists to increase the secretion of the aqueous humor in the treatment of glaucoma. The principle action of pilocarpine is the stimulation of the same autonomic effector cells which are acted upon by cholinergic
post-ganglionic nerve impulses. The compound is obtained from the leaflets of two South American shrubs (*Pilocarpus jaborandi* and *Pilocarpus microphyllus*) and is commercially available as pilocarpine nitrate or, as used by Goldenberg and associates, pilocarpine hydrochloride.

The pharmacologic properties of pilocarpine mimic, for the most part, a massive parasympathetic stimulation. Administration of 10-15 mg in man causes marked diaphoresis, salivation, lacrimation and increased secretion of the pancreas and gastrointestinal glands. Pilocarpine also causes a generalized increase in smooth muscle tone in most organs, including the gastrointestinal tract, respiratory tract, biliary system and urinary system. The only exception to its general parasympathetic-like effects are an increase in heart rate and hypertension. It is thought that this is due to a limited stimulation of sympathetic ganglia (Koelle, '75).

Goldenberg and associates ('69) injected adult fasted rats with a single subcutaneous dose (150 mg/kg) of pilocarpine and sacrificed them at .5, 1, 2, 4, 7, 10, 14, 18, 21 and 24 hours after injection. By light microscopy they observed a dilatation of the alveoli and the presence of apparently proteinaceous material within the bronchiolar and alveolar lumina soon after injection. By 24 hours post-injection the alveoli were either collapsed or dilated. Electron microscopy of the type II alveolar cells at .5 hours post-injection revealed a massively dilated rough endoplasmic reticulum which contained abundant granular material. At one hour they noted a significant increase in
the numbers of multivesicular bodies present within the type II cells. Examination at two hours following pilocarpine administration revealed large, highly osmiophilic lamellar inclusion bodies within the type II cells and an increase in the amount of intra-alveolar membranous material. Four hours post-injection the type II cells contained groups of large, mature inclusion bodies which were gathered at the apex of the cell. These were frequently observed to be discharging their contents into the alveolar space. The type II cells at seven hours contained relatively few inclusion bodies and thereafter returned to the appearance of controls. Based on these observations, Goldenberg and co-workers concluded that pilocarpine had a frank stimulatory effect on the exocrine secretory mechanism of the alveolar type II cells which was maximal at 2-4 hours after injection.

This stimulatory effect of pilocarpine was substantiated by Petrik's report ('69) of "grill structures" in the bronchioles of mice which had received the same dosage of pilocarpine used in Goldenberg's study. These structures were what are now termed tubular myelin arrays and represent surfactant. Kirkland ('70) reported a reduction in the surface tension of lung extracts in rats treated with pilocarpine, and concluded that this was due to an increase in the amount of pulmonary surfactant which had been released into the alveolar space in treated animals. At about the same time Ivemark and Robertson ('70), in a short report of a study dealing primarily with the effects of high vitamin A, mentioned an increased phospholipid content and lower minimal surface tension of lung extracts in rats treated with
pilocarpine. Morgan and Morgan ('73) administered 1.5 mg/kg pilocarpine intra-peritoneally to rats while simultaneously injecting $^{14}$C-palmitate into the left atrium via an in-dwelling catheter. The phospholipids from lung washes and homogenates of these rats were separated by thin layer chromatography and analyzed for lipid phosphorus and radioactivity. They found that pilocarpine significantly increased lecithin turn-over rates due to an increased secretory activity in type II cells. Massaro ('75), in order to study the secretion of the protein portion of pulmonary surfactant, injected radiolabelled leucine as well as 3 mg/kg pilocarpine to adult rabbits. By isolating the surface active fraction from lung lavages and assaying for protein, he found that pilocarpine caused a 2.5 fold increase in the percentage of radiolabelled surfactant protein.

The anatomical connection between the autonomic nervous system and the alveoli of the lung was first suggested by Meyrick and Reid ('71) who reported electron microscopic observations of nerves in the alveolar walls of rats. These were thought to correspond to those previously demonstrated by light microscopy (Hirsch et al., 68). Hung et al. ('72), using electron microscopy, demonstrated an abundance of nerves in the walls of alveolar ducts and alveoli of mice. They reported finding such nerves in every section of every block they examined from 18 different mouse lungs and, furthermore, reported finding two distinct types of nerve endings. The first type had an enlarged terminal containing many small mitochondria and was found in the pulmonary interstitium, either alone or related to type I pneumo-
cytes. The second type of nerve ending was described as being packed with large dense-core vesicles and forming an enlargement adjacent to type II pneumocytes.

The first type of nerve ending described by Hung and co-workers resembled typical sensory endings and were theorized as being such. The second type, although fewer in number than the first, are far more unique, since they provide the morphologic connection between the autonomic nervous system and surfactant secretion. This type of nerve ending in the lung most closely resembled a type of axon found in the pars tuberalis of the pituitary, as reported by Unsicker ('71). Hung and colleagues theorized that the substance in the dense-core vesicles of the terminal, upon vagal stimulation, would diffuse out of the axon and effect not only the type II pneumocyte adjacent to it, but also others at some distance away. The first type of ending, the sensory portion, was also thought to be of vagal origin, making both afferent and efferent pathways vagal. Stimulation of the left vagus in rabbits by Oyarzun and Clements ('77) resulted in a bi-lateral increase in alveolar phospholipids, apparently due to crossing over of fibers in the rabbit vagi. Oyarzun and Clements also reported a similar effect in rabbits subjected to substantial increases in ventilation. This effect could, however, be completely blocked by atropine which led them to conclude that, although the exact neural or humoral pathway for this reflex increase in surfactant following increased ventilation was unknown, it was reasonable to expect that the effect was mediated through cholinergic mechanisms.
In addition to pilocarpine, another parasympathomimetic, oxotremorine, has been implicated as having a stimulatory effect on surfactant secretion. Abdeblatif and Hollingsworth ('77) reported that an intra-peritoneal injection of oxotremorine to neonatal rabbits resulted in a significant increase in the phosphatidylcholine (PC) content of alveolar washes. Because they found no increase in the PC content of the residual lung, however, they concluded that the drug did not affect production. This conclusion, however, may not be valid since they sacrificed the animals thirty minutes after injection and an effect on production of surfactant would not be expected until perhaps two hours.

In 1976 Corbet et al. published the results of the first study dealing with the effects of pilocarpine on the surfactant system in the fetus. They injected 150 mg/kg pilocarpine in 0.1 ml of saline, or saline alone, intra-peritoneally to fetal rabbits of gestational ages 25.5, 26.5, 27.5, 28.5 and 29.5 days. This was accomplished by anesthetizing the pregnant does with methoxy-flurane, performing a laparotomy and injecting the fetus through the intact uterine wall. The abdomen of the does was then closed, to be re-opened 2.5 hours after injection. Fetal lung was then obtained and pressure-volume studies performed on intact lungs. Corbet and associates found that, in fetal lungs from rabbits of 25.5, 26.5 and 27.5 gestational days, this pilocarpine treatment resulted in substantial improvement of retention of air on deflation to low pressures. This would indicate an increase of surface activity within the alveoli, and thus an increased secretion
of surfactant. They found, however, no significant differences from controls in fetuses of 28.5 or 29.5 gestational days and interpreted this as indicating that the lungs were already functioning at minimal surface tension without the pilocarpine stimulus.

In a related study Pysher et al. ('77) using explants of fetal lung in culture observed that, at a concentration of $10^{-6}$ M, pilocarpine significantly increased the release of radiolabelled lecithin after three hours of incubation.

In order to clarify the mechanism of action of pilocarpine in the fetal lung Corbet et al. ('77) performed another study, this time using various autonomic agents and their antagonists. Employing the same operative procedure used in their earlier study, they administered saline, pilocarpine, muscarine (a parasympathomimetic similar in action to pilocarpine), atropine (a cholinergic antagonist), isoxsuprine (a β-adrenergic agonist), propranolol (a β-adrenergic antagonist), phenylephrine (an α-adrenergic agonist) and phenoxybenzamine (an α-adrenergic antagonist) alone or in appropriate combinations. They found the stimulatory effect of pilocarpine on the secretion of surfactant in the fetus to be blocked by either atropine or propranolol. The stimulatory effect was mimicked by isoxsuprine, which could not be blocked by atropine but could be by propranolol. Muscarine had no stimulatory effect, nor did phenylephrine. These and the results of other combinations, led Corbet and co-workers to conclude that the action of pilocarpine is due to a stimulation of β-adrenergic receptors, possibly on the surface of type II alveolar epithelial cells. The
nerve endings which were reported by Hung et al. ('72) would, in Corbet's view, then be sympathetic, β-adrenergic in nature.

This theory gains some measure of support from other reports of the action of isoxsuprine (the β-adrenergic agonist) in the fetal lung. Enhorning et al. ('77) injected isoxsuprine intra-muscularly to rabbit fetuses at the 28th gestational day and reported an increase in surface activity of the lung wash. Hayden et al. ('77) treated rabbit fetuses at 26.5 gestational days with three doses of isoxsuprine, eight hours apart, sacrificed the animals 26-30 hours after the last injection and observed an increase in lung lecithin over control values. In a clinical survey of newborn premature infants whose mothers had been treated with ritodine (another β-adrenergic drug) prior to delivery, Boog et al. ('75) reported a decreased incidence of RDS when compared to fetuses of similar birth weight born to mothers receiving no such treatment. In another clinical case review Hastwell ('77) reported a possible therapeutic benefit of salbutomol (another β-adrenergic agent) in preventing RDS in infants born prematurely.

Although the preceding studies seem to indicate that sympathetic activation would stimulate the secretion of surfactant, there exists some dispute in the literature. Bergren and Beckman ('76) reported decreased lung compliance, indicating decreased surfactant, in animals following sympathetic stimulation. Beckman et al. ('71) had previously found similar results in monkeys following head injury and reported that this effect was due to sympathetic activation, since it could be blocked by sympatholytic agents.
None of the studies to date dealing with the autonomic manipulation of the fetal lung surfactant system has described morphologic changes following administration of autonomic drugs. Furthermore, all of the previous studies have involved the direct injection of the autonomic agents to fetuses while in utero. This procedure involves surgical trauma to both the mother and fetus prior to the administration of the drug, in addition to possible effects of the anesthetic used. That this is not the ideal situation is pointed out by the findings of Robert et al. ('75) which indicated that the lungs of fetuses injected with saline in this manner showed evidence of a marked acceleration of maturation, when compared to similar fetuses of non-operated mothers.
MATERIALS AND METHODS

Animal Care and Mating

Large white New Zealand rabbits (average approximately 3.2 kg) were procured from Langshaw Farms (Augusta, Michigan) and used for all experiments. All animals were housed in the same room under identical conditions. They were fed a standard diet of approximately 90 g of lab chow (Wayne rabbit ration) per day and were allowed water ad libitum. In some cases the amount of water intake by a given rabbit was recorded daily. The external genitalia of the females were examined periodically in order to determine which animals would be receptive to the males. Females judged to be receptive were placed in the cage of a male and held in place while mating was accomplished. This procedure was then repeated with a second male. Mating was always completed within a fifteen minute period between the hours of 2 and 4 PM. The time of mating was recorded and considered to be time zero of gestation.

Experimental Protocols

Table I lists the types of experiments performed in this study along with the numbers of litters employed in each type.

Multiple Injections (Standard Regimen) --28 days

A solution containing 20 mg pilocarpine hydrochloride (Sigma
### TABLE I

**EXPERIMENTAL PROTOCOLS**

<table>
<thead>
<tr>
<th>Gestational Age at Sacrifice</th>
<th>Number of Litters</th>
<th>Pilocarpine Dosage</th>
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**Standard Regimen**

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<td>10,15 mg/kg</td>
<td>24 hours</td>
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**Injection of Fetuses in utero**

<table>
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<th>10 mg/fetus</th>
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Pharmaceutical) per ml of 0.9% saline was prepared fresh for each set of experiments. On the 24th gestational day pregnant female rabbits were weighed and a dose of 5 mg pilocarpine per kg of body weight was prepared by diluting the appropriate amount of the previously prepared stock solution of pilocarpine to 1 ml with 0.9% saline. This was then placed in a 1 ml tuberculin syringe and injected subcutaneously in the region of the upper back of the pregnant female rabbit. Controls were injected with 1 ml of saline alone. These injections were repeated daily on the 25th, 26th and 27th gestational days and are designated for convenience as the standard regimen.

At the 28th gestational day, at approximately the same time of day as mating occurred, the does were again weighed and in some cases venous blood was collected. This was accomplished by cutting the superficial vein at the lateral aspect of the ear and allowing the blood to drip into a collecting tube. This blood was sent to the clinical biochemistry laboratory at Loyola University's Foster McGaw Hospital and evaluated by the automated SMA-12 test series. Following collection of blood, the does were killed by injecting 30 ml of air into the same vein which was used to collect blood. This procedure resulted in a massive air embolism and the rapid death of the animal.

Immediately following death, the abdominal cavity of the animal was opened by a rapid mid-ventral incision. The fetuses were then obtained by a hysterotomy, removed from the amniotic sac and weighed. To prevent the fetuses from breathing, and thus becoming neonates, pressure was applied to the trachea, first by the thumb and then by
the rapid application of a ligature placed around the neck.

In order to maintain uniformity between experiments, fetuses of corresponding uterine placement were used for each procedure whenever possible. The most lateral fetus of the right uterine horn was removed first, as above, and the thorax opened by a mid-ventral incision. The entire upper and lower lobes of the right lung were removed, independently chopped into approximately 1 mm cubes and fixed in 2% glutaraldehyde buffered to pH 7.2 with .2M Sorenson's phosphate buffer. The left lung was fixed in toto by immersion in 10% buffered formalin. Simultaneously, the fetuses of the left uterine horn were removed, as above, and their lungs and livers frozen immediately by immersion in liquid nitrogen. In some cases the right lower lobe of the most lateral fetus in the left uterine horn was used for scintillation autoradiography as explained below.

Fetal lung tissue obtained on the 28th gestational day was studied using all of the procedures described below under the heading "Tissue Processing and Data Procurement" and constitute the bulk of the project.

In some cases portions of maternal lung were removed and fixed in 10% buffered formalin for paraffin embedding. In one case portions of various maternal organs, including liver, kidney, gastrointestinal tract, uterus and others, were similarly removed and fixed.

**Standard Regimen -- 31 days**

In two experiments (Table I) a delay was allowed between the
maternal administration of pilocarpine and sacrifice of the animal, in order to determine whether pilocarpine treatment resulted in any long term deleterious effects on the fetal lung and its maturation. The does were injected using the standard regimen, that is with 5 mg/kg pilocarpine or with saline on the 24th through the 27th gestational day. Subsequently these does were maintained without additional treatment until the 31st gestational day, at which time the experiments were terminated. Fetal lung tissue obtained at 31 days was fixed in 2% glutaraldehyde and analyzed using electron microscopy as well as electron microscopic stereology (see below). In addition, fetal and maternal lung tissue were fixed in formalin for paraffin embedding.

**Time-Course Studies**

In order to establish a time-course of the effects of maternal administration of pilocarpine, treatment was begun as with the standard regimen but the animals were killed before the 28th gestational day. pregnant does received pilocarpine or saline injections, beginning with the 24th gestational day, and were sacrificed at the 25th, 26th and 27th gestational day. Therefore, these does were treated for 1, 2 or 3 days at the time of sacrifice (Table I). Additional controls included fetuses of 29 and 30 days gestation obtained from non-injected does. Again, fetal lung tissue was analyzed by electron microscopy and stereology as explained below.

**Single Injection Studies**

Pilot studies early in the project included experiments dealing
with the short term effects of maternal administration of pilocarpine on the fetal lung. At different days of gestation, various strength doses (10-80 mg/kg) were administered to the pregnant females, which were then sacrificed at various times post-injection (2-24 hours). These experiments are summarized as part of Table I. Fetal lungs obtained in these studies were examined by routine light and electron microscopy.

**Injection of Fetuses In Utero**

In two cases pilocarpine was injected directly into the fetus, thus by-passing the maternal route used in the remainder of the experiments. In order to accomplish this, pregnant does (one at the 26th day and one at the 27th day) were given general anesthesia and a laparotomy was performed. The fetuses of the left uterine horn were injected intraperitoneally, through the uterine wall and amniotic sac, with 10 mg pilocarpine in 0.5 ml of 0.9% saline. Fetuses of the right horn were given 0.5 ml of saline alone, in the same manner. The abdominal incision of the doe was then sutured, and following the intramuscular injection of antibiotic the does were allowed to recover. At 24 hours post-injection the doe was killed and lung tissue obtained from the fetuses as explained above. This tissue was then analyzed using electron microscopy.

**Tissue Processing and Data Procurement**

**Glycogen Determination**

The lungs and livers of the 28 day fetuses which were frozen by
immersion in liquid nitrogen were used for the determination of glycogen content. This was accomplished using a modification of the method of Murat and Serfaty ('76). The entire lung or liver to be analyzed was first weighed, and then homogenized with 3 ml of citrate buffer (pH 4.2) in a Waring blender. For free glucose determination 0.1 ml of the homogenate was removed. The remainder was incubated at 37°C for two hours with the enzyme amyloglucosidase. This enzyme splits the glycogen present in the tissue into free glucose residues. Following the incubation period 0.1 ml of the homogenate was removed and, along with the 0.1 ml removed prior to incubation, a blank and a standard glucose solution were analyzed for glucose. Samples were deproteinated by adding 1.1 ml of water, 0.4 ml of barium hydroxide and 0.4 ml zinc sulfate. They were then centrifuged and 0.5 ml of the supernatant was removed. To this was added 2 ml of PGO (peroxidase and glucose oxidase) enzyme solution and 0.1 ml of the color reagent 0-dianisidine. The mixtures were then incubated at 37°C for 30 minutes and the absorbency readings were obtained using a Gilford 250 spectrophotometer. By using a standard curve the concentration of glucose in each sample was ascertained. Subtracting the free glucose value obtained from the original homogenate (prior to incubation) from that obtained following incubation with amyloglucosidase, a value of the amount of glucose due to glycogen breakdown was obtained. The amount of glycogen in each lung or liver was expressed as mg/gm tissue.

Paraffin Embedding

The lungs which were fixed in toto in formalin were processed for
paraffin embedding using an Autotechnicon Model 2A automated tissue processor timed as follows: 80% ethanol, .5 hr; 95% ethanol, 3 changes of 1 hr each; 100% ethanol, 3 changes of 1 hr each; toluene, 2 changes of 1 hr each; liquid Paraplast (Scientific Products), 2 changes of 1 hr each. Following this the tissue was placed in a vacuum oven for 1 hr and then embedded in Paraplast. Sections were cut at 4-6 μm on an American Optical rotary microtome and stained with either Harris hematoxylin and alcoholic eosin or Periodic acid-Schiff with (D-PAS) or without (PAS) diastase (malt U.S.P.) pretreatment.

**Electron Microscopy**

Lung tissue placed in glutaraldehyde was allowed to fix overnight in the original fixative solution. The following morning the tissue was washed in Sorenson's phosphate buffer for 4 changes of 15 minutes each and then post-fixed in 1% phosphate buffered osmium tetroxide for two hours. The tissue was then rapidly dehydrated as follows: one 15 minute change in each of 50%, 70% and 95% ethanol; two 15 minute changes in 100% ethanol; two 15 minute changes in propylene oxide. They were then placed in a 1:1 mixture of propylene oxide and Epon 812 mixture for 1 hour, in a 3:1 mixture of the same compounds overnight and then again overnight in a pure Epon 812 mixture. The following morning the tissue was embedded in fresh Epon 812 utilizing standard BEEM capsules. The Epon 812 mixture used throughout this project was prepared volumetrically as follows: Epon 812, 214 ml; DDSA, 216 ml; NMA, 71 ml; DMP-30, 7.5 ml. This mixture was stored at -40°C until used. Polymerization was carried out at 40°C.
for one day and then at 60°C for two days.

Using a Sorvall MT-1 ultramicrotome and glass knives, 1 μm sections were cut from the blocks, mounted on glass slides and stained with toluidine blue. These sections were used for orientation and trimming of the blocks for thin sectioning and, in some cases, for light microscopic morphometric analysis as explained below. Using the same microtome and a diamond knife, thin sections were cut from the trimmed blocks at a thickness of 600-800 nm. These sections were mounted on 200 or 300 mesh copper grids and stained with 5% uranyl acetate and Reynold's lead citrate ('63). Ultrastructural examination was then carried out using an RCA EMU-3H electron microscope with an accelerating voltage of 100 KV.

Light Microscopic Morphometric Analysis

The 1 μm Epon sections were used for the light microscopic morphometric analysis of the numeric distribution of mature, or well differentiated, type II alveolar epithelial cells in the lungs of fetuses obtained at the 28th gestational day from control and pilocarpine treated groups. Sections of randomly selected blocks were photographed at 400X magnification on a Zeiss Photomicroscope II using Kodak PCF-3 film. The transparencies thus obtained were used in a blind study where the slides were viewed randomly in a Kodak rear screen projector. The number of cells of any type, as well as the number of well differentiated type II cells in the unit area of the transparency (0.035 mm²) were recorded. Cells of airways other than
alveoli (bronchi, bronchioles, etc.) or cells of blood vessels larger than capillaries were not included in the cell count. The area occupied by these omitted cells was determined using a standard grid pattern and subtracted from the unit area of the slide. The results of this study were expressed as type II cells/unit area and type II cells/1,000 lung parenchymal cells.

Electron Microscopic Morphometry and Stereology

In 28-day fetuses treated with the standard regimen a morphometric analysis of the lamellar inclusion bodies and multivesicular bodies within the type II cells was undertaken. Randomly selected blocks of upper and lower lobe lung tissue from six fetuses in each group (all from different litters) were thin sectioned and evaluated in a blind study. All alveolar epithelial cells which could be identified as type II cells were photographed at a standard magnification of 5,800X. Identification of type II cells at this gestational age was based on cuboidal shape, presence of microvilli and, most importantly, the presence of at least one lamellar inclusion body. This process resulted in photographs of at least 120 cells from each of the upper and lower lobes of the two groups (control and pilocarpine treated). In these photographs, enlarged to a final magnification of 20,000X, the number of inclusion bodies and multivesicular bodies in each cell was recorded. In addition, the size of each lamellar inclusion body, measured at its maximum diameter, was recorded.

In all cases where electron microscopic stereology was undertaken
20-25 electron micrographs of each of the upper and lower lobes of both control and pilocarpine treated fetuses were obtained. For the 28-day fetuses the electron micrographs were selected at random from those used for morphometry as explained above. For the 31-day fetuses and the time-course study they were obtained in a similar fashion to those of 28-day fetuses. However, cells of fetuses with gestational age less than 28 days were not restricted to being type II cells specifically. Due to a distinct lack of recognizable type II cells, according to the identification criteria used at gestational age 28-31 days, any cuboidal cell lining the alveolar space was photographed and used for stereologic analysis. Following stereologic analysis a tally was made of all cells which contained lamellar inclusion bodies in any volume density. The latter cells were considered type II cells and the percent of all cuboidal cells which these constituted was calculated and recorded.

Stereologic analysis was performed in order to determine the volume density of major cell components, and employed the methods of Weibel ('73). The cell components which were analyzed in the present study included glycogen, lamellar inclusion bodies, rough endoplasmic reticulum, Golgi apparatus and mitochondria. A transparent acetate sheet imprinted with a 1/4 inch grid (Xerox Corp. #8R377) was placed over each photograph in turn and held securely in place. In this study the points of intersection of the grid lines were used as the reference points. Point "hits" then, were defined as the line intersections which fell over a given cell structure.
Point "hits" were tallied for each of the structures of interest, as well as for all other cytoplasmic components, taken as a group. Thus, any point not falling over one of the cell structures of interest was grouped under "other" and added to the point "hits" which did fall over one of the structures named above. This was done in order to obtain a total for all point "hits" falling over anything in the cytoplasmic portion of the cell. Point "hits" falling over the nucleus were not included.

Using the equation Point Intercept/Point Total \( \left( \frac{P_i}{P_t} \right) = \) Point Density \( (P_p) = \) Profile Density on a test area \( (A_a) = \) Volume Density \( (V_v) \) (Glagoleff, '33; Chalkley, '43; Weibel, '63, '72), volume densities were obtained for each of the structures of interest.

**Scintillation Autoradiography**

In some cases portions of lung approximately 1 mm x 1 mm x 5 mm were removed from the periphery of the right lower lobe of the most lateral fetus in the left uterine horn for evaluation of cell division using scintillation autoradiography. The pieces of lung were rapidly placed in culture media (Gibco minimum essential media, 20% fetal calf serum) containing \( 5\mu\text{Ci/ml} \) tritiated thymidine (Amersham, specific activity 20 Ci/mmol). These were then maintained at 37°C, using a standard tissue culture incubator, for two hours. Following incubation the tissues were fixed in cold 2% phosphate buffered paraformaldehyde and were allowed to remain in the fixative overnight. The following morning they were washed through three changes of Sorenson's buffer.
Dehydration was then carried out in a graded methacrylate (Polysciences JB-4 catalyzed solution A, prepared as per kit instructions) series as follows: 50% JB-4, two changes of .5 hour each; 70% JB-4, two changes of .5 hour each; 95% JB-4, two changes of .5 hour each; 100% JB-4, two changes of 1.5 hour each, this step corresponding to infiltration. The tissues were then embedded in a 25:1 mixture of JB-4 catalyzed solution A to JB-4 solution B, as per kit instructions. To accomplish polymerization a block molding cup was used and covered with paraffin.

Following a two day polymerization period sections were cut at 1-1.5 μm using a DuPont JB-4 microtome and glass knife, and mounted on glass slides. These slides were then coated, using standard techniques (Rogers, '67) with Kodak NTB-3 nuclear track emulsion. The emulsion was diluted 1:2 with distilled water, giving a very thin emulsion layer of approximately 1 μm thickness. The emulsion was then allowed to dry on a cooled metal plate for one hour and the slides were dipped for 10 seconds in a scintillation cocktail which was previously prepared by adding 10 grams of Packard Permablend I premixed scintillator to 1,000 ml of dioxane. Permablend I consists of 90% PPO (2,5-diphenyl-oxazole) and 9% dimethyl POPOP ([1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene]). The slides were then allowed to dry, placed in light proof boxes and allowed to expose for three days at 4°C. The exposure time was able to be shortened to three days because in scintillation autoradiography the β-particles emitted by the tritium, in addition to causing direct exposure of emulsion grains, also cause photon release from the scintillator, which further exposes the emulsion.
The autoradiographs were then developed for two minutes in Kodak Dektol developer diluted 1:2 with distilled water and fixed in Kodak Rapid fixer for 5 minutes. Following a 15 minute running water wash the slides were rinsed through a graded ethanol series, to remove the residue of the scintillation mixture, and stained lightly with Toluidine blue (stock solution diluted 1:10 with water). The autoradiographs were then photographed at 400X magnification using a Zeiss Photomicroscope II and Kodak PCF-3 film. Only the outer 0.2 mm of each tissue section were photographed since anoxic changes (including nuclear pyknosis) were frequently observed in the central portions of the tissue fragments. The transparencies thus obtained were viewed randomly in a rear screen projector. The numbers of labelled and unlabelled alveolar epithelial cells and interstitial cells in each slide were recorded so as to obtain labelled cell indices (labelled cells/1,000 cells) for both types of cells.

**Statistical Analyses**

All data which required statistical analysis were analyzed using the Student's t-test. This was accomplished using a Texas Instruments data terminal linked to Loyola University's main computer via an Anderson Jacobsen acoustic direct audio coupler. The program utilized was catalogued as "SNAP" or Statistical Numerical Analysis Package and is listed on the "Public" file of Loyola's available programs.
RESULTS

Injection of Fetuses In Utero

Light microscopic examination of paraffin sectioned fetal lung revealed no discernable differences between that of fetuses injected with pilocarpine in utero and those injected with saline in a like manner (figs. 1, 2). However, fetal lung in both groups appeared more mature than lung obtained from non-operated control fetuses of the same gestational age (fig. 9). Maturational changes observed in fetuses injected in utero included a loss of glycogen, thinner septa and the appearance of some surfactant material in the alveolar spaces (figs. 1, 2).

Electron microscopic studies corroborated these findings in that there was also ultrastructural evidence of apparent maturational changes present in the lungs of fetuses injected in utero with either saline or pilocarpine. The type II epithelium of both groups contained substantially less glycogen than that of non-operated controls and there appeared to be a stimulation of surfactant synthesis, as evidenced by the increased number of lamellar inclusion bodies and prominence of other organelles constituting the secretory apparatus of the type II cells (figs. 3, 4). Although these changes were present in both groups, ultrastructural evidence indicated that the effects were accentuated in the pilocarpine-treated group. There was no evidence of changes in the type I alveolar epithelial cells in
either pilocarpine or saline injected group, when compared to those of non-operated controls.

Single Injection Studies

Following a single injection of pilocarpine the pregnant does exhibited all the signs expected from the administration of a parasympathomimetic agent. Salivation, lacrimation and diarrhea began approximately 15 minutes post-injection and persisted for about three hours. In those experiments utilizing higher doses of pilocarpine (i.e. 20-40 mg/kg) these effects were more pronounced and persisted for a longer time period. In all cases the does appeared mildly distressed and their breathing was somewhat labored. Light microscopy, however, revealed no apparent deleterious changes in lung obtained from any of these does at various time intervals (2-24 hours) after pilocarpine injection. Electron microscopic evaluation revealed changes in the type II pneumocytes of these does which indicated a stimulation of the exocrine secretory function of these cells. These changes, which were particularly apparent at two hours post-injection, included the presence of dilated rough endoplasmic reticulum and increased lamellar inclusion bodies (compare figs. 5, 6).

Fetal lung from cases which utilized varying, single, doses of pilocarpine (10-40 mg/kg), varying post-injection sacrifice times (2-24 hours) and varying gestational ages (28-30 days) was also examined. Although there was some effect in all cases, the results of these experiments indicated more marked effects of single pilocarpine
injections in cases involving higher doses, later gestational ages and shorter post-injection sacrifice times. Although light microscopic examination in these cases revealed fetal lung virtually indistinguishable from controls, electron microscopy indicated changes in type II cells of these pilocarpine-treated fetuses which suggested an increased secretion of surfactant. These changes resembled those observed in adult lung, including increased numbers of lamellar inclusion bodies as well as increased multivesicular bodies and rough endoplasmic reticulum (compare figs. 7, 8).

**Standard Regimen--28 days**

Experiments involving 28-day fetuses obtained from mothers receiving daily injections of pilocarpine on the 24th through the 27th gestational day were performed in order to evaluate the effect of prolonged pilocarpine stimulation. These constituted the bulk of the present study.

**Maternal Effects**

As in the single injection studies, following each pilocarpine injection the pregnant does exhibited all the signs of parasympathetic stimulation. Food intake for both the control and pilocarpine-treated does was standardized and water intake was comparable between the two groups, averaging $360 \pm 55$ ml per day in the pilocarpine-treated group and $280 \pm 43$ ml in controls. These values, while somewhat higher in the pilocarpine-treated group, were not statistically different. During the four day injection period the pilocarpine-
treated does exhibited a mild weight loss (average of 3.8%) compared to a slight weight gain in control animals during the same period (average of 2.4%). This difference is thought to be due to diarrhea, since food and water intake was essentially the same in control and pilocarpine-treated animals. Furthermore, the results of the SMA-12 tests on the serum of the two groups revealed no significant differences in any of the twelve parameters tested (table II).

By light microscopy the lungs of the does which received pilocarpine exhibited some areas of minor atelectasis, but no other changes were observed. Additionally, in one case a survey of many organs, including uterus, liver, gastrointestinal tract and kidney, was performed by light microscopy which disclosed no apparent abnormalities in any organ from the pilocarpine-treated doe.

**Fetal Effects**

Fetuses of control and pilocarpine-treated mothers (hereafter for convenience referred to as control and pilocarpine-treated fetuses) were comparable grossly, with an occasional dwarf or dead fetus being found in each group. The average weight of pilocarpine-treated fetuses from eight representative does was 26.95 g (± 1.97 SEM) as compared with 35.42 g (± 1.42) for control fetuses. These values were significantly different (p < 0.001). Grossly the lungs of pilocarpine-treated fetuses appeared normal, as did all other organs.

Light microscopic evaluation of paraffin embedded fetal lungs...
TABLE II

RESULTS OF SMA-12 TEST SERIES ON MATERNAL SERUM

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<th>Test</th>
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<td>Calcium (mg%)</td>
<td>11.38 ± 0.46</td>
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<tr>
<td>Inorganic Phosphates (Mg% P)</td>
<td>3.45 ± 0.37</td>
<td>2.82 ± 0.19</td>
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<tr>
<td>Glucose (mg%)</td>
<td>130.00 ± 6.18</td>
<td>124.00 ± 1.81</td>
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<tr>
<td>Blood Urea Nitrogen (BUN) (mg%)</td>
<td>19.43 ± 4.05</td>
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<tr>
<td>Uric Acid (mg%)</td>
<td>0.57 ± 0.03</td>
<td>0.57 ± 0.05</td>
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<tr>
<td>Cholesterol (mg%)</td>
<td>27.43 ± 2.00</td>
<td>25.33 ± 3.13</td>
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<tr>
<td>Total Phosphorus (gm%)</td>
<td>5.00 ± 0.20</td>
<td>5.15 ± 0.08</td>
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<tr>
<td>Albumin (gm%)</td>
<td>0.68 ± 0.02</td>
<td>0.67 ± 0.02</td>
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<tr>
<td>Total Bilirubin (mg%)</td>
<td>0.23 ± 0.04</td>
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<tr>
<td>Alkaline Phosphatase (mU./ml)</td>
<td>27.71 ± 4.90</td>
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<tr>
<td>LDH (mU./ml)</td>
<td>181.85 ± 23.00</td>
<td>294.00 ± 71.7</td>
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<tr>
<td>SGOT (mU./ml)</td>
<td>36.00 ± 4.43</td>
<td>38.33 ± 13.56</td>
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revealed that the lungs of pilocarpine-treated fetuses gave an over-
all impression of an advanced maturational stage, when compared to
those of controls. This impression was based on general configuration
and alveolar septal thickness (compare figs. 9, 10). Examination of
PAS-stained paraffin sections showed a marked decrease in the amount
of PAS-positive material in the lung cells of pilocarpine-treated
fetuses. In slides stained with PAS after diastase pre-treatment
(D-PAS) no difference was noted between pilocarpine and control fetal
lungs, indicating that the difference originally noted in PAS staining
was due to glycogen content. The validity of this observation was
substantiated by the biochemical determination of glycogen content.
The results of this study (table III) indicated a significant decrease
in the glycogen content of the lungs of pilocarpine-treated fetuses,
when compared with controls. There was, however, no significant dif-
ference in the glycogen content of the fetal livers between the two
groups, which would indicate that the difference observed in the lungs
was not due to a generalized depletion of glycogen stores in the pilo-
carpine-treated group.

Light microscopic examination of toluidine blue-stained 1 μm
thick sections of Epon embedded fetal lung reinforced the impression
of an overall appearance of increased lung maturation in the pilo-
carpine-treated group, again based on alveolar configuration and
septal thickness. In addition, since the resolution of individual
cells was possible in these plastic sections, it was observed that the
alveoli of the pilocarpine-treated fetuses appeared, for the most part,
to be lined by cells which were fairly well differentiated into type I
### TABLE III

GLYCOGEN CONTENT (mg/gm tissue)

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<th>Number of Fetuses</th>
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<tr>
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<td>4.11 ± 0.33</td>
</tr>
<tr>
<td>PILOCARPINE</td>
<td>7</td>
<td>25</td>
<td>2.65 ± 0.13</td>
</tr>
</tbody>
</table>

p < .001  
n.s.
and type II cells (fig. 11). In contrast, the alveoli of control fetal lungs were lined by many apparently immature cuboidal cells, as well as type I and type II cells (fig. 11).

These findings were confirmed by light microscopic morphometric analysis of the distribution of mature type II cells in these lungs. This study revealed a significant increase in the number of mature type II cells in the lungs of pilocarpine-treated fetuses, both per unit area of section ($0.035 \text{ mm}^2$) and per 1,000 lung cells of any kind (fig. 12). Such an analysis was possible since mature, or well differentiated type II cells are readily recognizable in 1 $\mu$m thick sections by their size, shape, position and, most importantly, the presence of lamellar inclusion bodies (fig. 11).

Ultrastructural examination confirmed that the alveoli of 28-day control fetal lungs were lined by numerous immature cuboidal cells, as well as type I and type II cells in various stages of differentiation and maturation (fig. 13). Immature cuboidal cells are characterized by very large accumulations of glycogen and show little indication of differentiation into either type I or type II cells (fig. 26). In addition, the alveolar epithelial cells which had already differentiated into type I or type II cells appeared, in general, at a relatively immature stage of maturation. The nuclei of type I cells in these control fetal lungs were often surrounded by relatively abundant cytoplasm, indicative of an immature state. While the cytoplasmic extensions of these cells in some areas formed blood-air barriers, these extensions were frequently thick and contained, in addition to
accumulations of glycogen, mitochondria and rough endoplasmic reticulum (fig. 15). In addition, the nuclei of pulmonary capillary endothelial cells were often found positioned directly adjacent to developing type I cell cytoplasmic extensions, resulting in very thick, immature, blood-air barriers.

The majority of the type II cells present in 28-day controls were characterized by abundant glycogen, a poorly developed secretory apparatus and relatively few multivesicular bodies and lamellar inclusion bodies (figs. 17, 19). The inclusion bodies which were present in these cells often possessed a homogenous core surrounded by relatively few, or less intensely stained, lamellae (figs. 17, 19).

The alveolar epithelium of pilocarpine-treated fetuses demonstrated striking ultrastructural differences from controls in many respects. The cells lining the alveoli of these fetuses were, in the vast majority, well differentiated into type I or type II cells, with immature cuboidal cells being extremely rare (fig. 14). The most prominent change in these lungs was a striking reduction in the glycogen content of alveolar epithelial cells, with many of these cells containing virtually no glycogen.

Type I cells of pilocarpine-treated fetuses appeared, in general, to be at a more advanced stage of maturation than did those of controls. The nuclei were surrounded by a thin rim of cytoplasm containing sparse organelles and virtually no glycogen. The cytoplasm of these cells was most often attenuated to form mature, very thin, blood-air barriers (fig. 16).
When glycogen was present in the type II epithelial cells of pilocarpine-treated fetuses, it was usually in relatively small amounts and often associated with surfactant material (fig. 20). Additional findings in these cells involved maturational changes indicative of an increased activity of the secretory apparatus which produces pulmonary surfactant. These findings included an increase or dilatation of the rough endoplasmic reticulum associated with the presence of relatively numerous multivesicular bodies and large, densely osmiophilic, lamellar inclusion bodies which tended to aggregate toward the apex of the cell. The majority of these inclusion bodies possessed many well defined lamellae which, according to Kikkawa's classification ('68), would represent more mature forms (figs. 18, 20). Lamellar inclusion bodies in type II cells of pilocarpine-treated fetuses were also seen in the process of being, apparently, secreted into the alveolar space (fig. 18) with greater frequency than in controls, where this was rarely observed. Also observed more often in pilocarpine-treated fetuses were membranous whorls and segments of tubular myelin within the alveolar space (fig. 16). These are indicative of surfactant lipids and as such are the end-result of the secretion of lamellar bodies from the type II cells, as mentioned above. The remainder of the organelles within the type II cells were much the same in the two groups, with the exception of the Golgi apparatus, which appeared prominent, or multiple, with greater frequency in the type II cells of pilocarpine-treated fetuses.

The ultrastructural morphometric study dealing with the size and numerical distribution of lamellar inclusion bodies, as well as the
number of multivesicular bodies within the type II cells of the two groups substantiated the above interpretations of the electron microscopic findings. The results of this study (table IV) indicated that both the number of lamellar inclusion bodies per type II cell and the average size of the lamellar inclusion bodies were increased significantly in the pilocarpine-treated group when compared to controls. In addition, the number of multivesicular bodies present in the type II cells of pilocarpine-treated fetuses, expressed as number/ten type II cells, was also increased significantly over control values. Further quantification of the ultrastructural differences observed between type II cells of the two groups was obtained by stereologic analysis. The results of this study, expressed as percentages of total cytoplasmic volume, are listed in table V. These data indicated that the cytoplasmic volume density of lamellar inclusion bodies, rough endoplasmic reticulum and Golgi apparatus were increased significantly in the type II cells of pilocarpine-treated fetuses. The only exception was the volume density of the Golgi apparatus in the type II cells of the right lower lobe. In this case the mean value for pilocarpine-treated fetuses was higher than that of controls, but this elevation was not statistically significant. In addition to these increases in cytoplasmic components constituting the secretory apparatus and its product, there was a significant decrease in the volume density of glycogen in the type II cells of pilocarpine-treated fetuses. There was no difference in the values obtained for the volume densities of mitochondria in the two groups.
**TABLE IV**

RESULTS OF ELECTRON MICROSCOPIC MORPHOMETRIC ANALYSIS ON TYPE II CELLS OF 28-DAY FETUSES

(STANDARD REGIMEN)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Pilocarpine</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inclusion Bodies/Type II Cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>3.95 ± .48</td>
<td>5.36 ± .37</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>3.19 ± .39</td>
<td>5.02 ± .40</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Size of Inclusion Bodies (μm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>.692 ± .024</td>
<td>.813 ± .017</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>.674 ± .034</td>
<td>.836 ± .031</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Multivesicular Bodies/ Ten Type II Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>1.93 ± .45</td>
<td>3.02 ± .43</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>1.47 ± .29</td>
<td>3.43 ± .78</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>
TABLE V

RESULTS OF ULTRASTRUCTURAL STEREOLOGY OF 28-DAY (STANDARD REGIMEN) FETAL TYPE II CELLS

Expressed as Percent Cytoplasmic Volume

<table>
<thead>
<tr>
<th>Structure</th>
<th>Control</th>
<th>Pilocarpine</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamellar Inclusion Bodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>7.2 ± 0.9</td>
<td>10.6 ± 1.0</td>
<td>&lt;.025</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>5.6 ± 0.6</td>
<td>12.9 ± 1.2</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Glycogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>27.3 ± 1.7</td>
<td>13.7 ± 1.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>34.3 ± 2.2</td>
<td>17.6 ± 1.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Rough Endoplasmic Reticulum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>7.8 ± .6</td>
<td>10.9 ± .8</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>5.9 ± .6</td>
<td>14.3 ± 2.7</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Golgi Complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>2.0 ± .4</td>
<td>3.5 ± .6</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>2.6 ± .5</td>
<td>3.1 ± .7</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Lobe</td>
<td>8.0 ± .5</td>
<td>8.5 ± .7</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lower Lobe</td>
<td>6.4 ± .7</td>
<td>7.8 ± .4</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Electron microscopic stereologic analysis of type II cells from 29, 30 and 31-day control fetuses was undertaken in order to ascertain, by comparison, the overall maturational change in the type II cells, in terms of gestational days, of 28-day pilocarpine-treated fetuses. The volume density of lamellar inclusion bodies in the pilocarpine-treated group was not significantly different from values obtained in 29 or 30-day control fetuses (fig. 25). The glycogen content of the type II cells of 28-day pilocarpine-treated fetuses was significantly higher than that of 29, 30 or 31-day-controls. Rough endoplasmic reticulum in these cells was not significantly different from 31-day controls, while the volume density of Golgi apparatus did not differ significantly from controls of 30-days gestation (fig. 25).

Scintillation Autoradiography

Using scintillation autoradiography, adequate labelling of cells which had incorporated the 3H-thymidine was obtained after only three days of exposure. Cells were considered labelled if there were at least five emulsion grains over the nuclei. As shown in figure 21, the labelling obtained with this method was quite distinct and well localized over individual nuclei, while background grain exposure was minimal. Moreover, the use of 1 μm thick plastic sections, instead of thicker paraffin sections, enabled the identification of interstitial and alveolar epithelial cells, based on position, size and histologic features (fig. 21).

The blind study of the labelled cell index of alveolar epithelium revealed that the number of labelled epithelial cells was
decreased significantly in the pilocarpine-treated group when compared to controls (fig. 22). In addition, the number of labelled interstitial cells was also significantly lower in the pilocarpine-treated group than in controls (fig. 22).

**Standard Regimen--31 days**

Maternal lung tissue obtained at the 31st gestational day from does which had been treated with the same standard regimen (on days 24-27) as those killed at 28 days was studied by light microscopy. Examination of paraffin sectioned lung stained with H&E, PAS or D-PAS revealed no noticeable differences from adult controls obtained at the same gestational age. Fetal lung was similarly studied and again showed no differences between control and pilocarpine-treated groups. Fetal lung of both groups appeared mature, with thin septa, many blood-air barriers and very little, if any, glycogen within alveolar epithelium or cells of the septa. In addition, some alveolar spaces of both groups contained D-PAS positive granular material which is indicative of surfactant secretion.

Electron microscopic comparison of 31-day fetal lung demonstrated no observable damage in the lungs of fetuses which had been treated with pilocarpine on gestational days 24-27. Lung parenchyma appeared normal and comparable to controls and there was no evidence of any damage to type II alveolar epithelial cells (compare figs. 23, 24). Stereologic analysis revealed that the type II cells of pilocarpine-treated fetuses contained a significantly greater volume density of lamellar inclusion bodies than those of controls (fig. 25). Addition-
ally, although little remained in any cell of either group at 31 days, the amount of glycogen present in the type II cells of pilocarpine-treated fetuses remained significantly lower than control values (fig. 25). The volume densities of rough endoplasmic reticulum and Golgi complex were comparable to controls, however, the volume of mitochondria was significantly decreased in the pilocarpine-treated group.

**Time-Course Studies**

Fetal lung obtained from control and pilocarpine-treated fetuses at gestational ages 25, 26 and 27 days was examined by electron microscopy and the cuboidal cells lining the alveolar space were analyzed by electron microscopic stereology.

At the 25th gestational day the epithelium lining the control fetal lungs was quite immature, the cuboidal cells being 50% immature cuboidal cells (table VI). These cells contained much glycogen and some organelles but showed no signs of differentiation (fig. 26). The other 50% of the cuboidal cells photographed randomly for stereology were classified as type II cells since they contained very immature forms of lamellar inclusion bodies or, occasionally, a single multivesicular body. Stereologic analysis indicated that the cuboidal cells, as a group, contained roughly 34% (volume density of cytoplasm) glycogen and barely 0.3% inclusion bodies (fig. 25). Conversely, the cuboidal cells of pilocarpine-treated fetuses contained only 25% glycogen and 1.3% inclusion bodies, these values being significantly different from controls (fig. 25). In addition, these cells also
# TABLE VI

## PERCENT TYPE II CELLS OF SAMPLED CUBOIDAL CELLS

<table>
<thead>
<tr>
<th>Gestational Day</th>
<th>Control</th>
<th>Pilocarpine</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>50%</td>
<td>65%</td>
</tr>
<tr>
<td>26</td>
<td>70%</td>
<td>75%</td>
</tr>
<tr>
<td>27</td>
<td>78%</td>
<td>100%</td>
</tr>
</tbody>
</table>
contained greater amounts of mitochondria, rough endoplasmic reticulum and Golgi complex (fig. 25). Cells classified as type II cells comprised 65% of the cuboidal cells which lined the alveoli of these fetuses (table VI). These cells were, like those of controls, relatively immature (fig. 27).

Cuboidal cells present at the 26th gestational day in control fetal lungs contained slightly less glycogen and more lamellar bodies and Golgi than had those of 25 days (fig. 25). However, the overall appearance of the alveolar lining was still quite immature (fig. 28) even though 70% of the cells photographed were classified as type II cells (table VI). Stereology revealed that cuboidal cells of pilocarpine-treated fetuses at 26 days had changed little from those of the previous day (fig. 25). Thus, at 26 days, the only structure which differed significantly from control values was the rough endoplasmic reticulum, which was significantly greater in the pilocarpine-treated group (fig. 25). The majority (75%) of the cells lining the alveolar space in this group were characterized as type II cells (table VI).

The cuboidal cells lining the alveoli of 27-day controls were characterized by a large increase in lamellar bodies over the previous day, with a corresponding decrease in glycogen (fig. 25). Although 78% (table VI) of the cuboidal cells were classed as type II cells, the inclusion bodies within these cells were characteristically immature forms (fig. 30). 100% of the cuboidal cells which were randomly photographed in pilocarpine-treated fetuses of 27 days gesta-
tion were classed as type II cells (table VI). The volume density of lamellar inclusion bodies and rough endoplasmic reticulum were significantly greater than in controls while there was significantly less glycogen (fig. 25). The lamellar inclusion bodies within these cells were, for the most part, well developed with distinct and numerous lamellae (fig. 31).
DISCUSSION

The findings of this study indicate that maternal administration of pilocarpine results in stimulation of pulmonary surfactant synthesis and secretion in the rabbit fetus and, in multiple doses, in accelerated differentiation of alveolar epithelial cells and maturation of the fetal lung as a whole. Following pilocarpine administration on days 24 through 27 of gestation, morphologic correlates of increased maturation were apparent when fetuses were obtained on the 28th gestational day. In addition, time-course studies indicated that some evidence of maturational changes in the lungs of pilocarpine-treated fetuses were apparent as early as the 25th gestational day.

Based on electron microscopic examination of fetal rabbit lung, it was determined that the 28th gestational day was the best suited for the elucidation of changes occurring in the lungs of pilocarpine-treated fetuses. Thus, the bulk of this study utilized fetuses of this gestational age. On the 28th day of gestation control fetal lungs were found to be relatively immature, in that the glycogen content of alveolar epithelial cells was high and cuboidal cells lining the alveoli contained relatively few lamellar inclusion bodies. These findings are at variance to those of Kikkawa et al. ('68; '71) who observed no glycogen in alveolar epithelial cells on day 28 of gestation and concluded that the rabbit fetal lung was completely mature at this age. The reason for this discrepancy between the results of the present
study and those of Kikkawa is not clear, but may in part be related to differences in buffer or dehydration procedure used in tissue processing for electron microscopy. During the work involved in this project, it was observed that phosphate buffered glutaraldehyde and a rapid dehydration procedure are necessary for optimal preservation of the glycogen present in fetal rabbit alveolar epithelium. The common use of cacodylate buffer or prolonged dehydration results in a leaching of glycogen out of the alveolar epithelial cells and the appearance, by electron microscopy, of vacuoles indicating the location of previous glycogen deposits.

In the present study pilocarpine was administered by a maternal route to avoid the possibility of stimulatory or maturational changes in the fetal lung, brought about simply by the trauma inherent in the operational procedure necessary to inject drugs to fetuses in utero. Results of the pilot experiments in which pilocarpine or saline were injected in utero at days 26 and 27 indicated that the lungs of fetuses injected with only saline in this manner showed evidence of an acceleration of maturation when compared to similar fetuses of non-operated mothers. These results confirm those of Robert et al. ('75) who, using biochemical parameters, observed maturational changes in the surfactant system of fetuses injected with saline in utero. The reason for this apparent stimulation is unclear; it may, however, be related to endogenous corticosteroid release or autonomic activation resulting from the surgical trauma to both the mother and fetus.

An additional reason for administering the pilocarpine maternal-
ly was that a primary aim of the study was to evaluate the effect of repeated stimulation of the fetal alveolar epithelium over a period of several days. This involved multiple administrations of pilocarpine and, since daily surgical procedures would be impractical, necessitated employing the maternal route for delivery of the pilocarpine. It was assumed that pilocarpine would cross the placental barrier since it is well documented that the vast majority of compounds with molecular weights less than 600 readily cross this barrier (Mirkin and Singh, '76) and pilocarpine HCl has a molecular weight of 244.7. Furthermore, when Corbet et al. ('76) administered pilocarpine directly to fetuses in utero, they observed evidence of pilocarpine stimulation in the doe (salivation, lacrimation etc.) indicating that the drug had crossed the placenta from the fetus to the mother. Thus, it may be assumed that passage in the opposite direction, that is from the mother to the fetus, is also readily accomplished.

Based on the study by Goldenberg et al. ('69) using adult rats, initially 150 mg/kg pilocarpine was administered subcutaneously to the rabbits in the present study. Although this dose worked well in non-pregnant adult rabbits, when administered to pregnant does it resulted in immediate convulsions and the rapid death of the animal. In the single injection experiments of the present study, doses of pilocarpine ranging from 10-80 mg/kg were employed and the larger of these doses, at times, also resulted in the death of the pregnant doe. Subsequent experimentation revealed the dosage of 5 mg/kg to be optimal insofar as the pregnant females exhibited all the signs of parasympathetic stimulation, but neither the doe nor the fetuses suffered any
increase in mortality. In addition, electron microscopic results from the single injection experiments indicated that this dosage was capable of stimulating the surfactant system of the fetus while producing no apparent deleterious effects.

Results of the SMA-12 test series which was performed on the serum of the pregnant does showed no significant differences between pilocarpine and saline groups, indicating that pilocarpine administration had no serious deleterious effects on the health or well-being of the animals. This was substantiated by the light microscopic survey of various organs of a pilocarpine-treated doe which revealed no abnormalities. Data obtained in this study did, however, reveal that the body weight of the pilocarpine-treated fetuses was decreased somewhat compared to controls. The reason for this reduction in weight is unclear. However, it is apparently not directly related to malnutrition since the food intake and blood glucose level of the mother, as well as fetal liver glycogen, were not reduced from control values. Similar weight losses have been previously described in cortisol-treated rabbit fetuses (Motoyama et al., '71). Furthermore, Kotas et al. ('71) have demonstrated that the maturational stage of the lung is independent of body size in fetal rabbits; in fact, they observed that, within a given litter, smaller fetuses often had more mature lungs than larger littermates.

Light microscopic observations of the lungs of 28-day pilocarpine-treated fetuses were consistent with an acceleration of maturation, as judged by general architecture, septal thickness and, in PAS
stained sections, glycogen content. Similar parameters have been employed by many investigators involved in the study of maturational changes brought about pharmacologically (Kikkawa et al., '71; Wu et al., '73). In the present study, however, in order to substantiate the reduction in glycogen content observed morphologically, a biochemical determination of glycogen content was employed. This method yielded data which agreed with the morphologic observations while also indicating that, since fetal liver glycogen was not significantly reduced in the pilocarpine-treated group, the reduction in glycogen content of alveolar epithelial cells in these fetuses was due to increased maturation, not malnutrition.

Data obtained through light microscopic morphometric analysis demonstrated a significant increase in the number of mature, or well differentiated, type II cells per unit area as well as an increase relative to the total number of lung cells. It is of interest that Kauffman ('77), using similar morphometric techniques, obtained results much like those of the present study in an investigation of maturational changes in the lungs of fetal mice following maternal administration of dexamethasone, a glucocorticoid. Based on these findings Kauffman concluded that the alveolar epithelium in her experimental group underwent accelerated differentiation following dexamethasone administration. The results of the present study suggest that pilocarpine administration causes a shift away from immature cuboidal cells and toward differentiation into mature type II cells, capable of active synthesis and secretion of surfactant.
Electron microscopic examination of the lungs of 28-day pilocarpine-treated fetuses revealed ultrastructural differences from controls which indicated increased maturation of the alveolar epithelium. The observed epithelial changes are similar to those described in glucocorticoid (Kikkawa et al., '71) or thyroxine (Wu et al., '73) treated fetuses, as well as in other studies employing pharmacologic means of increasing the maturation of the fetal lung (for review see Avery, '75). In the present study quantification of these changes was aided by the use of ultrastructural morphometry, which indicated that the number and size of lamellar inclusion bodies, as well as the number of multivesicular bodies, were increased significantly in the pilocarpine-treated group. In order to eliminate possible bias due to differences in cell size or lamellar body distribution, as well as to quantify other cell structures, stereologic morphometry was performed. The goal of this method was to determine the cytoplasmic volume densities of the various cell components which have been established as playing a role in the synthesis and secretion of pulmonary surfactant in the fetus. These structures include glycogen, lamellar inclusion bodies, mitochondria, rough endoplasmic reticulum and Golgi apparatus.

Results of this study indicated that, at the 28th gestational day, the volume density of lamellar inclusion bodies and rough endoplasmic reticulum were increased in the pilocarpine-treated group, while glycogen was decreased compared to controls. Therefore, not only are there greater numbers of mature type II cells in the lungs
of pilocarpine-treated fetuses, as indicated by light microscopic morphometric analysis, but these cells contain a greater volume density of structures involved in the production, storage and secretion of pulmonary surfactant. Associated with these increases is a decrease in the glycogen content because it is being used for the synthesis of surfactant phosphatidylcholine as demonstrated by Gilden et al. ('77) and others.

Comparison of the stereologic results obtained from pilocarpine-treated fetuses at the 28th gestational day with those obtained from controls at 29, 30 and 31 days revealed that measured components of the type II cells in 28-day pilocarpine-treated fetuses did not correspond uniformly to those of any given gestational age in controls. One problem in interpreting the results of this study was that the control alveolar type II cells were functionally mature at 29 days. Therefore, changes in measured cytoplasmic components occurring at 29, 30 and 31 days, although indicating further maturation, would not alter the viability of the fetus.

When the stereologic data from all gestational ages are considered together (fig. 25) some trends become evident. The general trends observed in control fetal lung cell maturation from immature cuboidal cells to mature type II cells included a decrease in glycogen with advancing gestational age. The lamellar inclusion bodies increased gradually through day 28, exhibited a large increase at day 29 and thereafter gradually decreased. This decline late in gestation may be related to qualitative changes of surfactant synthesis,
storage and release, as well as increased turn-over of surfactant components. Associated with these changes was a sudden increase in rough endoplasmic reticulum at 31 days. The amount of Golgi complex within control cuboidal cells remained relatively constant throughout the time period studied.

It may be stated that, in general, these changes through time also occurred in pilocarpine-treated fetuses, but either occurred earlier or to a greater extent than in controls. At each day except the 26th gestational day, the cells of the pilocarpine-treated group contained a significantly greater volume density of lamellar inclusion bodies and significantly less glycogen. The reason for the lack of significant differences in these values at day 26 is unclear. However, it probably relates to the fact that on this day the amount of rough endoplasmic reticulum within the cells of the pilocarpine-treated group exhibited its largest increase, reaching a value nearly double that of controls.

The results of the electron microscopic studies would indicate that repeated stimulation of the exocrine function of fetal alveolar epithelium with pilocarpine causes not only an increased secretion of surfactant in mature cells, as does a single dose (Corbet et al., '76), but also an increased differentiation and maturation of type II cells. This process is marked by an increase in the secretory apparatus of these cells and by an increased utilization of glycogen stores for the synthesis, storage and secretion of increased amounts of surfactant.
It is appropriate to mention that certain forms of fetal distress may produce changes in the fetal lung similar in some respects to those observed in the present study. These changes include an initial stimulation of secretion and a transitory acceleration of maturation (Balis et al., '75). However, in the reaction to stress or injury, these changes are usually accompanied by septal edema, which was not observed in the present study. Additionally, it has been established that following an injurious episode the type II alveolar epithelium of the adult (Evans et al., '75; Witschi, '76) and fetal (Balis et al., '75) lung undergoes a proliferative response. The light microscopic morphometric results of the present study indicated an increase in the number of mature type II cells in the lungs of pilocarpine-treated fetuses. That this is not a response to an injurious effect of pilocarpine is confirmed by the results of the scintillation autoradiographic study. These results indicated that fewer lung cells in the pilocarpine-treated group were actively synthesizing DNA, in preparation for cell division, when the lung tissue was removed and placed in culture media containing $^3$H-thymidine; thus, they failed to incorporate the labelled precursor and therefore did not label the autoradiograph.

In addition to indicating changes following injury, cell proliferation has been demonstrated to be a useful criterion for judging maturation in the fetal lung. It has been established that cell proliferation in the fetal lung decreases suddenly in late gestation (Sorokin, '61; O'Hare and Townes, '70; Kauffman, '75). The results of the present study revealed a reduced proliferation of both alveolar
epithelial cells and interstitial cells in the lungs of pilocarpine-treated fetuses. It may be concluded, based on these results as well as morphologic criteria, that pilocarpine administration resulted in an acceleration of differentiation and maturation of alveolar epithelial cells, at the expense of proliferation. These epithelial changes are paralleled by decreased proliferation of mesenchymal cells, resulting in thinning of septa and maturation of the lung as a whole.

With respect to other factors thought to affect fetal lung maturation, it has been suggested that maternal malnutrition may have a stimulatory effect on surfactant secretion and lung maturation (Naeye et al., '74). Data obtained in a study related to the present one (Smith, in press) indicate that the lungs of fetuses obtained at the 28th gestational day from does which were fasted for 6-7 days do show signs of increased maturation. The type II cells of these fetuses, however, are characterized by many morphologically abnormal lamellar inclusion bodies. In addition to malnutrition, increased synthesis and secretion of surfactant was reported by Kikkawa and Motoyama ('73) in fetal rabbits treated with AY-9944, an inhibitor of cholesterol biosynthesis. This stimulation, however, was accompanied by retardation of the general maturation of the lung.

Pilocarpine has been reported to be capable of stimulating the secretion of surfactant by type II cells in adult animals (Goldenberg et al., '69; Morgan and Morgan, '73). Corbet et al. ('76) have demonstrated that direct administration of pilocarpine to fetuses results in a reduction of surface tension, indicating increased secretion of
surfactant. Morphologic evidence obtained in the single injection experiments of the present study suggests that single, maternally administered, doses of pilocarpine result in the stimulation of surfactant production and secretion in near-term fetal rabbit type II alveolar epithelial cells. The mechanism by which pilocarpine causes this stimulation is, however, unclear.

Pilocarpine is a parasympathomimetic cholinergic agent which acts primarily at muscarinic receptors of autonomic effector cells. However, ganglionic effects are also observed in some cases (Koelle, '75). As an example, the actions of pilocarpine on the cardiovascular system are complex and difficult to interpret. Following administration of pilocarpine there is, as one would expect, a drop in blood pressure. However, if administration is preceded by a nicotinic blocking agent, there is a marked increase in pressure. Atropine, a muscarinic blocker, abolishes both effects and the increase in pressure can be blocked by a $\beta$-adrenergic blocker. It is thought that this complex situation may occur because of an activation of excitatory muscarinic receptors on sympathetic ganglion cells, as well as adrenomedullary chromaffin cells, both of which receive sympathetic preganglionic fibers which employ acetylcholine as a transmitter substance. In addition, it is known that pilocarpine has various central nervous system effects, ranging from evocation of the cortical arousal system, to autonomic and somatic motor effects suggesting activation of paraventricular hypothalamic nuclei. The common bond which links the effects of pilocarpine is the ability of atropine to
completely block them all, which would indicate that they are all due to activation of muscarinic binding sites.

Hung et al. ('72) demonstrated axonal endings of an autonomic nature in close proximity to type II pneumocytes and concluded that these provided a morphologic connection between the autonomic nervous system and surfactant secretion. It has been demonstrated that surfactant secretion is deleteriously affected by vagotomy (Goldenberg et al., '67); conversely, stimulation of the vagus results in increased surfactant secretion (Oyarzun and Clements, '77). Judging by this, it is logical to conclude that the nerve endings observed by Hung and co-workers are of vagal origin; thus, the type II pneumocytes would possess muscarinic receptor sites.

In a pharmacologic study, Corbet et al. ('77) theorized that the action of pilocarpine on surfactant secretion may be due to sympathetic stimulation. He suggested that the nerve endings present in the lung alveolar wall are of sympathetic origin and that the type II cells possessed β-adrenergic receptors. However, studies by Beckman et al. ('71) and Bergren and Beckman ('76) suggested that sympathetic stimulation causes a decrease in surfactant activity. The very fact that vagal stimulation causes increased surfactant secretion makes it difficult to accept sympathetic, β-adrenergic, innervation of type II pneumocytes, as Corbet proposed. It may be speculated that pilocarpine, due to its action at the adrenal medullary or ganglionic receptors, is, in fact, acting through an entirely different mechanism than that activated through vagal efferent stimulation. However,
oxotremorine, a synthetic analog of muscarine which has none of pilocarpine's predilection for these sites, has also been demonstrated to stimulate alveolar type II cells (Abdeblatif and Hollingsworth, '77). As may be inferred from this discussion, the question of whether the effect of pilocarpine is due to parasympathetic or sympathetic activation remains unresolved. However, it seems highly probable that its action is related to the existence of an autonomic control mechanism for surfactant secretion.

The findings of the present study indicate that repeated pilocarpine stimulation of the fetal rabbit surfactant system results in increased maturation of the type II epithelial cells of the alveolus, and the lung as a whole. The results have much in common with those obtained through glucocorticoid administration (Kikkawa et al., '71, and others) and the protocol of the present study does not absolutely rule out the possibility of endogenous corticosteroid influence. The same, however, may be said of any study, to date, which has attempted to pharmacologically enhance fetal lung maturation. The results of the single injection experiments of the present study, as well as the results of Pysher ('77) employing fetal lung in culture which was thus obviously separated from corticosteroid influence, have demonstrated that fetal lung is responsive to pilocarpine. Furthermore, in the present study stereologic analysis revealed maturational changes 24 hours after a single pilocarpine injection at the 24th gestational day while most studies using corticosteroids cite results 48-72 hours after multiple injections.
It is probable that normal lung maturation is controlled through many inter-acting mechanisms and systems, since it is possible, experimentally, to influence lung maturation through a variety of means. Although the natural role of the autonomic nervous system in the developing lung is unknown, the results of the present study indicate that the maturation of the fetal lung can be influenced through autonomic manipulation. Respiratory distress syndrome of the newborn has been linked to immaturity of the surfactant system of the lung. The results of this study contribute to the knowledge available concerning factors influencing or controlling the maturation of the fetal lung and its surfactant system, knowledge essential to promote development of rational modes of therapy.
SUMMARY

Pilocarpine HCl, a parasympathomimetic drug, was administered to pregnant white rabbits at various times late in gestation. Morphologic studies indicated that this resulted in a stimulation of surfactant synthesis and secretion. The same drug was then administered in a daily subcutaneous injection of 5 mg/kg on day 24 through day 27 of gestation. Fetuses from these animals and from saline injected controls were obtained by cesarean section on day 28 of gestation, term being 31 days in the rabbit. Histologic examination revealed thinner alveolar septa, and in sections stained with periodic acid-Schiff (PAS), a decrease in PAS positive material in the lungs of pilocarpine-treated fetuses when compared with controls. Morphometric evaluation indicated a significant increase in the number of mature type II cells in the alveolar epithelium of pilocarpine-treated fetuses, both per unit area and per 1,000 lung cells of any kind. Scintillation autoradiography using 1 μm thick methacrylate sections of fetal lung, from both groups, which was incubated with $^{3}\text{H}$-thymidine in vitro, revealed a significant decrease in the labelled cell indices of alveolar epithelial cells, as well as interstitial cells, in the lungs of pilocarpine-treated fetuses.

Electron microscopic studies revealed that the alveoli of the pilocarpine-treated group were lined by epithelial cells at a more advanced stage of maturation than those of controls. Type I cells in the pilocarpine-treated group were consistently well differentiated,
forming thin blood-air barriers. Ultrastructural findings in type II cells of this group included a substantial reduction in glycogen content, well developed or dilated rough endoplasmic reticulum and a significant increase (as determined by morphometric analysis) in the number and size of lamellar inclusion bodies, as well as the number of multivesicular bodies. Electron microscopic stereology indicated a significant increase in the cytoplasmic volume density of lamellar inclusion bodies, rough endoplasmic reticulum and Golgi complex in the pilocarpine-treated group while indicating a significant decrease in glycogen content. This observation of decreased glycogen was substantiated by the biochemical determination of glycogen content in the fetal lungs.

Stereologic analysis of type II cells from fetuses treated with pilocarpine as above but maintained, after cessation of drug administration at the 27th day, till the 31st day, showed no deleterious effects of pilocarpine treatment; in fact, this study indicated a sustained stimulation of surfactant synthesis and storage. Similar studies of type II cells from fetuses treated with pilocarpine for 1, 2 or 3 days and obtained at day 25, 26 and 27 of gestation respectively, yielded a time-sequence of the effect of pilocarpine which indicated that maturational changes were evident as early as the 25th gestational day.

The findings of this study provide evidence that maternal administration of pilocarpine results in accelerated differentiation and maturation of fetal type II alveolar epithelial cells from immature
cuboidal cells, at the expense of proliferation. This results in an increased activity of the type II cells in the synthesis, storage and secretion of pulmonary surfactant. These changes are accompanied by decreased proliferation of mesenchymal cells and increased maturation of type I epithelial cells, leading to thinner alveolar septa and blood-air barriers and, therefore, increased maturation of the lung as a whole.
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Figure 1. Light micrographs of paraffin embedded lung from a 28-day fetus injected with saline in utero. Sections are stained with periodic acid-Schiff (PAS) and thus dark area represent PAS-positive material: 1a; Bronchiolar cells (B) contain dark staining representing PAS-positive glycogen. Septa are relatively thin with far less PAS-positive staining than similar lungs of non-operated controls (compare with figure 9a) X 270. 1b; Higher magnification of same lung. While bronchiolar cells (B) contain abundant darkly stained glycogen many alveolar epithelial cells contain little or none (arrows). Contrast this with figure 9b. X 1060.
Figure 2. Light micrographs of paraffin embedded lung from a 28-day fetus injected with pilocarpine in utero. Sections are stained, photographed and enlarged exactly as those of figures 1, 9 and 10. 2a; Bronchiolar cells (B) contain PAS-positive staining. This lung is much like those of the controls illustrated in figure 1, with the exception that following pilocarpine injection, the lungs contain even less PAS-positive glycogen. Note surfactant material (S) in alveolar space. X 270. 2b; Higher magnification of the same lung. Arrows indicate alveolar epithelial cells containing little or no glycogen. X 1060.
PLATE III

Figure 3. Electron micrograph of a type II cell from the lung of a 28-day fetus injected with saline in utero. Lamellar inclusion bodies (L) are relatively numerous while glycogen (G) is scant. Compare with figure 17, which shows a similar cell from a non-operated control. R, rough endoplasmic reticulum; Go, Golgi complex; A, alveolar space. X 15,000.
PLATE IV

Figure 4. Electron micrograph of a type II cell from a fetus injected with pilocarpine in utero. Note large lamellar inclusion bodies (L) aggregated at the apex of the cell with one (arrow) apparently about to be discharged into the alveolar space (A). X 15,000.
Figure 5. Electron micrograph of a typical adult rabbit type II alveolar epithelial cell. Note the vacuolar appearance of lamellar inclusion bodies (L) and inconspicuous rough endoplasmic reticulum (R). Go, Golgi complex; M, Mitochondria; A, Alveolar space. X 21,000.
Figure 6. Electron micrograph of a type II cell from an adult rabbit which received a single injection of pilocarpine (150 mg/kg) two hours prior to sacrifice. Large lamellar inclusion bodies (L) are located apically and rough endoplasmic reticulum (R) is dilated. A, Alveolar space. X 31,000
PLATE VII

Figure 7. Electron micrograph of a type II cell from the lung of a 29-day control fetal rabbit obtained early in the project. Note greater development of lamellae within the lamellar inclusion bodies (L) when compared to those of the adult. Glycogen (G), which is virtually absent in adult type II cells, is also present within this cell. A, Alveolar space; M, mitochondria; Go, Golgi complex. X 16,800.
Figure 8. Electron micrograph of type II epithelial cells from a 29-day fetus whose mother had received 40 mg/kg pilocarpine six hours earlier. Rough endoplasmic reticulum (R) is dilated, glycogen is very scant and lamellar inclusion bodies (L) are apically located with one in each cell being apparently secreted (arrows) into the alveolar space (A). X 21,000.
Figure 9. Light micrographs of paraffin embedded lung from a 28-day control fetus. 9a; Note relative thickness of alveolar septa and heavy PAS staining in bronchiolar (B), septal and alveolar epithelial cells. X 270. 9b; Higher magnification of same. Alveolar epithelial cells (arrows) contain large amounts of PAS-positive glycogen. X 1060.
Figure 10. Light micrographs of paraffin embedded lung from a pilocarpine-treated 28-day fetus. 10a; Alveolar septa are, for the most part, thinner than controls while PAS-positive staining is negligible. X 270. 10b; Higher magnification of same. Alveolar epithelial cells (arrows) contain little or no PAS-positive glycogen X 1060.
PLATE XI

Figure 11. Light micrographs of 1 μm thick toluidine blue stained sections of 28-day fetal lungs. 11a; Low power of control. Note thick alveolar septa and relatively sparse blood-air barriers. X 550. 11b; Higher magnification of same. A mature type II cell is shown at the arrow. Portions of the alveolar space are lined by immature cuboidal cells (c) which contain abundant darkly stained glycogen. X 2,200. 11c; Low power of pilocarpine treated fetal lung. Note thinner alveolar septa and better development of blood-air barriers. X 550. 11d; Higher magnification of same. Mature type II cells containing intensely stained lamellar inclusion bodies are noted at the arrows. X 2,200.
Figure 12. The histogram on the left illustrates the numerical distribution of mature, or well differentiated type II alveolar epithelial cells per unit area (0.035 mm²) of a 1 µm thick section of fetal lung from the upper and lower lobes of each group. On the right is a similar histogram of the number of mature type II cells per 1,000 lung parenchymal cells. P, pilocarpine-treated; C, control.
TYPE II CELLS / UNIT AREA (0.35 mm²)

RIGHT UPPER LOBE
- C
- P

RIGHT LOWER LOBE
- C
- P

TYPE II CELLS / 1000 CELLS

RIGHT UPPER LOBE
- C
- P

RIGHT LOWER LOBE
- C
- P

P < 0.05

P < 0.005
Figure 13. Survey electron micrograph of a 28-day control fetal lung. Type II cells (II) contain large amounts of glycogen and few lamellar bodies. Blood-air barrier (BAB) is very thick and the epithelial component contains a heavy accumulation of glycogen. A, alveolar space; RBC, red blood cell in alveolar capillary lumen; I, interstitial cell. X 6,000
Figure 14. Survey electron micrograph illustrating alveolar epithelial cells of a 28-day pilocarpine-treated fetus. Type II cells contain little glycogen and relatively numerous lamellar inclusion bodies. Blood-air barriers (BAB) are well developed and thin. E, endothelial cell of capillary; C, capillary lumen; A, alveolar space. X 6,000.
Figure 15. Type I alveolar epithelial cell from a 28-day control rabbit fetus. Nucleus (N) is surrounded by abundant cytoplasm containing much glycogen (G). Cell extension is thin on the right and forms blood-air barrier over capillary (C). On the left side the cell extension is thick, contains mitochondria (M) and rough endoplasmic reticulum (R) and overlies a capillary endothelial cell (E). A, alveolar space. X 14,000.

Figure 16. Type I cell of a 28-day pilocarpine-treated fetus. Nucleus (N) is surrounded by a thin rim of cytoplasm containing virtually no glycogen. Cell extensions are attenuated to form blood-air barriers over capillary lumens (C). Note surfactant material (S) in alveolar space. X 10,000.
Figure 17. Type II cell of a 28-day control rabbit fetus. The cytoplasm contains abundant glycogen (G) and few lamellar inclusion bodies (L), usually containing sparse lamellae. A, alveolar space. X 13,000.

Figure 18. Type II cell of a 28-day pilocarpine-treated fetus. Glycogen is virtually absent while lamellar inclusion bodies (L) are numerous and show well developed, darkly staining lamellae. Notice lamellar body apparently in the process of being secreted (S) into the alveolar space (A), as well as a multivesicular body (MV) and well developed cisternae of rough endoplasmic reticulum (R). X 10,500.
PLATE XVI

Figure 19. Portion of a 28-day control type II cell. Note glycogen (G) and indistinct lamellae of inclusion body (L). M, mitochondrion; N, nucleus. X 30,000.

Figure 20. Portion of a type II cell from a pilocarpine-treated fetus. Glycogen (G), when present, is usually found in association with surfactant material (L). Note transitional form of inclusion body (T). M, mitochondrial. X 33,000.
Figure 21. Light micrograph of a 1 μm thick plastic section of a control fetal lung illustrating the labelling obtained through scintillation autoradiography. Labelled alveolar epithelial cells (short arrows) and interstitial cells (long arrows) are readily identified. X 1,380.
Figure 22. Histogram illustrating the results of the \(^{3}\text{H}\)-thymidine incorporation study on the alveolar epithelium and lung interstitium of pilocarpine-treated (P) and control (C) rabbit fetuses. Results are expressed as labelled cells per 1,000 cells and the small lines represent the standard error of the mean.
Labeled Cells / 1000 Cells

ALVEOLAR EPITHELIUM  INTERSTITIAL CELLS

C  P  C  P

p < .001
PLATE XVIII

Figure 23. Electron micrograph of a type II cell from a 31-day control fetus. Glycogen (G) is, by now, scarce and lamellar inclusion bodies (L) well developed. A, alveolar space. X 10,500.

Figure 24. Electron micrograph of a type II cell from a 31-day fetus which was treated with pilocarpine on days 24 through 27 of gestation. No evidence of damage is apparent. A, alveolar space. X 14,000.
Figure 25. Histograms illustrating the results of the electron microscopic stereologic analysis of various cell components involved in the synthesis and secretion of surfactant. Results are expressed as percentages of cytoplasmic volume and the small lines represent the standard error of the mean. Numbers at the bottom of each graph represent gestational age. C, control; P, pilocarpine-treated.
Figure 26. Electron micrograph of an immature cuboidal cell lining the alveolus of a 25-day control fetus. Note lack of lamellar inclusion bodies and abundant glycogen (G). A, alveolar space. X 13,000.

Figure 27. Electron micrograph of an immature type II cell from a 25-day pilocarpine-treated fetus. Glycogen (G) is less abundant than in controls, while the secretory apparatus is more apparent. Note immature form of inclusion body (IF). Go, Golgi complex; A, alveolar space. X 14,000.
Figure 28. Electron micrograph of an immature type II cell from a 28-day control fetus. Glycogen (G) has decreased from 25-day levels. Note immature forms of inclusion bodies (IF). A, alveolar space. X 13,500

Figure 29. Electron micrograph of an immature type II cell from a 26-day pilocarpine-treated fetus. These cells differed little from similar cells of 25-days. G, glycogen; Go, Golgi complex; IF, immature inclusion body; A, alveolar space. X 13,500
Figure 30. Electron micrograph of a type II cell from a 27-day control fetus. Glycogen (G) is fairly abundant. Note intermediate form of lamellar inclusion body (arrow) as well as mature forms (L). X 13,000.

Figure 31. Electron micrograph of a type II cell from a 27-day pilocarpine-treated fetus. Lamellar inclusion bodies (L) are mature and relatively numerous while glycogen (G) is becoming scant. Note dilated rough endoplasmic reticulum (R). A, alveolar space. X 11,000.
The dissertation submitted by Dennis M. Smith has been read and approved by the following committee:

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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