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Biochemical Maturation Changes in Developing Lung Surfactant in Rabbits

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BIOCHEMICAL MATURATION CHANGES IN DEVELOPING LUNG SURFACTANT

IN RABBITS

by

Peter Pagast

A Thesis Submitted to the Faculty of the Graduate School

of Loyola University in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

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Life

Peter Pagast was born in Konigsberg, Germany, on April 7, 1940.

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CHAPTER I
INTRODUCTION

Pattle, in 1955, was the first who demonstrated that the bubbles present in foam obtained from mammalian lungs are stable because they contain a potent surface active material. (1)

Pattle obtained foam by producing acute pulmonary edema in animals and by washing these lung in vivo and in vitro with saline solution. The bubbles which made up this foam had certain characteristic properties.

1. They were highly resistant against antifoaming agents.

2. The bubble size were extremely stable when placed in an air saturated medium.

From these properties Pattle felt that the lining film of the bubbles either were characterized by extremely low surface tension or were highly impermeable to air. By placing this foamy material into air-free water Pattle further demonstrated that the bubbles rapidly dissolved and, therefore, are not impermeable to air. From these findings Pattle concluded that the bubbles were lined by a strong, surface active film which lowered the surface tension of the bubbles to zero. He further suggested that the lining film of the bubbles originated from the lung alveoli which he assumed that they are also coated by a surface active material similar to that of the bubbles.

In 1957, Clements (2) confirmed Pattle's statements and made further important observations: lung extract which he obtained by rinsing lung with 0.9% NaCl were tested for surface tension with a modified Langmuir-Wilhemy surface balance. Tension was measured during the cyclic compression and expansion of the surface film. During compression and expansion of the
film a wide separation of the compression and expansion isotherms which he called "surface hysteresis loop" was noted.

An analogous situation was known to exist in excised lung specimens which characteristically show a hysteresis loop following lung deflation. The static volume pressure behavior of an air filled lung is due to tissue elastic and surface tension forces. The finding of Pattle and Clements suggested that during expiration, the surface tension is abolished due to the surface activity of a lung surfactant and, therefore, the normal lung is capable to retain air at low inflational pressure, resulting in a characteristic hysteresis loop. It is now generally accepted that the surfactant is a complex noncellular, surface active lipoprotein which lines the alveoli of a normal mammalian lung (4,5,6,7). Klaus and his co-workers (8) were the first who showed that a high phospholipid content was mainly responsible for the surface activity of the surfactant. Lipid extracts of lung foam from beef lung contained about 74% phospholipids and some cholesterol, triglyceride and free fatty acids. The phospholipids, but not the phospholipids combined with the neutral lipids were strongly surface active. Similar observations were made by Buckingham on sheep lung (9). Buckingham showed also that phosphatidylcholine (lecithin) was the predominant component of phospholipid extract obtained from lung foam. Fujwara and Einhörning, (10,11,12) examined the phospholipids of the surfactant-rich extracts from total lung tissue and washing from several species. They found that all extracts contained lecithin, sphingomelin and phosphatidylethanolamine and that the lecithin content was two to three times as high as the other two components combined. Morgan (13,14) reported a detailed
survey on pulmonary phospholipids obtained from washing of living dogs. He separated the phospholipids from the total lipids and the individual phospholipids were separated by thin layer chromatography. The surface activity and the fatty acids of each phospholipid were determined. His findings showed that the lipids of extracts obtained by alveolar lavage, which apparently contained primarily material lining the alveoli, represented a relatively small fraction of the total lipids of the lung. The composition of whole lung lipids were similar to those of other body tissues, whereas the lipids from lung washing were quite different. Thus, extracts from homogenized lung contained proportionally more cholesterol and less lecithin than the washings. The fatty acid residues of the lecithin obtained from washing were 78% saturated (66% palmitic acid), whereas the percent of saturated fatty acids in lecithin from homogenized lung was only 50%. Moreover, lecithin isolated from the whole lung homogenate showed less surface activity than highly saturated lecithin recovered from washings. Early studies by Popjak (16) suggested that the adult rabbit lung tissue readily synthesizes long chained fatty acids which are preferentially incorporated into the lecithin. He injected intravenously $^{14}$C-acetate in rabbits, and the animals were sacrificed nineteen to forty-eight hours after the injection of the isotope. In the liver the labeling was in the cholesterol fraction of the extracted lipids, while in lung it was found in the fatty acids of the lecithin. The specific activity of the pulmonary phospholipid fatty acids were extremely high, even higher than that of the cholesterol of the liver. When glycerol-$^{14}$C were incubated with lung tissue, the specific activity of glycerol was essentially the same for triglycerides
and phospholipids. By contrast the activity of fatty acid ester were higher in phospholipids than in triglycerides. This suggested that a common intermediate (diglyceride) is utilized for the synthesis of the lung triglycerides and phospholipids, but the phospholipids incorporate fatty acids preferentially. Chida investigated the synthesis of long chain fatty acids and especially palmitic acid in fetal sheep lung in vitro (31). He incubated lung tissue of different late gestational stages in a 75 ml Erlenmeyer flask equipped with a center well. The incubations were carried out for 3 hours at 38°C. In addition to several other compounds acetate 1-C\textsuperscript{14} was added to the lung tissue. The incubations were terminated by placing the flask into dry ice. His biochemical results showed that the fetal lung is capable of synthesizing long chain fatty acids. The distribution of the labeled material suggested that the majority of acetate incorporated into palmitic acid was through total synthesis via malonyl-coenzyme A, and only a relatively small amount of palmitic acid was formed by the elongation of short chain fatty acids. Felts (30) studied the in vitro synthesis of phospholipids in the lung and liver slices using palmitate-C\textsuperscript{14}. His results indicated that the incorporation of palmitic acid into phospholipids was greater in lung slices than in liver slices. Most of the phospholipid radioactivity (85%) was found in the lecithin fraction.

Fujiwara studied the in vivo incorporation of labeled palmitate into phospholipid components of the surfactant of fetal sheep (32). All of the fetal lambs were twins, near term; and were delivered by cesarean section under spinal anesthesia. With the placental circulation intact and the fetus in utero, one of the twins was given palmitate 1-C\textsuperscript{14} via the cotyledon
vein. After one hour, the fetus was delivered, the umbilical cord tied and the lamb was allowed to breathe normally for 10 minutes. Labeled palmitate similar to that given to its twin was injected via isolated vein into the other fetal lamb and killed after an hour without allowing it to breathe. Fujiwara's biochemical results showed that fetal lamb in vivo are able to incorporate palmitate into surface active phospholipids, chiefly phosphatidylcholine. The whole lung phosphatidylcholine contained 74-88% of the palmitate $^{14}C$ incorporated into the phospholipids. In the fetal lamb which breathed for 10 minutes he found that there was threefold increase in alveolar lecithin obtained from lung washing. These results were confirmed by Gluck, et al(18).

Nasr and Heinemann (17) studied the in vitro synthesis of lung phospholipids in adult rabbits. They either incubated minced lung tissue or perfused for 1-3 hours the intact lung with $C^{14}$ labeled acetate as a precursor. In the perfused lung, 75% of the incorporated activity from $C^{14}$ acetate was recovered in the phospholipid fraction. After separation of the phospholipids by thin layer chromatography 64% of the radio-activity was detected in lecithin, 5% in phosphatidylethanolamine and 3% in sphingomyelin. These data suggest that in comparison with other organs the lung has reserved a specific purpose for the synthesis of phospholipids.

Felt (15) showed in an in vitro study that with addition of glucose and labeled acetate to the incubation medium of adult rabbit lung slices, the fatty acid synthesis increased by 400%. He incubated also lung slices with glucose evenly labeled with $C^{14}$ and found that most of the isotope appeared in the phospholipid fraction with 90% of the radioactivity being in the glycerol of the phospholipids. From these findings Felt concluded
that the role of glucose is to provide an acceptor molecule (α-glycero-
phosphate) for the fatty acid synthesis of the phospholipids. It is also 
expected that another key role of glucose in the lung is the formation of 
NADP via the hexose-monophosphate shunt, which is used for the reductive 
synthesis of fatty acids.

The formation of lecithin, the principal phospholipid in the lung, 
commonly proceeds by incorporation of cytidine diphosphate choline and 
diglycerides. (Tri-glyceride + CDP-Choline → Lecithin + CMP). An 
alternate pathway involves the initial formation of phosphatidyl ethanolamine, 
which is derived from the incorporation of cytidine diphosphate ethanolamine 
and diglyceride. The phosphatidyl ethanolamine is converted to lecithin by 
N-methylation. (Phosphatidyl Ethanolamine +CH3 +CH3 +CH3 → PME → PDME → Lecithin).

Lecithin formation by methylation of phosphatidyl ethanolamine is 
appreciable in the fetal lung of the rabbit and reaches its peak activity 
shortly before birth (18). There appears to exist species differences in 
the prenatal pattern of lipid synthesis because a high rate of lecithin 
formation via phosphatidyl ethanolamine has not been observed in the fetal 
or adult rat (20). It has also been proposed that phosphatidyl ethanolamine 
within the alveolar lining layer not only contributes to the surface tension 
lowering properties of surfactant, but also acts as a precursor for 
extracellular formation (in the lining layer) of dipalmitoyl lecithin.

Morgan (21) suggested that under conditions of hypoperfusion or hypoxia the 
N-methyltransferase pathway may become relatively important for the biosynthesis of dipalmitoyl phosphatidylcholine. Thus, during fetal development the
low uterine PO₂ may give the methyltransferase lecithin synthesis relative preponderance over the CDP-choline pathway. Land (1963) described a third pathway for the formation of lecithin by the acylation of lysolecithin, but this has not been traced in the lung in vivo (19). The synthesis of the other surface active phospholipids, phosphatidylethanolamine and sphingomyelin, have a very close interrelation to lecithin synthesis. Phosphatidylethanolamine is formed from CDP-ethanolamine and D-α-β-diglyceride. Sphingomyelin from palmityl-CoA + serine and fatty acyl CoA + CDP-choline. Phosphatidylinositol is formed from CDP-diglyceride and inositol. Weinhold (22) studied the in vitro biosynthesis of phosphatidyl choline in lung of rats during prenatal development and his results suggested that the rate of the phosphatidylcholine synthesis is governed either by the formation of CDP-choline from phosphorylcholine and CTP or the combination of CDP-choline and diglyceride to form phosphatidyl-choline.

From various lines of evidence it is generally believed that the large alveolar cells (granular pneumocytes or type II cells) synthesize surfactant or precursors of the surfactant, which is stored in the inclusion bodies of these cells and eventually secreted into the air space (23,8,24,25). Originally, it was suggested that the lung surfactant were the products of the biosynthetic activity of the mitochondria of these type II cells. This hypothesis was based on circumstantial evidence suggesting surfactant activity of the washed mitochondrial fraction. Tombropoulos (26) suggested that both pathways for lecithin synthesis take place in the mitochondrial and microsomal fraction of the lung cells, but that the microsomal fraction was more active. Microsomal and microchondrial fractions were incubated
with palmitic acid labeled with $^{14}C$ in the carboxyl group. The microsomal fraction incorporated more $^{14}C$ from palmitic-$^{14}C$ than did the mitochondrial in the esterification process to lipids. The distribution of $^{14}C$ among the various lipid fractions were similar in the mitochondrial and microsomal fraction. Lecithin had the highest specific activity followed by triglycerides and diglycerides. Since Coenzyme A and adenosinetriphosphate were needed for the incorporation of $^{14}C$ from palmitate into the glycerides and phospholipids, this reaction represented not an exchange reaction but a synthetic process.

Gluck, et al, performed a series of studies concerning the biosynthesis and composition of surfactant phospholipids in fetal rabbit lung (30,18,28). In his experiments lung washings were obtained from rabbit fetuses of 21-31 gestational ages and from newborn rabbits. For each fetus five gentle lung washings were performed using 2 ml of 0.9 saline solution per washing. The cells and debris in the lavage fluid were removed by centrifugation (1000 g at 2°C). (From our experiments it seems to be questionable that 2 ml of saline solution could have been injected into a 21-day rabbit fetus since we found that for a 26-day rabbit fetus only up to 1 ml Ringer's lactate could be injected because of the small volume of the lungs. In addition, from previous work done in this laboratory (41) it has been established that centrifugation of washing at a centrifugation speed higher than 450 g results in significant sedimentation of surfactant into the cellular sediment, even when centrifugation time is only 5 min.) The results of these investigators showed that surface active lecithin appeared in washings after the 28th day of gestation, but with a special procedure they
were able to isolate some surface active lecithin from fetal lung parenchyma for the first time about the 23rd day of gestation. These findings suggested that surface active lecithin was stored in lung parenchyma a long time before its first appearance in the alveolar space. In newborn rabbits which breathed for several hours, a 3-5 fold increase in surfactant lecithin was detected in lung washing, while at the same time the percentage of surface active lecithin obtained from the residual (previously washed) lung, decreased. This suggests that with the onset of breathing large amounts of surface active lecithin are released into the alveolar space from the epithelial cells. In a recent study the different pathways for the synthesis of lecithin and phosphatidylethanolamine in the developing fetal rabbit were investigated in vitro (18): Lung homogenates when incubated with (a) CDP-(1,2-14C) choline for the CDP-choline pathway (CDP-choline + D-αβ-diglyceride \[\rightarrow\] lecithin) and (b) \(^{14}\)CH\(_3\)-S-adenosyl-L-methionine for the transmethylation pathway (phosphatidylethanolamine + 3(CH\(_3\))-S-adenosyl-L-methionine \[\rightarrow\] lecithin). The formation of phosphatidylethanolamine was investigated by incubating of lung homogenate with CDP-(1,2-14C) ethanolamine. All incubation procedures were stopped after one hour and the lipids were extracted and chromatographed on DEAE cellulose acetate. The individual phospholipids were separated on thin layer plates. Gluck data obtained from these studies indicated that the same pathways which are active for the de novo synthesis of lecithin and phosphatidylethanolamine in adult lung are also active in the fetal lung of rabbits. However, the incorporation of CDP-(1,2-14C)-ethanolamine was more active during fetal development with a peak activity at 25 and 26 gestational days.
The CDP-choline pathway was overall the most active during fetal development although a strong rate of transmethylation at 28-29 days of gestation from phosphatidylethanolamine to lecithin was found. In a more recent publication Gluck, et al, studied the fatty acid composition of lecithin during late fetal development (12). The total fatty acids of lecithin obtained from lung homogenate showed no tendency toward greater saturation, while the lecithin obtained from lung washings showed increase in the percentage of saturation of fatty acids during later stages of development. After the 28th day of gestation both \( \alpha \) and \( \beta \) component palmitic acid of lecithin rose abruptly from 45% to 67% and from 48% to 60% for \( \alpha \)- and \( \beta \)-carbon, respectively. This coincides with the first appearance of surface activity found in lung lavages from fetal rabbits. Thus, the synthesis of dipalmitoyl lecithin seems to contribute a great deal to the normal development of surface active material in the lung. Following observation that acetone-precipitated lecithins are surface active while acetone soluble lecithin are surface inactive, Gluck, et al, separated two presumably distinct types of phosphatidylcholine. The surface active acetone precipitated lecithin and the surface inactive acetone soluble lecithin had a high percentage of saturated fatty acids on the \( \alpha \)-carbon but a difference in fatty acid saturation was shown for the \( \beta \)-carbon. The surface active, acetone precipitated lecithin had 70% saturated fatty acids on the \( \beta \)-carbon, while there was only 25% of saturated fatty acids on the \( \beta \)-carbon of the surface inactive, acetone soluble lecithin.

Hyaline membrane disease of the newborn (HMD) is the most important illness with an underlying surfactant defect. It is believed that the pathogenesis of this disease is related to lung immaturity and fetal distress (29,38,39). Several biochemical studies indicated that the lung of infants
with this disease show a marked decrease in the concentration of the
lecithin, which is the major surface active phospholipid of the surfactant
(37,38). Earlier studies by Brumley et al, (39) indicated that the
phospholipid concentration was reduced in lung of infants with HMD. In
infants with HMD the lung phospholipid content was below 12.6 mg/gm wet weight,
while infants without hyaline membrane disease had a high concentration of
phospholipids, 22.3 mg per gram wet weight.

Adams (38) recently described for the first time the phospholipid content
and composition of human fetal lung tissue during normal maturation. Lung
of infants, which died from HMD were also investigated and were found to have
phospholipid values similar to that of normal fetuses of 6 months gestation.
In addition, the percent of palmitic acid in β-position of surfactant
lecithin was low in infants with HMD. This was in agreement with Gluck
studies (37). Adams further noted a similarity in the distribution of
lung phospholipid between infant with HMD and prematurely delivered lambs,
which developed postnatally respiratory distress and changes of the lung
resembling HMD (32). From these findings Adams suggested that HMD is
developed only in those infants with low concentration of lung phospholipids
regardless of birth weight or gestational age. It is known, however, that
in addition to lung immaturity other factors relating to in utero environment
of the fetus (maternal diabetes, various forms of fetal distress) may play
an important role in the etiology and pathogenesis of HMD (39,7). The
mechanism by which such factors alter the biosynthesis of surfactant is
unknown.

This project was undertaken to establish baseline, quantitative
biochemical parameters for the developing rabbit lung surfactant in order to subsequently investigate the mechanism of prenatal and postnatal surfactant changes induced experimentally. Total lipid, phospholipid and protein determinations were performed on fractions of lung washings from 26, 28 and 31 day old rabbit fetus and newly born rabbits using techniques, which were developed in our lab. The washings recovered from each litter were combined, fractionated and lyophilized. Biochemical values for the surfactant and other fractions were expressed per mg dry wt. of the lyophilized material. In addition, the amount of lipid, phospholipid and protein of the various fractions recovered per gram of lung weight was determined.

Finally, a pilot study was performed to evaluate the effect of fetal distress induced by maternal injection of E. coli endotoxin on the surfactant system of rabbit fetus.
CHAPTER II
MATERIALS AND METHODS

Seventy pregnant albino rabbits with fetuses of known gestational age were used for the experiment series. The animals were divided in four groups:

26 days gestation
28 days gestation
31 days gestation
Newborn rabbits

The pregnant rabbits were anesthetized by intravenous injection of sodium pentobarbitol. A cesarean incision was done and the pregnant rabbits were exsanguinated by cutting the aorta and inferior vena cava. The fetuses were killed by craniotomy and they exsanguinated by cutting the aorta and inferior vena cava. The fetuses were then weighed. A longitudinal mid-line cervical incision was made and the trachea was exposed and canulated with polyethylene catheter. Ringer's lactate solution was injected with a syringe connected to the above catheter into the trachea and the lungs were fully expanded. The amount of each single injection varied from .8 ml to 1.8 ml depending on the size of the lung. After slightly massaging the chest the buffer solution was drawn back. Several alveolar lavages were performed for each fetus until a total of 0.2 ml of buffer solution/gm of fetal body weight was introduced into the lungs. Subsequently the washing obtained from each litter were pooled. Ringer's lactate rather than saline solution was used because of studies in our laboratory have shown that saline solution were damaging the alveolar macrophages.
The lung washing contained cells (macrophages) and surfactant, which were separated using differential centrifugation. A sample of washing (3 ml) was lyophilized and the remainder was centrifuged at 450 g for five min. to obtain the cellular sediment A. The cell depleted supernatant (40 ml) was further centrifuged at 100,000 g, 4°C for 90 min. to obtain the surfactant sediment B and the final supernatant C. The sediments A and B were lyophilized following resuspension in 4 ml Ringer's lactate solution. Samples, 3 ml each, of the supernatant C were also lyophilized. The vials used for lyophilization were dehumidified and preweighed. Since all the different fractions were suspended in an exactly known amount of Ringer's lactate solution before lyophilization, the dry weight of the Ringer's lactate could be determined and subtracted from the total weight in order to obtain the absolute weight of the different fractions of the washing.

**EXTRACTION OF LIPIDS:** Lipids were extracted by the method of Folch, et al (40). Two ml of a 2:1 chloroform-methanol mixture (C-M) was placed in each lyophilized vial and left for sixteen hours at room temperature. The samples were then transferred to centrifuge tubes and the vials were washed with 2 ml of the C-M one at a time and added to the centrifuge tubes. After centrifugation the supernatants were placed in test tubes and the residues were reextracted with one ml C-M for one hour, centrifuged and the supernatants were added to the previous supernatants.

One ml of 0.37% KCL was added to each test tube containing the C-M extract and the content was mixed and centrifuged to separate layers. The upper phase was then discarded and the interface was rinsed carefully, so
that the lower phase was not disturbed, three times with pure solvent upper phase (chloroform - 3 ml; methanol - 48 ml; water - 47 ml) containing 0.37% KCl. The samples were then transferred to volumetric flasks (10 ml) and diluted with C-M to 10 ml.

**PHOSPHOLIPID DETERMINATION:** A modified phosphorus analysis by Martin and Doty was used (35). Desired volumes of lipid extract in 15 ml test tubes were evaporated to dryness with nitrogen. Standards were prepared as followed: One gram NaH₂PO₄ x H₂O was added to one liter H₂O, the solution having a concentration of 224.5 mg P/ml. This stock solution was then diluted 1 to 20. Six standards were prepared for each phosphorus determination using two 0.05 ml, two 0.1 ml and two 0.2 ml of the diluted stock solution. To each sample (unknown, standard and blank) 0.5 ml of 70% perchloric acid containing one gram of ammonium-molybdate/liter were added and heated in a sand bath at 180°C for about one hour or until the solutions were colorless. After cooling 1.5 ml water, 0.25 ml 10% ammonium molybdate and 1.5 ml benzene-isobutanol (1:1) were added to each test tube. The contents of the tubes were then mixed and centrifuged to separate phases. Aliquots (one ml or less if the samples appeared very yellow) of the upper phase were transferred to other tubes and the volumes were made up to 3.0 ml with absolute ethanol-conc. sulfuric acid (96.8:3.2.)

Samples were then mixed and 0.5 ml of stannous chloride reagent (10% in conc. HCC and diluted 1:100 with 1:N sulfuric acid just before using) was added to each tube and mixed again.

After 5 min. the absorbance was read at 725 nm with the Beckmann DB spectrophotometer. The phospholipid content was calculated assuming the phospholipids contain 4% phosphorus.
Fig. 1. Standard curve for determination of phospholipid.
SEPARATION OF PHOSPHOLIPID CLASSES: The phospholipid classes were separated by paper chromatography using the method of Wuthier (34). Whatmann papers SG-81 were cut out to 15.3 x 19 cm size. Five to fifteen µg phosphorus in chloroform-methanol solvent were applied on a spot near one corner, 2 cm from the edge, and were dried with a hair dryer. Solvent 1 (chloroform-90 ml; methanol - 30 ml; disobutyl-ketone - 60 ml; pyridine-40 ml and 8 ml distilled water) was used in the long direction. After developing in this solvent for 2 1/2 hours the papers were dried for 1/2 hour and developed in solvent 2 (chloroform - 60 ml; methanol - 35 ml; disobutylketone - 50 ml; pyridine - 70 ml and 12 ml of 0.5 M, pH-10.4 ammonium chloride buffer) in the other direction for 1 1/2 hours. The papers were dried for at least two hours, washed with distilled water, dipped into 0.005% solution of rhodamine 6G and washed again with distilled water. For the identification of the phospholipid spots the papers were examined under U.V. light. The phospholipid spots were cut out and each spot was weighed. Blanks were also cut out for correction of the background. For the quantitative determination of the single spots the phosphorus determination were used as described before.

DETERMINATION OF TOTAL LIPIDS: The calculation of the total lipids were done with the method of Marsh and Weinstein (33). Aliquots of lipid extract from the different fractions of the fetal lung washing were placed in 15 ml tubes. The C-M solvent was removed from the liquid sample under flow of nitrogen. One ml of concentrated sulfuric acid was added to each tube. The test tubes were placed for exactly 15 min. (stop watch) into a sand bath at 180°C. The tubes were left to cool for about
one minute and placed into ice water for 4 minutes. 1.5 ml distilled water were added to each tube, the contents were mixed thoroughly and the tubes were left to cool in ice water. After five minutes the optical densities were taken with a Beckman spectrophotometer at 375 nm.

As standard reference curve the relationship between optical density at 375 nm and the quantity of lipids in the different fractions of washing obtained from weanling rabbits was used.

The combined bulk - A - B and C fractions of lung washings of weanling rabbits were placed in preweighed bottles, evaporated to dryness and weighed. From the different combined fractions aliquots were taken and diluted to a concentration of 100 µg/ml with C-M.
Fig. 2. Standard curve for determination of total lipids in lung washings.

A - Total lipid in sediment A, obtained by fractionation of lung washing
B - Total lipid in sediment B, obtained by fractionation of lung washing
C - Total lipid in supernatant C, obtained by fractionation of lung washing
Bk - Total lipid in bulk
Fig. 3. Standard curve for determination of total lipids
**PROTEIN DETERMINATION:** The method of Lowry, et al was used (36). The method is based on the reaction of protein solution treated with phenol reagent of Folin Ciocalteau, which is due to:

1. Biuret reaction - peptide bonds with copper alkaline solution.
2. Reduction of phosphomolybdic acid and phosphotungstic acid by tyrosine and tryptophane.

As reagents a 2% Na$_2$CO$_3$ in 0.10 N NaOH (A) a 0.5% Cu SO$_4$.5 H$_2$O in 1% Na-tartrate (B) were used. Of solution A 50 ml were mixed with 1 ml B to make solution (C). A diluted Folin reagent, 1 N phenol, was prepared (solution D). In addition, a protein stock solution (crystallized human albumin, Dade - 8g/100 ml) was made for standardization. This stock solution was further diluted with distilled water 1:100.

In samples of 1 ml protein solution 5 ml of reagent C were added and mixed. The samples were allowed to stand at room temperature for 10 min. Of the diluted Folin reagent .5 ml were added rapidly and mixed within a second or two. After 30 min. the optical densities of the samples and the standards were measured at 540 nm.
Fig. 4. Standard curve for determination of total proteins.
RADIOISOTOPE STUDIES: In a series of pregnant rabbits (2 rabbits 26-day gestation, 2 rabbits 28-day gestation and 1 rabbit 31-day gestation) 10.4 mC (spec. activity 3.2 C/mM) tritiated sodium acetate was injected intravenously in each animal one hour prior to sacrifice. Lung washing of the fetuses were obtained and processed using the above procedures. In addition, samples of the previously washed lungs were homogenized. C-M solvent (20 ml) was added to the homogenate of the lungs, the mixture was left for 24 hrs and centrifuged for 10 min. at 1500 RPM. The lipids from the supernatant were extracted as described before. Samples of lipid extract dissolved in C-M solvent of the lung and the surfactant fraction of the washing were evaporated to dryness in scintillation vials. 0.1 ml of Chl:M=2:1 and 15 ml of fluor was added. (The fluor consisted of 5.5 gm of premixed M or perma blend T.M.J. in one liter toluene). The radioactivity was measured using a Packard Tri-Carb liquid scintillation counter.

EXPERIMENTAL MODEL FOR HYALINE MEMBRANE DISEASE: Six pregnant rabbits were injected intravenously at the 29 gestational day with 2-7 µg/kg body weight of E. coli endotoxin. Following cesarean section of the pregnant rabbits at the 31st gestational day, the fetuses were killed immediately. Lung tissues from the experimental groups were processed for phospholipid and protein analysis using the same techniques described for normal fetuses.
CHAPTER III
EXPERIMENTAL RESULTS

DETERMINATION OF LUNG WASHING RECOVERED PER WET GRAM LUNG WEIGHT

For each group of animals the average fetal lung weight and the average amount of lung washing recovered per fetus was determined. From the fetal lung weight and the corresponding washing recovered per fetus the amount of washing recovered per gram of wet lung weight were then calculated (Table I). From the above relationship it was possible to express the biochemical values of lung washing and its fractions for each gestational age per gram of wet lung weight.

LIPID AND PHOSPHOLIPID CONTENT OF LUNG WASHINGS (BULK)

The total lipids and the phospholipids of lung washing (bulk) for each gestational group were determined in order to find the relationship of phospholipid to total lipid content during development. The total lipids progressively increased during the late stages of gestation and a sharp further increase occurred after delivery (Table II). The percent of the phospholipid fractions of the total lipids also progressively increased but the degree of this increase was substantially greater from 28 to 31 days of gestation. The percent of phospholipids in the lung washing (bulk) was low for 26- and 28-day rabbit fetuses (6.77% and 9.8%) while after 28 days the percent of phospholipid rose to 67.80 for 31-day rabbit fetuses and 70.72% for newborn rabbit.
### TABLE I
PARAMETERS USED FOR DETERMINING THE AMOUNT OF WASHING RECOVERED PER WET GRAM LUNG WEIGHT

<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>Body Weight per fetus (g)</th>
<th>Lung Weight per fetus (g)</th>
<th>Washing, in ml, recov. per fetus</th>
<th>Washing, in ml, recov. per g of lung wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>21.57 ± 0.2</td>
<td>0.65 ± 0.022</td>
<td>3.11</td>
<td>4.75</td>
</tr>
<tr>
<td>28</td>
<td>28.38 ± 0.6</td>
<td>0.83 ± 0.021</td>
<td>4.81</td>
<td>5.77</td>
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<tr>
<td>31</td>
<td>50.92 ± 0.5</td>
<td>1.25 ± 0.019</td>
<td>7.04</td>
<td>5.61</td>
</tr>
<tr>
<td>Newborn</td>
<td>42.27 ± 0.8</td>
<td>0.70 ± 0.021</td>
<td>8.32</td>
<td>11.90</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>Total Lipid* (µg/g wet lung)</th>
<th>Phospholipid* (µg/g wet lung)</th>
<th>Phospholipid (Percent of TL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>176.10</td>
<td>26.00</td>
<td>6.77</td>
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<tr>
<td>28</td>
<td>596.19</td>
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<td>9.89</td>
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<tr>
<td>31</td>
<td>630.28</td>
<td>427.34</td>
<td>67.80</td>
</tr>
<tr>
<td>Newborn</td>
<td>2922.00</td>
<td>2071.20</td>
<td>70.72</td>
</tr>
</tbody>
</table>

*Each measurement was made on pooled lung washing from 15-30 fetuses.
PHOSPHOLIPID AND PROTEIN CONTENT OF CENTRIFUGATION FRACTIONS OF LUNG WASHINGS

The phospholipid content of the fractions A, B and C was initially expressed per 40 ml of lung washing (Table III). From the calculated relationship of lung washing recovered per gram of wet lung weight the phospholipid content of each fraction could be expressed per gram of wet lung weight (Table IV). The phospholipid content of the surfactant fraction B was low 8 and 10 µg/g lung in 26- and 28-day fetuses but it rapidly rose to 234.0 and 1350 in 31 day fetuses and newborns respectively, and had its highest value in the adult rabbit (Tables IV, V). This indicated that the above described rapid rise of phospholipid in the lung washing (bulk) was largely due to the increase in the surfactant fraction B. As other investigators have previously noted the results also showed that the onset of breathing is followed by a multifold increase in the content of surfactant phospholipids. In 26- and 28-day fetuses most of the noncellular phospholipids of the lung washing were found in the supernatant fraction C. In 31-day fetuses and newborns there was no further significant increase in the phospholipid content of fraction C. Most of the protein was found in the supernatant fraction C (Table IX, X, Fig. 5). With advancing maturation the percent of protein recovered in supernatant C gradually decreased due to corresponding increase in the protein content of both cellular fraction A and surfactant fraction B.
<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>Centrifug. Fraction</th>
<th>36</th>
<th>48</th>
<th>45</th>
<th>44</th>
<th>59</th>
<th>Average</th>
<th>%Average</th>
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</thead>
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<tr>
<td></td>
<td>Sed. B</td>
<td>90.84</td>
<td>77.43</td>
<td>73.65</td>
<td>60.29</td>
<td>51.89</td>
<td>70.82</td>
<td>17.33</td>
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<td>Sup. C</td>
<td>342.74</td>
<td>407.00</td>
<td>250.53</td>
<td>289.73</td>
<td>216.00</td>
<td>305.75</td>
<td>14.80</td>
</tr>
<tr>
<td></td>
<td>BK</td>
<td>461.83</td>
<td>524.28</td>
<td>349.36</td>
<td>373.16</td>
<td>308.45</td>
<td>408.16</td>
<td>100.00</td>
</tr>
<tr>
<td>28</td>
<td>Sed. A</td>
<td>39.00</td>
<td>36.39</td>
<td>60.61</td>
<td>64.19</td>
<td>41.89</td>
<td>48.41</td>
<td>11.55</td>
</tr>
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<td>Sed. B</td>
<td>58.88</td>
<td>45.52</td>
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<td>97.28</td>
<td>69.40</td>
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<td>Sup. C</td>
<td>355.42</td>
<td>220.76</td>
<td>380.00</td>
<td>350.00</td>
<td>200.00</td>
<td>301.24</td>
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<td>719.65</td>
<td>515.20</td>
<td>682.08</td>
<td>697.25</td>
<td>1109.74</td>
<td>744.78</td>
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<td>1590.43</td>
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<td>2298.85</td>
<td>1328.74</td>
<td>1819.32</td>
<td>1671.80</td>
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<td>Sup. C</td>
<td>355.42</td>
<td>325.23</td>
<td>312.86</td>
<td>341.45</td>
<td>203.93</td>
<td>307.78</td>
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<td>2665.50</td>
<td>2162.10</td>
<td>3293.79</td>
<td>2367.46</td>
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<td>Sup. C</td>
<td>316.00</td>
<td>426.90</td>
<td>543.80</td>
<td>777.29</td>
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<td>4992.11</td>
<td>6663.14</td>
<td>6600.83</td>
<td>5548.28</td>
<td>100.00</td>
<td></td>
</tr>
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</table>

* Underlined number means case number.  
  a Average is obtained from at least four different cases; each case was obtained from the pooled washings of 8 - 15 fetuses.
<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>Centrifug. Fraction</th>
<th>36*</th>
<th>48</th>
<th>45</th>
<th>44</th>
<th>59</th>
<th>bAverage %Average</th>
</tr>
</thead>
<tbody>
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<td>3.35</td>
<td>4.73</td>
<td>3.11</td>
<td>2.75</td>
<td>4.82</td>
<td>3.75±0.02</td>
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<td>Sed. B.</td>
<td>10.79</td>
<td>9.19</td>
<td>8.75</td>
<td>7.16</td>
<td>6.16</td>
<td>8.41±0.3</td>
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<tr>
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<td>Sup. B</td>
<td>40.70</td>
<td>48.33</td>
<td>29.75</td>
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<td>25.65</td>
<td>36.31±3.6</td>
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<td>54.84</td>
<td>62.26</td>
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<td>36.62</td>
<td>48.54 100.00</td>
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<td>28</td>
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<td>5.63</td>
<td>5.25</td>
<td>8.74</td>
<td>9.26</td>
<td>6.04</td>
<td>6.98±0.3</td>
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<td>8.49</td>
<td>6.57</td>
<td>11.31</td>
<td>9.65</td>
<td>14.03</td>
<td>10.01±0.4</td>
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<tr>
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<td>Sup. C</td>
<td>51.27</td>
<td>31.84</td>
<td>54.82</td>
<td>50.49</td>
<td>28.85</td>
<td>43.45±4.8</td>
</tr>
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<td>65.39</td>
<td>43.66</td>
<td>74.87</td>
<td>69.39</td>
<td>48.93</td>
<td>60.45 100.00</td>
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<td>155.64</td>
<td>104.46±12.3</td>
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<td>223.06</td>
<td>185.36</td>
<td>322.41</td>
<td>186.36</td>
<td>255.16</td>
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<tr>
<td></td>
<td>Sup. C</td>
<td>49.85</td>
<td>45.61</td>
<td>43.88</td>
<td>47.89</td>
<td>28.60</td>
<td>43.17±3.4</td>
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<td>111.51</td>
<td>139.76</td>
<td>189.24</td>
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<td>146.72±11.2</td>
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<td>965.74</td>
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<td>1963.75</td>
<td>1650.61</td>
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* Underlined number means case number.

b-Averages obtained from at least four different cases.

a: Indicates standard error.
TABLE V
PHOSPHOLIPID CONTENT OF SURFACTANT (B) AND OTHER FRACTIONS OF WASHING (EXPRESSED IN µg PL/g WET LUNG)

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>26</td>
<td>3.75 ± 0.02</td>
<td>7.73</td>
<td>8.41 ± 0.32</td>
<td>17.33</td>
<td>36.31 ± 3.62</td>
<td>74.80</td>
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<tr>
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<td>6.98 ± 0.39</td>
<td>11.55</td>
<td>10.01 ± 0.49</td>
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<td>43.45 ± 4.83</td>
<td>71.88</td>
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<tr>
<td>31</td>
<td>104.46 ± 12.33</td>
<td>30.27</td>
<td>234.47 ± 22.08</td>
<td>57.21</td>
<td>43.17 ± 3.41</td>
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<tr>
<td>Newborn</td>
<td>146.72 ± 8.88</td>
<td>8.88</td>
<td>1350.37 ± 136.5</td>
<td>81.81</td>
<td>153.52 ± 25.5</td>
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</table>

* indicates standard error

Each value is the average of measurements on 4-5 different cases. Each case was obtained from the pooled washing of 8-15 fetuses.
### TABLE X.

PERCENT PROTEIN IN DIFFERENT FRACTIONS OF LUNG WASHING

<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.23</td>
<td>1.98</td>
<td>96.77</td>
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<td>1.21</td>
<td>3.92</td>
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<td>4.11</td>
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<tr>
<td>Newborn</td>
<td>6.19</td>
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<td>80.51</td>
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</table>
TABLE IX

PROTEIN CONTENT IN SUPERNATANT C-FRACTION

<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>µg Pr/mg D.W.</th>
<th>Average</th>
<th>mg Pr/g wet lung</th>
<th>Average</th>
</tr>
</thead>
<tbody>
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<td>368.6</td>
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<td>195.2</td>
<td>.855</td>
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<td>1.334 ± .162</td>
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<td>376.5</td>
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<td>.855</td>
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<td>1.732 ± .212</td>
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<td></td>
</tr>
<tr>
<td>230.3</td>
<td>1.855</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290.0</td>
<td>2.512</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Each measurement was done on cases of 8-20 pooled fetuses.

*b* Indicates standard error.
Fig. 5. Shows protein content in supernatant fraction C.
DISTRIBUTION OF PHOSPHOLIPIDS IN SURFACTANT FRACTION B AND SUPERNATANT C

The composition of the phospholipids in the surfactant fraction B and supernatant C was obtained by two dimensional paper chromatography (Table VII, Fig. 9, 10). Figures 9 and 10 represent examples of the distribution of the different phospholipids for newborn obtained by paper chromatography. Table VII shows average values of the phospholipid distribution in surfactant fraction B obtained from at least 4 different cases for each gestational age. Phosphatidylcholine was the predominant phospholipid (90%) in the surfactant fraction B. Other phospholipid found in this fraction were sphingomyelin, phosphatidylethanolamine, phosphatidylinositol and unidentified phospholipids. In 31-day fetuses the distribution of the phospholipids of the surfactant fraction B was similar to that found in newborn and adult. The non-sedimentable phospholipids of the supernatant C were entirely composed of lecithin in 26-day fetuses (Table VIII). Other phospholipids including phosphatidylethanolamine were found in the more mature fetuses.
### TABLE VII
PERCENT OF TOTAL PHOSPHOLIPIDS
FOR SURFACANT FRACTION B

<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>Sphingomyelin</th>
<th>Phosphatidyl Choline</th>
<th>Phosphatidyl Ethanolamine</th>
<th>Phosphatidyl Inositol</th>
<th>Unidentified PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>1.4</td>
<td>88.1</td>
<td>3.1</td>
<td>4.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Newborn</td>
<td>0.5</td>
<td>84.9</td>
<td>3.5</td>
<td>5.2</td>
<td>5.5</td>
</tr>
<tr>
<td>Adult</td>
<td>1.0</td>
<td>90.2</td>
<td>2.4</td>
<td>4.3</td>
<td>4.4</td>
</tr>
</tbody>
</table>
Fig. 9 Phospholipid distribution in the surfactant fraction-B for new-born rabbit obtained by paper chromatography.
1- sphingomyelin
2- lecithin
3- phosphatidylethanolamine
4- phosphatidylinositol
5- unidentified phospholipids
Fig. 10. Phospholipid distribution in C-fraction of 31-day rabbit fetus obtained by paper chromatography.
1 - lecithin
2 - phosphatidylinositol
3 - unidentified phospholipids
### TABLE VIII

PERCENT OF TOTAL PHOSPHOLIPID FOR SUPERNATANT FRACTION C

<table>
<thead>
<tr>
<th>Gestation Days</th>
<th>Sphingomyelin</th>
<th>Phosphatidyl Choline</th>
<th>Phosphatidyl Ethanolamine</th>
<th>Phosphatidyl Inositol</th>
<th>Unidentified</th>
<th>PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>--</td>
<td>100</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>28</td>
<td>--</td>
<td>54.27</td>
<td>--</td>
<td>--</td>
<td>56.72</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>--</td>
<td>58.55</td>
<td>5.76</td>
<td>--</td>
<td>35.76</td>
<td></td>
</tr>
<tr>
<td>Newborn rabbit</td>
<td>--</td>
<td>74.78</td>
<td>7.91</td>
<td>--</td>
<td>17.39</td>
<td></td>
</tr>
</tbody>
</table>
RADIOISOTOPE STUDIES

In following injections of pregnant rabbits with tritiated sodium acetate the incorporation of the radioisotope into the lipid of the fetal lung increased progressively from 26-day of gestation to term which is in agreement with the general concept that lung synthesizes and stores intracellularly increasing amounts of surfactant until birth (Fig. 7). By contrast, the incorporation of tritiated sodium acetate into the surfactant fraction of the lung washing was insignificant in 26 and 28-day fetuses while a large incorporation of H-acetate was found in 31-day fetuses (Fig. 8).
Fig. 7. Incorporation of $^3$H-acetate into lipids of developing rabbit lung. The fetus were sacrificed one hour after intravenous maternal injection of the radioisotope.
Fig. 8. Incorporation of $^3$H-acetate into lipids of the B-fraction. The fetus were sacrificed one hour after intravenous injection of the radioisotope.
QUANTITATION OF PHOSPHOLIPID AND PROTEIN OF THE SURFACTANT FRACTION

The phospholipid and protein values were expressed not only per wet lung weight but also per mg dry weight of the lyophilized material. On a dry weight basis (µg/mg dry weight) the phospholipid values were low in 26 and 28 fetuses (8 and 10). After 28-days gestation the phospholipid content sharply increased. The phospholipid content for 31-day fetuses and newborns were 360 and 458 respectively and had its highest value in 721 for the adult rabbits. By contrast the protein values expressed on a dry weight basis (µg/mg dry weight) decreased from 238.00 in 28 day fetus to 190.00 in 31 day fetus, to 125.00 in newborn rabbit, and to 81.30 in the adult rabbit. From the above inverse relationship between phospholipid and protein it was possible to establish a reliable maturation index for the developing surfactant. Thus, the phospholipid to protein ratio was 0.13 and 1.94 on days 28 and 31 fetuses, 3.80 and 8.90 in newborn and adult rabbits, respectively (Table VI, Fig. 11).
TABLE VI
QUANTITATIVE PHOSPHOLIPID AND PROTEIN VALUES FOR SURFACTANT (B) FRACTION

<table>
<thead>
<tr>
<th>Gest. Age</th>
<th>µg/gm lung weight</th>
<th>c</th>
<th>µg/mg dry weight</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phospholipid a</td>
<td>Protein b</td>
<td>PL/Protein</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>26 - day rabbit fetus</td>
<td>8.81</td>
<td>42.27</td>
<td>.20</td>
<td>2.83</td>
</tr>
<tr>
<td>28 - day rabbit fetus</td>
<td>10.01</td>
<td>67.92</td>
<td>.15</td>
<td>24.36</td>
</tr>
<tr>
<td>31 - day rabbit fetus</td>
<td>240.00</td>
<td>89.33</td>
<td>2.68</td>
<td>360.03</td>
</tr>
<tr>
<td></td>
<td>1200.00</td>
<td>249.66</td>
<td>4.84</td>
<td>458.38</td>
</tr>
<tr>
<td>New Born Rabbit</td>
<td>1200.00</td>
<td>249.66</td>
<td>4.84</td>
<td>458.38</td>
</tr>
<tr>
<td>Adult rabbit</td>
<td>721.25</td>
<td>81.30</td>
<td>8.90</td>
<td></td>
</tr>
</tbody>
</table>

a - Phospholipid values at each gest. age are mean values from 5 different cases
b - Protein values at each gest. age are mean values from 3 different cases
c - PL/protein-ratio obtained for µg/g lung weight values
d - PL/protein-ratio obtained from the µg/mg dry weight values
Fig. 11. Shows the ratio of phospholipid against protein in surfactant fraction against gest. age.

$P_L_B = \text{Phospholipid B expressed in } \mu g/mg \text{ D.W. of washing.}$

$P_B = \text{Protein B expressed in } \mu g/mg \text{ D.W. of washing.}$
Fig. 6. Relationship of phospholipid and protein content between sedimentable fraction B and non-sedimentable supernatant C during development.

--- phospholipid C
- phospholipid B
O protein C
A protein B
EXPERIMENTAL MODEL FOR HYALINE MEMBRANE DISEASE

Some preliminary studies for the development of an experimental model for hyaline membrane disease were done. This exploratory study was undertaken in order to evaluate the effects of in utero induced fetal distress on developing fetal lung. Pregnant rabbits were injected intravenously at the 29 day gestation with endotoxin ranging from 2 µg/kg to 7.5 µg/kg body weight and the animals were sacrificed at 31 day of gestation when lung washings of the distressed fetuses were obtained. The calculated phospholipid values in surfactant fraction B of fetuses from three endotoxin injected pregnant rabbit were much higher than the control values, while the protein values were within normal limits (Table V, XL). The calculated maturation index of surfactant lipoprotein in the above three cases were 2.91, 3.49 and 4.92, which falls within the range found in the newborn rabbit.
**TABLE XI**

EXPERIMENTAL CASES*

<table>
<thead>
<tr>
<th>P.L(µg/40ml)</th>
<th>Protein(µg/40ml)</th>
<th>µg PL/g wet lung</th>
<th>µg Pr/g wet lung</th>
<th>P.L.B/Pr</th>
<th>(Surfac. Index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2.0µg endot./hg)</td>
<td>Sed. A</td>
<td>1110.0</td>
<td>-</td>
<td>158.4</td>
<td>-</td>
</tr>
<tr>
<td>Sed. B</td>
<td>2911.0</td>
<td>1000</td>
<td>-</td>
<td>415.5</td>
<td>142.7</td>
</tr>
<tr>
<td>Sup. C</td>
<td>174.0</td>
<td>-</td>
<td>-</td>
<td>24.8</td>
<td>-</td>
</tr>
<tr>
<td>(2.0µg endot./hg)</td>
<td>Sed. A</td>
<td>907.1</td>
<td>-</td>
<td>129.4</td>
<td>-</td>
</tr>
<tr>
<td>Sed. B</td>
<td>2435.7</td>
<td>698.0</td>
<td>-</td>
<td>341.6</td>
<td>97.8</td>
</tr>
<tr>
<td>Sup. C</td>
<td>280.0</td>
<td>9208.0</td>
<td>-</td>
<td>39.9</td>
<td>1291.0</td>
</tr>
<tr>
<td>(3.0µg endot./hg)</td>
<td>Sed. A</td>
<td>6408.8</td>
<td>-</td>
<td>914.8</td>
<td>-</td>
</tr>
<tr>
<td>Sed. B</td>
<td>4663.3</td>
<td>948.0</td>
<td>-</td>
<td>654.0</td>
<td>132.9</td>
</tr>
<tr>
<td>Sup. C</td>
<td>300.0</td>
<td>14473.0</td>
<td>-</td>
<td>42.8</td>
<td>2029.8</td>
</tr>
<tr>
<td>(3 x 30 ml dextrose-subcut)</td>
<td>Sed. A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sed. B</td>
<td>884.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sup. C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Phospholipid fraction B/Protein fraction B - Obtained from µg/g lung values.

Phospholipid and protein values from lung washings and their relationship to each other of 31 gestation days rabbit fetuses.

Normal development of the rabbit fetuses were impaired by intravenous injection of endotoxin to the mother rabbit two days before the animals were sacrificed.

Case 4-represents the phospholipid value of the surfactant fraction from fetuses which were kept under hyperglycemic condition for 2 days. The phospholipid value is about half of the phospholipid values of control animals.
CHAPTER IV

DISCUSSION

The primary objective of this research work was to establish baseline quantitative parameters for the biochemical maturation of normal surfactant in developing rabbit lung. Surfactant is a complex lipoprotein substance and, therefore, maturation changes during lung development may occur not only in the phospholipids but also in other constituents of the surfactant molecule. The system of quantitative evaluation described in this study, therefore, was designed to provide information relating to 1) quantitative and qualitative changes of the various components of the surfactant lipoprotein molecule which may occur with advancing maturation of the surfactant system and 2) to compare and correlate changes of the surfactant and other fractions recovered from the alveolar space. Thus, fractionation of lung washing was made in a manner to provide information relating to the surfactant fraction as well as to the alveolar macrophages and those non-cellular constituents of the alveolar space which do not represent an integral part of the surfactant system. In addition, biochemical values were expressed per gram of lung weight, to evaluate changes in the phospholipid and protein content of the alveolar spaces, and per mg dry weight of each fraction in order to obtain data reflecting changes in the composition of the surfactant lipoprotein with advancing maturation. With the above new system of quantitative evaluation it was possible to obtain data strongly suggesting that the composition of surfactant lipoprotein undergoes significant changes with lung maturation. Thus, from the findings of this work it appears that during late stages of lung development, rapid increase in the concentration
of surfactant phospholipids in alveolar spaces is accompanied by a more gradual increase in the amount of the protein component of the surfactant system. Since the rate of phospholipid increase was found to be faster than that of protein, it would be expected that the surfactant lipoprotein molecule will progressively contain relatively more phospholipids than proteins with advancing maturation of the surfactant. This interpretation is supported by the finding that on a dry weight basis, the maturing surfactant was characterized by progressive increase in the phospholipid, in association with gradual decrease in its protein content. From the above inverse relationship between phospholipids and proteins of the developing surfactant, a maturation index was established. From this index, it appears that at least in rabbits, maturation of the surfactant lipoprotein proceeds even postnatally since the ratio of phospholipids to proteins is highest in the adult rabbit lung surfactant.

It is well established from the findings of Gluck, et al, and Adams, et al, (27,38) that saturation of the fatty acids on the \( \alpha \) and \( \beta \) carbon of the surfactant lecithin progressively increases with maturation of the fetal lung. The above changes in the composition of surfactant lecithin, therefore, could be used as a maturation index for the mammalian surfactant since these changes have already been observed in lambs, human and rabbit fetuses. More recently, Gluck, et al, provided evidence that for human surfactant the ratio of sphingomyelin to lecithin can be used as a reliable index of surfactant maturation (53). Whether this index can be also used for other mammalian species is not known...
If it should be mentioned at this point that since surfactant is a lipoprotein (apparently in all mammalian species) it would be logical to expect that the maturation index in this work may prove to be not only more convenient to perform but also more reliable in providing information relating to the state of surfactant-maturation under normal and pathologic conditions. In this respect, some pilot studies have been performed and although it is still early to make a definite statement the findings are very encouraging as to the significance of the system for quantitative surfactant evaluation as well as the maturation index described in this study. Thus, following maternal injection of E. coli endotoxin to induce fetal distress, lung washings of the distressed fetuses were investigated. The results suggested that the surfactant fraction of the washings were characterized by a higher phospholipid content as compared with the normal fetus of the same gestational age, and the maturation index was similar to that of more mature animals (newborn rabbits) under normal conditions. By electron microscopy the alveolar spaces of distressed fetuses revealed an excessive accumulation of myelin figures which represent structural counterparts of surfactant lipoprotein. It is known that the developing lungs continuously store surfactant intracellularly even after the 29th day of gestation, at which time the surfactant begins to appear in the alveolar space. It is tempting to speculate, therefore, that in utero distress resulted in premature (prior to delivery) release of stored surfactant into the air spaces leading to exhaustion of the intracellular surfactant reservoir. It is generally accepted that newborn animals need large amounts of surfactant for the commencement of the normal respiratory function, and
in utero exhaustion of the surfactant stores could interfere with the above prenatal event. This hypothesis could explain the apparent paradox that while the lung of distressed full term fetuses contained more surfactant phospholipid; similarly endotoxin treated fetuses developed after delivery by cesarean section respiratory distress and edematous changes of the lung.

Since maternal diabetes is a known predisposing factor for hyaline membrane disease of the newborn a pilot experiment was also performed to evaluate the effect of maternal hyperglycemia on the maturation of developing lung and surfactant. The results of this pilot study suggested that intra-alveolar release of surfactant is delayed following repeated subcutaneous injections of glucose to pregnant mothers. Thus, the phospholipid values of surfactant fraction recovered from full term, treated fetuses were significantly lower than normal fetuses of the same gestational age. These biochemical findings were in agreement with the result of electronmicroscopic studies which suggested that the type II epithelial cells of treated animals revealed changes compatible with arrested secretory activity (42).

Previous investigation in the developing lung surfactant have been mainly concerned with changes of the surfactant phospholipids, especially lecithin. The results of these studies indicated that phospholipid metabolism does change during functional development of the lung. But since the surface active material of the lung is a complex lipoprotein, further biochemical work should be directed in developmental changes of other surfactant lipoprotein constituents using quantitative and qualitative parameters. Furthermore, by correlating these parameters with enzymatic changes involved in the biosynthesis of developing surfactant, it would be possible to obtain
a basic understanding as to the nature and mechanism of surfactant damage in hyaline membrane disease of the newborn.

ABSTRACT

This study was undertaken to establish quantitative biochemical parameters for developing rabbit lung surfactant. Lung washings from 26-31 days gestation fetuses, newborn and adult rabbits were fractionated into cells, sedimentable surfactant and supernatant using techniques designed to characterize and quantify distinct constituents of the alveolar space. In 26 and 28 days fetuses more than 70% of the phospholipids (composed almost entirely of phosphatidylcholine) were found in the supernatant fraction of the lung washing. After day 28 the phospholipid content of the sedimentable surfactant fraction per gram lung weight revealed a rapid increase in association with a more gradual rise in the protein content of the same fraction. Since the rate of increase of phospholipids was significantly greater than that of proteins, the amount of protein per mg dry weight of the surfactant fraction decreased with advancing maturation of the fetus. From the above inverse relationship between phospholipid and protein it was possible to establish a reliable maturation index for the developing surfactant. Thus, the phospholipid to protein ratio was 0.13 and 1.94 on days 28 and 31 fetuses, 3.80 and 8.9 in newborn and adult rabbits, respectively.

The findings indicate that the composition of surfactant lipoprotein undergoes significant changes during late stages of fetal development. The results also suggest that the phospholipid to protein ratio is a reliable and convenient parameter to evaluate the state of surfactant maturation.


34. Wuthier, R.E.: Two-dimensional chromatography on silica gel-loaded paper for the microanalysis of polar lipids. J. Lipid Res. 7: 544, 1966


42. Balis, J.U.: Unpublished data.
APPROVAL SHEET

The thesis submitted by Peter Pagast has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 16, 1972
Date

[Signature of Advisor]