

A REPORT ON THE SURFACTANT SYSTEM OF THE LUNG

by

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W

B.S., University of Southern California, 1975

A MASTER'S REPORT

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas
1979

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I. INTRODUCTION

In the past 10-15 years, basic biological research has progressed greatly in defining the pulmonary surfactant system. The reason for specialized focus on this system is because it plays a particularly significant role in the survival of newborn infants. The lack of pulmonary surfactant is intimately linked to the respiratory distress syndrome (RDS) in infants which is also called the hyaline membrane disease (HMD). This disease appears to be the cause of 30% of all neonatal deaths and 50-70% of premature infant deaths (Crofton and Douglas, 1969). According to Northway and Daily (1972), 25% of "liveborn premature infants," and 1-2% of all newborn infants develop this disease. Because this disease accounts for such a large portion of infant deaths, scientists are involved in research which may allow better treatment and perhaps prevention of this disease.

The basic cause of this disease is not known. However, it appears as though the "maturity" of the pulmonary surfactant system at birth is an important factor in this disease. From the statistics cited above, it appears as if prematurity predisposes an infant to develop this disease. As will be shown in this report, in premature animals the surfactant system is not fully developed. However, treatment with corticosteroids appears to accelerate the development of the system and may prolong survival of animals (Avery, 1973).

Avery (1973) constructed a table from various sources showing things which are known for certain, or are probable, or are possible in relation to this disease. A few things which are known with certainty will be mentioned here. The disease occurs near the time of birth, and death or recovery occurs in 3-5 days. Reduced lung compliance (elasticity), low systemic blood pressure, reduced effective pulmonary blood flow, and cyanosis are additional characteristics. Pathological observations include regions of atelectasis (collapse) of alveoli, and formation of hyaline membranes. The hyaline membrane lines the alveoli and is made up of "sloughed cell debris in a protein matrix" (Crofton and Douglas, 1969). These membranes contain fibrins (Avery, 1973). The etiology of the disease is definitely linked to surfactant deficiency (Avery, 1973). Evidence for this comes from the "effectiveness of continuous distending airway pressure" (Avery, 1973). These and other clinical and pathological symptoms are characteristic of this disease. The general consensus appears to be that alveolar collapse due to a lack of pulmonary surfactant is a primary factor of the disease. Bates et al. (1971) (citing various sources) gave possible causes of this disease which include "asphyxia, hypoperfusion of the pulmonary vasculature, or a fibrinolytic-enzyme defect."

This report will focus on the pulmonary surfactant system itself. Four basic topics will be covered. The first topic will mainly define the system which includes the

composition, structure, and synthesis of surfactant. Secondly, the discussion will center on the type II cell and model systems. The third topic will deal with various treatments of animals and/or lung tissues in order to determine what agents or conditions might control surfactant synthesis and secretion. A discussion of proposed research will be the fourth topic. Due to the rather broad scope of this paper, an attempt will be made to cover significant points about this subject. The reader should keep in mind that this report, as with any publication, is limited by the author's biases toward certain information and is limited to this author's knowledge of the field. Even though some portions of this subject are covered superficially, the report should provide a thorough background for anyone interested in studying the pulmonary surfactant system.

References for Introduction

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II. LUNG DEVELOPMENT

Before plunging into a discussion of pulmonary surfactant, lung development will be briefly outlined. This will provide some orientation as to the time and place of the events leading up to and following surfactant production.

Lung development as described here will be very general. Development will be discussed mainly in terms of the human lung. (Most of the material here comes from books or review articles). Most animal lungs share the same general pattern of development with some variation (Williams, 1977). Engel (1962) has compared the alveolar diameter in lungs of different species (see Table 1) and he found that the diameter range is very small even though they differ greatly in body size. He (Engel, 1962) also noted that rat and mouse lungs have "true acinar structure," but the mouse acini are primitive. Engel (1962) stated that the lungs of mice and rats did not require a bronchial tree due to their small size, and termed their branched structure as a "bronchiolar tree."

In humans, the lung first appears as a shallow groove in the ventral wall of the foregut, just cephalad to the liver and stomach, by 22-26 days after fertilization (5mm long embryo) (Strang, 1977). The lung epithelial lining cells are derived from endoderm, and the "auxilliary" tissues such as pleura, muscle, cartilage, and blood vessels are mesodermal and mesenchymal derivatives (Williams, 1977). Pulmonary elastic tissue and lymphatic tissue are also derived from mesenchyme (Emery, 1969a). In humans, the caudal end of the groove forms a single small pouch which later divides (Emery, 1969a; Strang, 1977). However, in the mouse, there are initially two separate lung buds which later fuse at their bases forming a "primary branch point" (Spooner and Wessells, 1970). Later, the gut tube gives rise to the dorsal esophagus and ventral trachea at the primary branch point (Spooner and Wessells, 1970). The epithelial component of these buds form a sheet of cells which grow more rapidly than the surrounding mesoderm, and branch to form the bronchial tree (Strang, 1977). The branching pattern of lungs depends on the interaction of epithelium and its surrounding mesoderm (Williams, 1977; Strang, 1977). For a short review of how mesoderm, and collagen produced by the mesoderm, influence lung branching, see the review by Strang (1977). The formation of alveoli is the final and one of the most important steps in lung development. The type II alveolar epithelial cell, believed to produce surface-active material which prevents collapse of alveoli in the air-breathing infant, appears at various stages of development depending on the animal studied. Osmiophilic inclusion bodies (OIB's), presumably the surfactant storage sites in type II cells, appear at the following times:

- 18 days in the mouse fetus (19 days = term)
- 120 days in the lamb fetus (147 days = term)
- 24 days in the rabbit fetus (31 days = term)

22-26 weeks in the human fetus (40 weeks = term)
(Strang, 1977).

Much alveolar growth apparently occurs postnatally in humans but some alveolar ducts, alveolar sacs, and alveoli appear before birth (or term) (Northway and Daily, 1972; Emery, 1969b).

Lung development is generally broken down into three stages: 1) The glandular period - characterized by respiratory tubules lined by tall columnar epithelia, unassociated with capillaries (Williams, 1977). This stage occurs up to about the fourth month of human fetal gestation (Emery, 1969a). 2) The canalicular period - characterized by respiratory tubules with broad lumens becoming "apposed to the capillary network," according to Williams (1977). Emery (1969a) described this as a period during which bronchi are dividing, sometimes "dichotomously," and often monopodially. There is increased vascularization but capillaries are not yet "intimately" in contact with the epithelium. 3) The alveolar period - type I cells apparently form thin cytoplasmic extensions which are in contact with capillaries to form "attenuated blood-gas barriers" (Williams, 1977). In humans, this period begins around six months (Emery, 1969a) and alveolarization continues through to postnatal life (Northway and Daily, 1972; Emery, 1969b). For a view of human lung anatomy at the cellular level (electron microscopic) throughout development see Conen and Balis (1969).

Table 1. (Engel, 1962)

<u>Animal</u>	<u>Alveolar Diameter</u>
Mouse	0.005
Rat	0.1
Guinea Pig	0.05
Rabbit	0.1
Goat	0.07
Man	0.2
Elephant	0.25

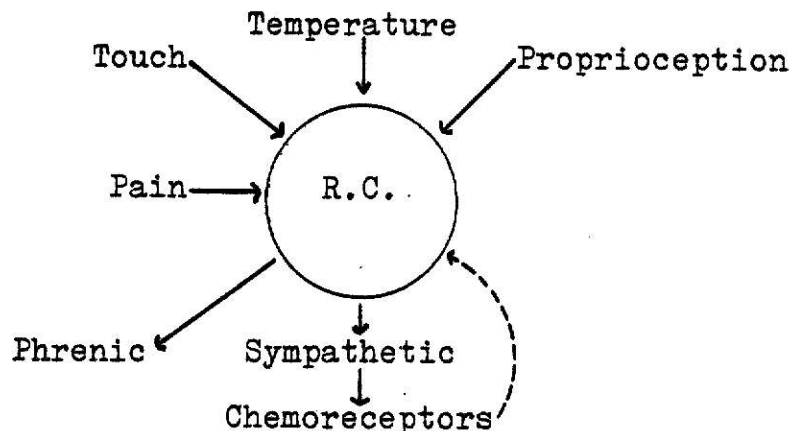
The factors which control the onset of respiration are not fully understood. During delivery, lung fluid is expelled and the remaining fluid in the lungs is thought to be removed through the lymphatic (Northway and Daily, 1972; Strang, 1977) and capillary circulations (Northway and Daily, 1972). Purves (1967) has performed studies on the initiation of respiration. Figure 1 after his drawing (Purves, 1967) diagrams the proposed events at the onset of respiration. The hypothesis involves chemoreceptors in the regulation of breathing. A number of stimuli apparently activate the respiratory center of the brain at birth, then the sympathetic activity results in activation of the chemoreceptors (Purves, 1967). The review by Strang (1977) provides more up-to-date information on the initiation and control of neonatal breathing

which includes evidence that chemoreceptors are depressed in the fetus.

The whole of lung development is much more complex than presented here. However, it gives one an idea of where the events of surfactant production fit in to the overall pattern of development. This report will deal mainly with the activities of alveolar type II cells and the surface-active material which these cells produce.

Figure 1. (after Figure I from Purves, 1967)

Sequence of Events at Onset of Respiration



"The respiratory centre (R.C.) is activated by sensory impulses from the periphery which, in turn, causes rhythmic activity in phrenic and efferent sympathetic nerves. One consequence of this may be activation of the peripheral chemoreceptors."

(Note: Much of the material used in the introduction was derived from reviews and books - original papers were not cited here).

References for Lung Development

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III. DEFINING THE SURFACTANT SYSTEM

In the past several years, the biochemical make-up of pulmonary surfactant has been studied carefully. Pulmonary surfactant is a combination of mainly lipid and protein molecules (Farrell and Avery, 1975) which are secreted by the type II alveolar epithelial cell during late gestation in most experimental animals, as will be seen in future sections (OIB's occur around mid-gestation in humans - see Strang (1977) from the development section). The purpose of the surfactant appears to be that of lowering surface tension at the air-liquid interface in the alveoli, thus preventing their collapse (King, 1974).

In this section of the report, the phospholipid and protein compositions of adult lung surfactant will be discussed, with brief attention to carbohydrate composition. A monograph written by Clements and King (1976) provides a good overview of the lipid and carbohydrate composition but emphasizes the protein composition. After establishing the composition, proposed structures for intracellular and extracellular surfactant will be covered. Finally, ideas on the synthesis (including enzyme and lipid profiles during development) of surfactant will be presented.

A. Phospholipid Composition of Adult Lung

The isolation and identification of surface-active material from lung washings has made it possible to determine the content of pulmonary surfactant itself. Lung wash fractions with surface tension lowering capabilities appear to be primarily lipid in nature and, as will be seen in the protein section, there may be specific proteins associated with surfactant (King, 1974).

According to a review by Farrell and Avery (1975) surfactant is composed of 85% lipid, 13% protein, <1.7% hexose, <0.7% nucleic acid, and <0.5% hexosamine. Phosphatidylcholine (PC) makes up 75% of the surfactant lipid (Farrell and Avery, 1975). This class of lipids has been studied to a much greater extent than the other lipid classes of surfactant. Other phospholipids include phosphatidylethanolamine (PE), sphingomyelin (SP), and lysolecithin (lyso-PC) which make up 6.3, 2.1, and 0.9% of surfactant lipid, respectively (Farrell and Avery, 1975). Neutral lipids account for 5-13% of surfactant lipid, which is mostly cholesterol (King, 1974). For an example of the surfactant components from canines, see Figure 2.

The following material was derived from a few studies of the surfactant lipid composition. They yield examples of the types of data obtained when analyzing surfactant lipids. Many studies similar to these have been performed.

Because phospholipids are the primary lipid component of surfactant, most of the material presented in this section

will deal with this class of lipids. The phospholipids will be viewed from three different levels - phospholipid classes, degree of saturation and unsaturation as indicated by fatty acid (FA) composition, and molecular classes. The biological half-life of these phospholipids will also be covered. It is important to note that the information found in the following studies is derived from adult animals. A discussion on the synthesis of these lipids especially during development, will be found in the synthesis section of this report.

Table 2 contains data on the surfactant phospholipids found in various cell fractions. Compositions of the alveolar wash (extracellular surfactant) and lamellar body (surfactant storage sites) fractions are considered to be indicative of surfactant. It is interesting to note that some of the minor surfactant components such as SP, PS, and PE are found in greater relative abundance in other cell fractions.

Phosphatidylcholine is the most abundant phospholipid in surfactant or in any other lung fraction studied (Hallman and Gluck, 1975; Jobe et al., 1978a). In general, PG appears to be the next most abundant phospholipid of surfactant based on a comparison of alveolar wash and lamellar body fractions (Hallman and Gluck, 1975; Jobe et al., 1978a). (Note: PI $\cong$ PG in alveolar wash from the Jobe et al., 1978a study). PE appears to rank third based on the same comparison (Hallman and Gluck, 1975) or PI may rank third according to Jobe et al. (1978a). Both PI and PE comprise a very similar percentage of lamellar body and alveolar wash phospholipid (Hallman and Gluck, 1975; Jobe et al., 1978a). Bis-(monoacylglyceryl)-phosphate, cardiolipin, PS, lyso-PC, phosphatidic acid, and SP make up the rest of surfactant phospholipids as far as the studies cited in Table 2 show.

Figure 2. (after Figure 2 from Clements, 1973)

Composition of Canine Surfactant

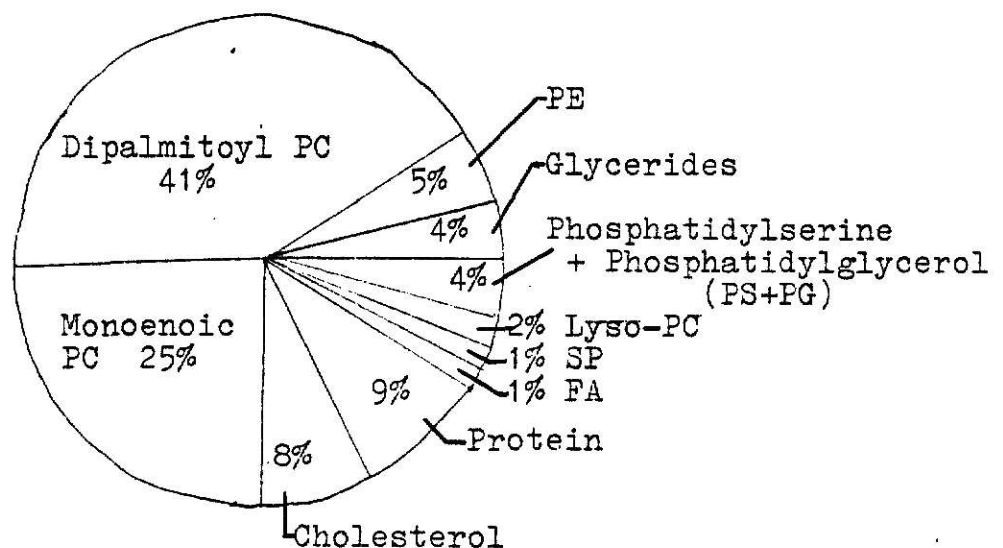


Table 2. Phospholipid Composition in Subcellular Fractions of Lung
(Note: Applies to Adult Lungs)

Phospholipid	Lung Homogenate	Microsomes (endoplasmic reticulum)	Lamellar bodies	Alveolar wash	Mitochondria	Ref.
Phosphatidylcholine (PC)	0.06 μ mol/ mg protein	0.17 μ mol/ mg protein	2.6 μ mol/ mg protein	7 μ mol	0.19 μ mol/ mg protein	a
	11.01 $\pm$ 1.16 μ mol/ g wet tissue	---	---	---	---	b
	57.5 $\pm$ 1.4% *	41.9 $\pm$ 1.7% **	77.0 $\pm$ 1.7% ***	75.5 $\pm$ 1.7% ***	44.8 $\pm$ 1.8% ****	c
	52.5 $\pm$ 2.0% *	51.8 $\pm$ 4.0% **	86.0 $\pm$ 5.1% ***	83.8 $\pm$ 2.2% ***	---	d
Phosphatidylglycerol (PG)	0.08 $\pm$ 0.07 μ mol/g wet tissue	---	---	---	---	b
	3.3 $\pm$ 0.5% *	1.7 $\pm$ 0.3% **	11.2 $\pm$ 1.2% ***	11.0 $\pm$ 0.9% ***	1.7 $\pm$ 0.2% ****	c
	1.6 $\pm$ 0.5% *	1.2 $\pm$ 0.6% **	5.0 $\pm$ 1.1% ***	4.7 $\pm$ 1.9% ***	---	d
Bis-(monoacyl-glyceryl)-phosphate (Structural relative of PG)	1.0 $\pm$ 0.2% *	0.7 $\pm$ 0.2% **	1.5 $\pm$ 0.1% ***	1.7 $\pm$ 0.4% ***	0.5 $\pm$ 0.3% ****	c
Diphosphatidylglycerol (cardiolipin)	1.4 $\pm$ 0.2% *	0.2 $\pm$ 0.1% **	0.3 $\pm$ 0.2% ***	0.1 $\pm$ 0.0% ***	7.3 $\pm$ 1.4% ****	c

Table 2. (Continued)

Phospholipid	Lung Homogenate	Microsomes (endoplasmic reticulum)	Lamellar bodies	Alveolar wash	Mitochondria	Ref.
Phosphatidyl-ethanolamine	5.29 ± 0.11 μmol/g wet tissue	---	---	---	---	b
	16.6 ± 0.7% *	24.6 ± 1.0% **	4.2 ± 0.2% ***	4.6 ± 0.2% ****	26.5 ± 1.4% *****	c
	18.4 ± 1.7% *	20.4 ± 5.3% **	3.5 ± 0.5% ***	3.6 ± 1.3% ****	---	d
Phosphatidyl-serine (PS)	9.3 ± 0.6% *	12.8 ± 1.0% **	1.2 ± 0.1% ***	1.9 ± 0.5% ****	8.9 ± 0.5% *****	c
	3.2 ± 0.9% *	4.7 ± 1.7% **	0.4 ± 0.1% ***	0.4 ± 0.2% ****	---	d
Phosphatidyl-inositol (PI)	0.93 ± 0.09 μmol/g wet tissue	---	---	---	---	b
	2.6 ± 0.4% *	3.1 ± 0.2% **	3.2 ± 0.2% ***	3.0 ± 0.4% ****	2.2 ± 0.1% *****	c
	3.1 ± 0.4% *	3.6 ± 0.8% **	4.2 ± 1.9% ***	5.2 ± 1.6% ****	---	d
Lyso-PC	0.5 ± 0.2% *	0.5 ± 0.2% **	0.4 ± 0.4% ***	0.2 ± 0.0% ****	0.0 ± 0.0% *****	c
	1.3 ± 0.6% *	not detected (ND)	0.7 ± 0.3% ***	ND	---	d
Phosphatidic acid (PA)	0.3 ± 0.1% *	0.8 ± 0.1% **	0.3 ± 0.3% ***	0.5 ± 0.2% ****	0.6 ± 0.1% *****	c

Table 2. (Continued)

Phospholipid	Lung Homogenate	Microsomes (endoplasmic reticulum)	Lamellar bodies	Alveolar wash	Mitochondria	Ref.
Sphingomyelin (SP)	7.5 ± 0.2% *	13.7 ± 0.6% **	0.7 ± 0.3% ***	1.5 ± 0.2% ****	7.5 ± 0.2% ****	c
	13.2 ± 2.3% *	10.8 ± 1.4% **	0.5 ± 0.4% ***	0.6 ± 0.4% ****	---	d
Diacylglycerol (not a phospholipid)	0.34 ± 0.03 μmol/g wet tissue					b

* % of phospholipids in homogenate
 ** % of phospholipids in microsomes
 *** % of phospholipids in lamellar bodies
 **** % of phospholipids in alveolar wash
 ***** % of phospholipids in mitochondria

Refs.

- a - Jobe (1977)
- b - Okano et al. (1978)
- c - Hallman and Gluck (1975)
- d - Jobe et al. (1978a)

A note about PG should be made here concerning its significance in surfactant. PG is second in relative abundance to PC as seen above. PG is also found in surfactant from other mammalian species (other references cited by Hallman and Gluck, 1975). PG is present in trace amounts in animal tissues, mostly in the mitochondria (Strickland and Benson, 1960; Gray, 1964; Ray et al., 1969 - from Hallman and Gluck, 1975). These observations led Hallman and Gluck (1975) to the conclusion that PG is a characteristic component of surfactant in adult animals. The importance, if any, of the structural relatives of PG such as bis-(monoacylglyceryl)-phosphate as found in lamellar bodies and alveolar wash is not known (Hallman and Gluck, 1975). Its presence in lavage has not been detected by some investigators (Pfleger et al., 1972 - from Hallman and Gluck, 1975). Further discussion on the significance of PG will be seen later in this discussion on lipids.

Table 3 shows the percentages of different fatty acids attached to the major phospholipids of surfactant and gives an overall percentage saturation of those phospholipids or of fatty acids in the various lung fractions.

Focusing again on the lamellar body and alveolar wash fractions, one can see that PC contains over 50% palmitic acid and around 67% of its fatty acids are saturated (Jobe et al., 1978a). In general, PC has the highest percentage of palmitic acid and highest percentage of saturated fatty acids compared to the other surfactant phospholipids (see Table 3). PG, the second most abundant phospholipid contained similar amounts of palmitic acid in the lamellar body and alveolar wash fractions, compared to PC (Hallman and Gluck, 1975; Jobe et al., 1978a). Fatty acids of PG were about 61% saturated in lamellar body and alveolar wash fractions (Jobe et al., 1978a). A relatively high percentage of palmitic acid was found in PG from mitochondria, also (Hallman and Gluck, 1975). PG found in mitochondria is thought to contribute to cardiolipin formation according to references cited by Hallman and Gluck (1975). Both PC and PG contained relatively high percentages of oleic acid (18:1) in the lamellar body and alveolar wash fractions (Jobe et al., 1978a; Hallman and Gluck, 1975). PC contained high amounts of linoleic (18:2) acid in these two fractions (Jobe et al., 1978a). PI contained about 42% saturated fatty acids in the lamellar body and alveolar wash fractions, and palmitic acid and oleic acid were the most abundant FA's of this phospholipid in these fractions (Jobe et al., 1978a). Arachidonic (20:4) acid was the most plentiful FA of PI in the lung homogenate and microsomal fractions (Jobe et al., 1978a). Stearic (18:0) acid was the major FA at the 1-position of PI and arachidonic at the 2-position (Okano et al., 1978) in lung homogenates. PI contained about 30% saturated FA's in the homogenate and microsomal fractions (Jobe et al., 1978a). PE plus PS had mostly oleic and linoleic FA's associated with them in the alveolar and lamellar body fractions (Jobe et al., 1978a). Large amounts of palmitoyl- and stearyl-aldehydes were found at the 1-position of PE and mostly polyunsaturated FA's at the

Table 3. Fatty Acid Composition and Percentage Saturation

Lipid	FA	Lung Homogenate (Parenchyma)		Microsomes (endoplasmic reticulum)		Lamellar bodies		Alveolar wash	
		Ref. A	Ref. B	Ref. B	Ref. C	Ref. B	Ref. C	Ref. B	Ref. B
PC	14:0	3.0	3.6 $\pm$ 0.6	3.2 $\pm$ 1.1		5.1 $\pm$ 0.4		5.0 $\pm$ 1.0	
	16:0	53.9	50.1 $\pm$ 2.8	38.4 $\pm$ 2.5		58.0 $\pm$ 5.0		60.4 $\pm$ 3.6	
	16:1	8.3	5.7 $\pm$ 2.2	4.5 $\pm$ 0.9		7.9 $\pm$ 1.6		7.3 $\pm$ 1.9	
	18:0	6.8	5.3 $\pm$ 0.7	6.9 $\pm$ 1.5		3.4 $\pm$ 2.4		3.3 $\pm$ 1.3	
	18:1	9.9	12.7 $\pm$ 1.2	14.6 $\pm$ 1.1		11.3 $\pm$ 1.8		10.7 $\pm$ 1.0	
	18:2	9.2	17.7 $\pm$ 2.6	23.4 $\pm$ 2.3		11.8 $\pm$ 2.6		11.2 $\pm$ 2.3	
	18:3 + 20:1		0.8 $\pm$ 0.5	0.6 $\pm$ 0.7		2.6 $\pm$ 2.5		0.7 $\pm$ 0.5	
	20:3	(+)				(-)		1.3 $\pm$ 2.0	
	20:4	5.2	4.0 $\pm$ 2.7	8.4 $\pm$ 1.8					
	20:5	0.8							
% Saturation	22:4	(+)							
	22:5	0.7							
	22:6	1.7							
		33.5 $\pm$ 3.8	59	48.5		66.5		68.7	
	14:0	(+)	2.8 $\pm$ 0.8	4.6 $\pm$ 1.5	1.6 $\pm$ 0.2	4.2 $\pm$ 3.5	1.5 $\pm$ 0.1	2.5 $\pm$ 1.1	
	16:0	46.6	47.8 $\pm$ 3.1	38.0 $\pm$ 3.7	49.9 $\pm$ 2.1	53.9 $\pm$ 5.5	51.0 $\pm$ 2.8	55.5 $\pm$ 1.9	
	16:1	2.5	8.3 $\pm$ 1.5	5.6 $\pm$ 0.6	4.7 $\pm$ 0.2	7.0 $\pm$ 2.4	6.5 $\pm$ 0.4	8.5 $\pm$ 1.6	
	18:0	7.3	4.2 $\pm$ 0.7	6.3 $\pm$ 0.2	5.1 $\pm$ 0.3	4.0 $\pm$ 1.8	6.7 $\pm$ 0.6	2.8 $\pm$ 0.4	
	18:1	18.4	24.1 $\pm$ 3.9	23.7 $\pm$ 0.7	21.7 $\pm$ 1.6	23.6 $\pm$ 6.7	26.9 $\pm$ 1.2	23.2 $\pm$ 1.9	
	18:2	15.9	9.2 $\pm$ 3.3	19.4 $\pm$ 0.2	9.4 $\pm$ 0.9	6.4 $\pm$ 1.7	4.2 $\pm$ 0.4	6.1 $\pm$ 1.7	
% Saturation	18:3 + 20:1		1.7 $\pm$ 0.4	0.5 $\pm$ 0.7	5.2 $\pm$ 0.5	0.8 $\pm$ 0.9	1.0 $\pm$ 0.1	1.2 $\pm$ 0.6	
	20:3	(+)				(-)		(-)	
	20:4	5.1	1.8 $\pm$ 0.2	1.8 $\pm$ 0.2					
	20:5	(+)							
	22:4	(+)							
	22:5	1.4							
	22:6	2.2							
		22.3 $\pm$ 2.3	54.8	48.9		62.1		60.8	

(For mitochondrial PG (Ref. C) 14:0-1.9 $\pm$ 0.1, 16:0-31.2 $\pm$ 2.4, 16:1-5.4 $\pm$ 0.3, 18:0-4.1 $\pm$ 0.4, 18:1-17.5 $\pm$ 1.8, 18:2-17.1 $\pm$ 1.1, 18:3+20:1-10.3 $\pm$ 1.5)

Table 3. (Continued)
(FA's ranked according to mol% or mean % composition)

Lipid	Lung Homogenate (Parenchyma)	Microsomes (endoplasmic reticulum)	Lamellar bodies	Alveolar wash
PI	20:4>18:0>18:1>18:2≤16:0	20:4>18:0>18:2≡18:1>16:0	16:0>18:1>18:2>16:1>18:0	18:1>16:0>20:4≡18:2≡18:0
% Saturation	31.1 (Ref. B)	30.9 (Ref. B)	44.5 (Ref. B)	41.4 (Ref. B)
% Saturation	18:0>20:4>16:0>18:1>18:2			
% Saturation	4.1 (Ref. A)			
PE	20:4≡16:0>18:0>ald 18:1>18:0 ald (Ref. A)			
PE + PS	20:4>18:1>18:2>18:0>others	20:4>18:2>18:1>others>16:0≡18:0	18:2>18:1>16:0>16:1>18:0	18:2≡18:1>20:4>16:0>18:0
% Saturation	23.9 (Ref. B)	19.0 (Ref. B)	25.9 (Ref. B)	21.4 (Ref. B)
Diacylglycerol	16:0>18:0>18:1>18:2>20:4			
% Saturation	24.1 + 3.9 (Ref. A)			

Ref. A - Okano et al. (1978) - measured FA's in mol %

Ref. B - Jobe et al. (1978a) - measured FA's as % mean composition

Ref. C - Hallman and Gluck (1975) - measured FA's as % FA Composition

% Saturation from Ref. A - % of that phospholipid which was saturated

% Saturation from Ref. B - % of FA's which were saturated

(+) = less than 0.5%
(-) = not detected
or trace amt.

2-position (Okano et al., 1978) in lung homogenate. The table demonstrates the point that there is variation in the degree of saturation and in the types of FA's associated with the same phospholipid class derived from different subcellular fractions. Generally, higher percent saturation is found in lamellar body and alveolar wash fractions for major surfactant phospholipids (Jobe et al., 1978a).

Analysis of phosphoglycerides which are naturally-occurring in mammalian tissues, reveals a predominance of saturated fatty acids at the 1-position and unsaturated fatty acids at the 2-position of the glycerol moiety (Hanahan, 1960; Montfoort et al., 1971 - from Okano et al., 1978). This was demonstrated in the lung lipids studied by Okano et al., (1978). For example, they found palmitic acid predominantly at the 1-position of PC, PG, PE, and PI rather than at the 2-position. Palmitic acid was also significantly linked at the 2-position of PC and PG (Okano et al., 1978). They found oleic acid primarily at the 2-position of PC, PG, and PE rather than at the 1-position. It is important to note that palmitic acid was the primary FA of both the 1- and 2-positions of PC (Okano et al., 1978). PC, PG, and diacylglycerol had similar FA compositions (Okano et al., 1978). As shown earlier, palmitic acid was the major FA of both PC and PG and Okano and co-workers (1978) also found that it was the major FA of diacylglycerol. The FA patterns found by Okano et al. (1978) for PC and PG agreed with previous studies for rat (Toshima and Akino, 1972; Sanders and Longmore, 1975 - from Okano et al., 1978) and rabbit (Abe et al., 1973 - from Okano et al., 1978). It should be kept in mind that the Okano et al. (1978) study was done on lung homogenate.

In comparing PC, PG, and diacylglycerol, one finds that all three glycerolipids had similar molecular class compositions (Okano et al., 1978). PC, PG, and diacylglycerol were 33.5%, 22.3%, and 24.1% saturated, respectively (Okano et al., 1978). PI was only 4.1% saturated, and only a trace of saturated molecules were seen in the PE class (Okano et al., 1978). PI and diacyl- and alkenyl-acyl PE's had the highest percent total of tetraenes while diacylglycerol had about half the percent total of tetraenes of these molecules, but twice as much as PC and PG (Okano et al., 1978). The lipid plus the molecular class which had the highest percent total for that lipid are listed here:

- PC - saturated
- PG - monoenes
- diacylglycerol - tetraenes = saturated
- diacyl- and alkenyl-acyl PE, PI - tetraenes (Okano et al., 1978).

Disaturated or anenoic PC molecules of lung surfactant are unusual in that they are present in such high concentrations in surface-active material as compared to other body tissues (King, 1974). The dipalmitoyl PC appears to account for a large portion of the saturated PC class. Okano and

co-workers (1978) found that the saturated class made up 33.5% of PC from lung homogenate, and 25.1 mol% of the total PC fraction was made up of the dipalmitoyl species.

Okano et al. (1978) found significant amounts of saturated species, mainly dipalmitoyl, in PG and diacylglycerol in lung homogenate. PG has been found in "uniquely high concentrations in the lung, particularly in the surfactant fraction" (Pfleger et al., 1972; Henderson and Pfleger, 1972; Godinez et al., 1975 - from Okano et al., 1978). Okano et al. (1978) gave some ideas on the means of dipalmitoyl PG formation, but these will not be covered here.

In addition to knowing the composition of surfactant phospholipids at a given point in time, it is important to know the dynamics of surfactant phospholipids in various subcellular lung fractions. Table 4 summarized two different studies using injection of a variety of labels to study the biological half-life of lung phospholipids in adult rabbits. This type of study reveals information on the metabolic characteristics of lung phospholipids rather than their static characteristics.

In both of the studies cited in Table 4, PC has a two component decay curve with all of the labels used, in both the lung parenchyma and microsomal fractions (Jobe et al., 1978a; Jobe, 1977) (with the exception of the [^{32}P]phosphate label (Jobe, 1977)). In the two fractions more indicative of surfactant (lamellar bodies and alveolar wash), the half-life is about 17 hours using the glycerol and palmitic acid labels (Jobe, 1977; Jobe et al., 1978a). Disaturated phosphatidylcholine (DSPC) showed a half-life similar to PC in the parenchymal and alveolar wash fractions (Jobe, 1977). PG had a two component decay curve in the microsomal fraction, but a half-life of eight hours in the lamellar body fraction and ten hours in the alveolar wash fraction using the glycerol label (Jobe et al., 1978a). Values were slightly higher than this using the [^{14}C]palmitic acid label (Jobe et al., 1978a). PI has a two component decay curve in microsomes and a half-life of 20 hours using the [^3H]glycerol label and 36 hours using the [^{14}C]palmitic acid label in alveolar wash (Jobe et al., 1978a). PE plus PS had a very long half-life when they were compared with the initial or single decay components of the other phospholipids labeled with glycerol or palmitate (see Table 4).

Jobe (1977) found rapid [^{14}C]palmitic acid labeling and decay of PC in microsomes which he noted as consistent with the idea that the endoplasmic reticulum is the principal site of PC synthesis. He further suggested that the rapid disappearance of labeled PC from the microsomal fraction may represent movement of newly synthesized PC from microsomes toward lamellar bodies and other cellular organelles (Jobe, 1977).

In both studies presented in Table 4, there were two biological half-lives derived from decay curves for microsomal

Table 4. Biological Half-life of Lung Phospholipids (in hours)

Phospholipid	Label	Ref.	Lung parenchyma	Microsomes	Lamellar	Alveolar wash
PC	[³ H]glycerol	A	8	8	17	16
	[¹⁴ C]palmitic acid	A	30* 10 34*	64 7 57*	18	17
	[¹⁴ C]palmitic acid	B	9 26*	4 34*	16	17
	[³ H]choline	B	13 59*	4 77*	33	35
	[³² P]phosphate***	B	88	103	104	61
DSPC		B	DSPC showed similar decay curves to total PC			DSPC showed similar decay curves to total PC with [¹⁴ C]palmitic acid or with [³ H]-choline labels
PG	[³ H]glycerol	A	11	2 11*	8	10
	[¹⁴ C]palmitic acid	A	18	4 29*	10	14
PI	[³ H]glycerol	A	29	5 200*	**	20
	[¹⁴ C]palmitic acid	A	39	7 80*	**	36
PE + PS	[³ H]glycerol	A	80	57	**	57
	[¹⁴ C]palmitic acid	A	124	75	**	88

Notes for Table 4:

* Second decay component

** Inadequate data

*** [^{32}P]phosphate label not used in most text comparisons.

Refs.:

A = Jobe et al., 1978a

B = Jobe, 1977

and parenchymal PC using glycerol, palmitic acid, or choline labels (Jobe, 1977; Jobe et al., 1978a). Decay curves for PC in lamellar bodies and alveolar wash were single component curves with similar half-life values between these fractions. Using the initial decay component for comparison, PC half-life in the subcellular fractions was smallest in the microsomes (see Table 4). Decay curves for choline labeled PC were similar to those for palmitic acid labeled PC but half-life values were greater than those for palmitic acid labeled PC by a factor of about two (Jobe, 1977) (see Table 4). Comparison of the decay curves and half-life values of DSPC and total lung PC indicated that in the rabbit, DSPC and total lung PC appear to be metabolized in a similar manner (Jobe, 1977).

Specific activity of palmitate labeled PC in lamellar bodies increased as the specific activity of the microsomal PC decreased (Jobe, 1977). Jobe (1977) stated that this was a pattern consistent with electron microscopic autoradiographic studies (Chevalier and Collet, 1972 - from Jobe, 1977). It was not possible to determine the "absolute amount of microsomal or lamellar body PC" in the lung because there are no biochemical factors which could be used to indicate losses (Jobe, 1977). Therefore, "the actual radioactivity in PC disappearing from the microsomal fraction could not be correlated with the radioactivity in PC appearing in the lamellar body fraction" (Jobe, 1977).

Autoradiographic studies support the idea that PC in microsomes enters the lamellar bodies continually during "development of mature lamellar bodies rather than at a discrete point in the sequence" of lamellar body maturation (Jobe, 1977). This was supported by Jobe's (1977) study in which he found "prolonged maintenance of high specific activity in lamellar body and alveolar wash PC," and that PC of the lamellar bodies or alveolar wash were not "cleanly" pulse labeled as the microsomal PC was with palmitic acid (Jobe, 1977).

Jobe's (1977) study supported those who suggest that lamellar bodies do not play a role in PC synthesis. It has already been determined that many of the PC synthesis enzymes are absent (Garcia et al., 1976 - from Jobe, 1977) in lamellar bodies. However it is possible for palmitic acid to be directly incorporated into lamellar body PC by deacylation-reacylation or transacylation reactions not requiring de novo synthesis (Mason, 1976 - from Jobe, 1977). Jobe (1977) found maximal incorporation of palmitic acid occurred before the lamellar bodies had significant amounts of radioactive PC.

Jobe (1977) found that there was a delay in the time pulse-labeled PC appeared in alveoli. This was also found in other studies cited by Jobe (1977). He concluded that "the delay in appearance of pulse-labeled PC in alveoli measures the minimal time for synthesis and delivery of surfactant PC to the alveolus in the rat and rabbit."

In general, initial decay rates were quite rapid for PC, PG, and PI in lung microsomes compared to lamellar body and alveolar wash fractions (see Table 4). PC and PG labeled with glycerol or palmitic acid may be "turned over" as intact molecules in lamellar bodies because the labeled acyl groups (palmitate) and the "labeled backbone glycerol of the phospholipids had similar half-life values" (Jobe et al., 1978a). The order of biological half-lives from alveolar wash was: PG<PC<PI<PE+PS (Jobe et al., 1978a).

As was stated earlier, lung tissue and alveolar wash contain much more PG than other mammalian tissues (Rooney et al., 1975; Hallman et al., 1976 - from Jobe et al., 1978a). Also, it appears to be synthesized mainly in lung microsomes as are other phospholipids (Hallman and Gluck, 1975), and it may be a precursor for cardiolipin when synthesized by mitochondria (Hallman and Gluck, 1975 - from Jobe et al., 1978a). The function of PG may be "as a stabilizer of the surfactant lipoprotein complex" and it may be necessary for "surface-active characteristics of the lung" (Pfleger et al., 1972 - from Jobe et al., 1978a). In the human neonate, PG appearance "coincides with mature lung function" (Hallman and Gluck, 1976 - from Jobe et al., 1978a). In the Jobe et al. (1978a) study, [¹⁴C]palmitic acid appeared to label PG and PC to the same extent (at least in lung parenchyma).

PG is not as integral to general cellular metabolism as other phospholipids, and its half-life may be more representative of surfactant metabolism than values for other phospholipids (Jobe et al., 1978a). PG disappeared from microsomes, parenchymal, lamellar body, and alveolar wash fractions faster than other phospholipids (except for the initial decay of PC in lung parenchyma) (see Table 4) (Jobe et al., 1978a). Therefore, PG is "an actively metabolized phospholipid with probably no pulmonary function other than as a component of surfactant" (Jobe et al., 1978a). PG may be as good as DPPC in serving as a biochemical marker for surfactant metabolism due to its (PG's) uniqueness in surfactant (Jobe et al., 1978a).

Phosphatidylinositol showed a slight increase in percent of saturated fatty acids in the alveolar fractions over the microsomal fractions (Jobe et al., 1978a). It was unique in that it remained at 3-5% composition in the lung fractions studied (Jobe et al., 1978a). In general, PI had a longer measured half-life in lung fractions than PC or PG (see Table 4) (Jobe et al., 1978a). The apparent importance of PI to surfactant function was shown by developmental data which indicated that PI was involved in maturation of surfactant in preparation for birth (Hallman and Gluck, 1976 - from Jobe et al., 1978a).

PE and PS were studied together because they "represent highly unsaturated phospholipid classes that are preferentially excluded from surfactant" (Jobe et al., 1978a). PE and PS are not surface-active according to Lusted (1973 - from Jobe et al., 1978a), but are considered to be "integral membrane components" (Jobe et al., 1978a). They have longer half-life values and lower initial specific activities which indicates that they are less metabolically active than the other phospholipids studied (Jobe et al., 1978a).

PG, PC, and PI all exhibited two component decay curves in the microsomal fraction (Jobe, 1977; Jobe et al., 1978a). In general, the two component curves may represent metabolism of these phospholipids in cells other than the type II lung cell (Jobe, 1977; Jobe et al., 1978a). Recall that these experiments were done with a heterogeneous cell population. The two decay rates for PC are not due to differences in metabolism between unsaturated PC and DSPC (Jobe et al., 1978a) because total PC and DSPC have similar half-life values in parenchymal and alveolar wash fractions (Jobe, 1977). About 50% of palmitic acid label originally "incorporated into lung PC disappeared by 7 h," but only 30-40% of labeled PC was recovered in the alveolar space (Jobe, 1977). Young and Tierney (1972 - from Jobe, 1977) found similar results working with DSPC in the rat. According to Jobe (1977) the rapid decay component of microsomal PC may indicate that around 50% of the newly synthesized PC goes to other cellular organelles, while the slower component may be from PC incorporated into microsomal membrane structures which are more permanent.

In summary, a few significant details need to be emphasized. The major phospholipid of surfactant is PC (Hallman and Gluck, 1975; Jobe et al., 1978a). This lipid is highly saturated overall (Jobe et al., 1978a). The dipalmitoyl PC abundance in lung is unusually high compared to other tissues (Montfoort et al., 1971; Fujiwara et al., 1968 - from Okano et al., 1978). DSPC synthesis appears to require a "special" mechanism for its synthesis because saturated FA's are usually found at the 1-position and unsaturated FA's at the 2-position of the glycerol moiety of phosphoglycerides naturally present in most mammalian tissues (Hanahan, 1960; Montfoort et al., 1971 - from Okano et al., 1978). (Possible mechanisms will be discussed in the synthesis section). According to King (1974), the saturated PC species lower "alveolar interfacial tension." The monoenoic PC's and cholesterol appear to enhance the adsorption of surfactant to the alveolar interface (King, 1974). The unusually high relative abundance of PG in lung compared to other tissues (Rooney et al., 1975; Hallman et al., - from Jobe et al., 1978a) plus the idea that its half-life may be more indicative of surfactant metabolism than values for "phospholipids more integral to general cellular metabolism" (Jobe et al., 1978a) may make this a good indicator of surfactant metabolism. This lipid may stabilize the surfactant lipoprotein complex, also (Pfleger et al., 1972 - from Jobe et al., 1978a). Both PC and PG appear to be concentrated in those fractions indicative of surfactant material (lamellar bodies

and alveolar wash) (see Table 2). Also, both PC and PG appear to be highly saturated, even more so in fractions from lamellar bodies and lung wash (see Table 3). Palmitic acid appears to be the major FA of PC and PG (see Table 3). Finally, labeling studies reveal that PC, PG, and PI have faster turnover in microsomes than in alveolar wash and lamellar body fractions as indicated by their initial half-life values (see Table 4). (There is no half-life for PI in lamellar bodies given). Jobe (1977) found some evidence that the decrease in specific activity of microsomal palmitate labeled PC may be associated with an increase in specific activity of lamellar body PC. He also noted a delay in the "appearance of pulse-labeled PC in the alveoli." Both of these observations are consistent with a transfer of newly synthesized PC from microsomes to lamellar bodies to the alveolar surface which is part of the proposed "route" of surfactant synthesis (see section on synthesis).

References for Introduction to Section III and Phospholipids

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B. Protein Composition

As was stated in the introduction to this section on surfactant composition, protein appears to be a component of the surfactant material. The molecular weights and other characteristics of these proteins may vary between different studies, as will be evident in the following discussion. Due to the broad scope of this report, it is not possible to cover all of the studies directed at surfactant protein. Several studies which provide examples of the types of investigations carried out on surfactant proteins, have been selected for discussion here. The first few papers to be covered will contain information derived from some of the more widely used methods of protein study. These will be followed by discussion of studies using immunological techniques. Studies on the possible glycoprotein character of surfactant protein will be presented. Investigations on the specificity of surfactant proteins will also be covered. A brief discussion on the proposed roles of surfactant protein will conclude this section on protein composition.

The reader is encouraged to look over the monograph by Clements and King (1976) on surfactant composition. This review provides a good overview of material on surfactant proteins, including evidence for a specific association between apoprotein and surface-active material, as well as discussion of immunological studies.

Sawada et al. (1977) isolated a protein from bovine lung surfactant which had a MW of 36,000 when sodium deoxycholate was used to remove lipids from the protein prior to gel filtration and other analytical procedures. The MW determination was made on sodium dodecyl sulfate (SDS) polyacrylamide gels after the protein had been isolated by gel filtration on Sephadex G-75 and Sepharose 6B. The electrophoretic pattern of the isolated protein was the same as that obtained for protein taken directly from surfactant (Sawada and Kashiwamata, 1977). Protein from Sephadex G-75 and Sepharose 6B columns were studied by analytical ultracentrifugation and were found to be almost identical. The protein from Sephadex G-75 columns had a sedimentation rate of 12.1S.

Sawada et al. (1977) concluded that there may be proteases in the lung surfactant material because addition of protease inhibitors prevented tailing of the protein peak on the Sephadex G-75 column. They also suggested that the apoprotein was lipophilic because there was a "relatively large amount of deoxycholate bound to protein" (Sawada et al., 1977) (w/w of deoxycholate/protein was 0.28). The MW of the apoprotein was estimated to be about 20×10^4 , "assuming a spherical protein and considering the amount of sodium deoxycholate bound to the protein" (Tanford, 1961 - from Sawada et al., 1977). Finally they concluded that the apoprotein isolated by gel filtration in the presence of sodium deoxycholate was an apoprotein of lung surfactant and the major band of 36,000 MW may be its "main constituent or its major protein subunit" (Sawada et al., 1977).

In an earlier study, Sawada and Kashiwamata (1977) isolated protein from bovine lung surfactant having a MW of 36,000 on SDS gels, as stated previously. Seventy to 80% of the protein from surfactant which was applied to the gel (this author's interpretation) had a MW of 36,000 (Sawada and Kashiwamata, 1977). The ratio of phospholipid to protein in surfactant material isolated after the initial centrifugations and washes was 4.98 $\mu\text{mol}/\text{mg}$. Lipid analysis of this surfactant material revealed 59.1 PC, 3.4 PE, 10.5 PG, and 15.0 cholesterol in molecular ratio. The density of this surfactant after centrifugation on a continuous sucrose density gradient was 1.081 g/ml with an occasional trace amount of phosphorus and protein at 1.061 g/ml. The phosphorus:protein ratio of the 1.081 g/ml band was 18.48 $\mu\text{mol}/\text{mg}$. Note the over three-fold increase in the ratio when the protein is isolated ("purified"). Shelley et al. (1975) obtained a similar increase in the PL/protein ratio after a portion of the phospholipid band from their density gradient was run on an additional gradient. Aqueous suspensions of surfactant before and after density gradient centrifugation were tested for surface activity. Only the material obtained after density gradient centrifugation satisfied King and Clement's criterion for surface activity (minimum surface tension of less than 12 dynes/cm) (Sawada and Kashiwamata, 1977).

The band of surfactant isolated on the continuous density gradient was characterized by protein and phosphorus analysis as lipoprotein. The band occasionally seen at 1.061 g/ml had a phosphorus:protein ratio close to that of the surfactant band at 1.081 g/ml, and had essentially the same SDS disc gel pattern as the surfactant in the 1.081 g/ml band. Thus, the lighter band may be fragments, due to preparation methods, of surfactant from the heavier band. The phosphorus:protein ratio increased in the heavier band with long term centrifugation on continuous density gradients, and this ratio approached the value for canine surfactant material isolated by King and Clements (1972a - from Sawada and Kashiwamata, 1977). Protein diffused out of the main peak leaving behind phospholipids in the main band. They found little if any albumin in bovine lung surfactant, contrary to other reports (Maguire et al., 1977).

King et al. (1973 - from Sawada and Kashiwamata, 1977) found an apoprotein of canine surfactant lipoprotein to have a MW of 10,000 - 11,000 and a large number of hydrophobic amino acids. In a later paper, King (1974 - from Sawada and Kashiwamata, 1977) reported a protein with a MW of 34,000. King et al., according to Sawada and Kashiwamata (1977), have classified the material separated of SDS disc gels as an apoprotein. Recall, Sawada et al. (1977) used sodium deoxycholate gel filtration to separate protein completely from phospholipids and found the MW of this protein was several times larger than that of the major protein subunit of lung material (Sawada and Kashiwamata, 1977). Sawada and Kashiwamata (1977) again concluded that "the native apoprotein of surfactant lipoprotein is probably a combination of a few protein subunits." Bhattacharyya et al. (1975) found a 36,000 dalton glycoprotein from rabbit lungs only after lavage with hypotonic EDTA

solution. This protein may be more tightly bound to the glycocalyx of the underlying alveolar epithelium in rabbit lung surfactant (Maguire et al., 1977).

Studies such as those above are less costly and still provide much needed initial data on surfactant proteins. However, one of the most effective methods for analyzing surfactant material for specific proteins has been the radioimmunoassay (RIA). This method is more sensitive to minute quantities of protein and therefore allows analysis of fetal surfactant for the presence of protein.

In 1974, King et al. published a paper which described the use of a solid-phase RIA for studying the protein(s) of sheep lung surfactant. This technique appears to be particularly valuable in that it allows study of surfactant presence during development when quantities of protein may be minute.

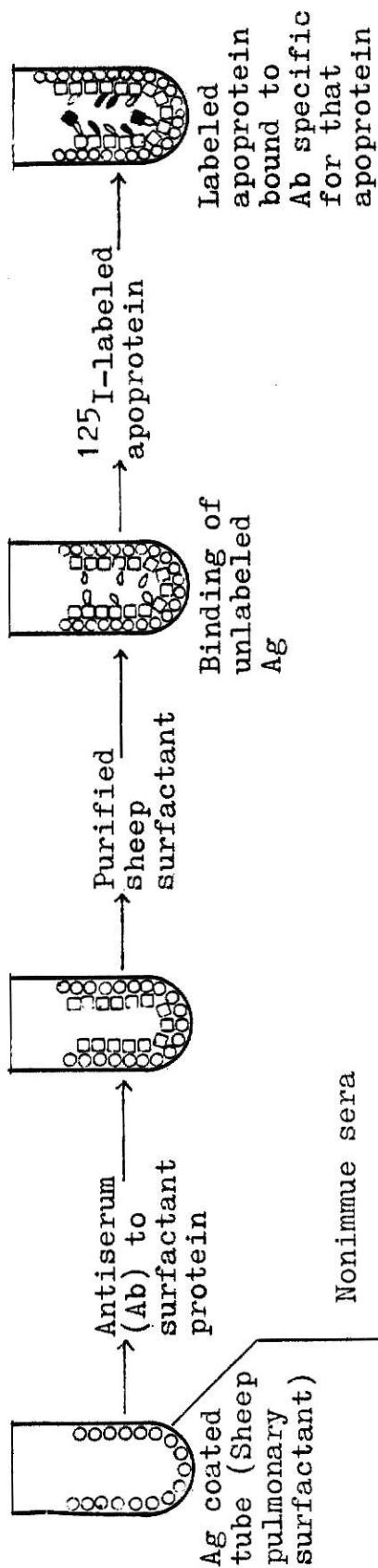
In general the technique involves coating test tubes with surfactant (antigen). Tubes were then coated with anti-serum specific for surfactant protein(s) or with nonimmune rabbit sera as a control. Purified sheep surfactant was then added to control and experimental tubes followed by application on nonimmune sera to make the total volume 1ml. Labeled apoprotein was then added to the tubes, followed by gamma-particle scintillation counting of tubes only (King et al., 1974). (See Figure 3).

The procedure for isolation of one nonserum apoprotein found in lung surfactant is outlined in Figure 1 from King et al. (1974; Marchesi and Andrews, 1971 - from King et al., 1974). It is interesting to note that the pulmonary surfactant, from which the apoprotein was isolated, had an isopycnic density of 1.083 g/ml in sodium bromide. This density is almost identical to the density of surfactant after centrifugation on a continuous sucrose density gradient found by Sawada and Kashiwamata (1977). King et al. (1974) report here that the apoprotein has a 34,000 dalton MW according to SDS gels. This figure differs from the results of Sawada and Kashiwamata (1977) and Sawada et al. (1977) which show that the apoprotein has a MW of 36,000.

Protein which precipitated in ethanol is soluble in water, has a MW of 34,000 daltons according to SDS polyacrylamide gel electrophoresis, and this protein makes up about 85-95% of the total protein in solution. The protein preparation was free of phospholipids as indicated by the fact that no phosphorus was detected. The protein formed an identity precipitin pattern against anti-pulmonary surfactant antiserum "when tested in a well adjacent to this surfactant" in a double-diffusion immunoprecipitation experiment in 0.75% agarose gels. The protein was not a serum protein as indicated by the fact that it "did not react with antiserum to sheep serum." Note that Harwood et al. (1975 - from Maguire et al., 1977) found a small amount of 34,000 dalton protein in rabbit and sheep surfactant, but ox surfactant contained a large amount of this protein.

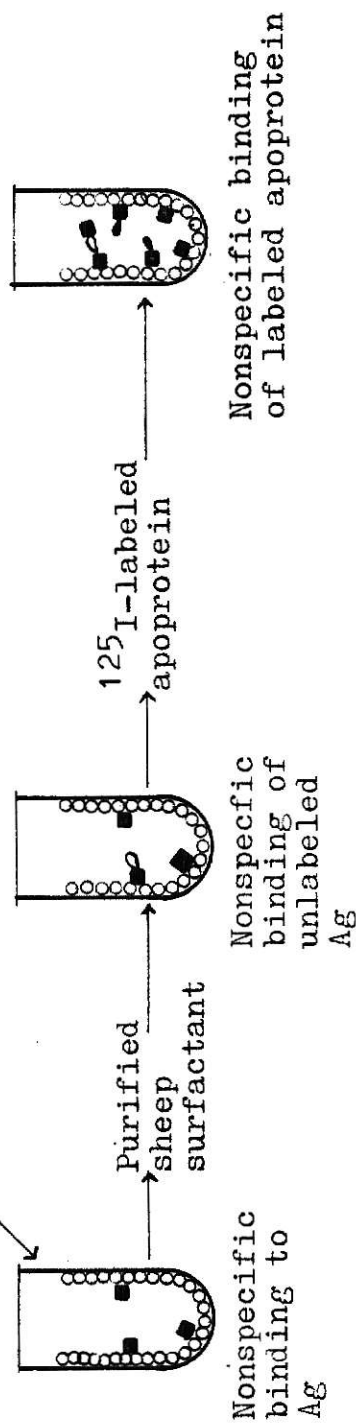
Figure 3. (Based on Figures 2 and 3 of King et al. (1974))

Experimental



Nonimmune sera

Control



KEY: ○Antigen, □Specific Ab, ■Nonspecific Ab, ◐Unlabeled Ag, ◑Labeled Ag

Figure 1. (from King et al., 1974)

KING, GIKAS, RUCH, AND CLEMENTS

EXTRACTION OF APOPROTEIN

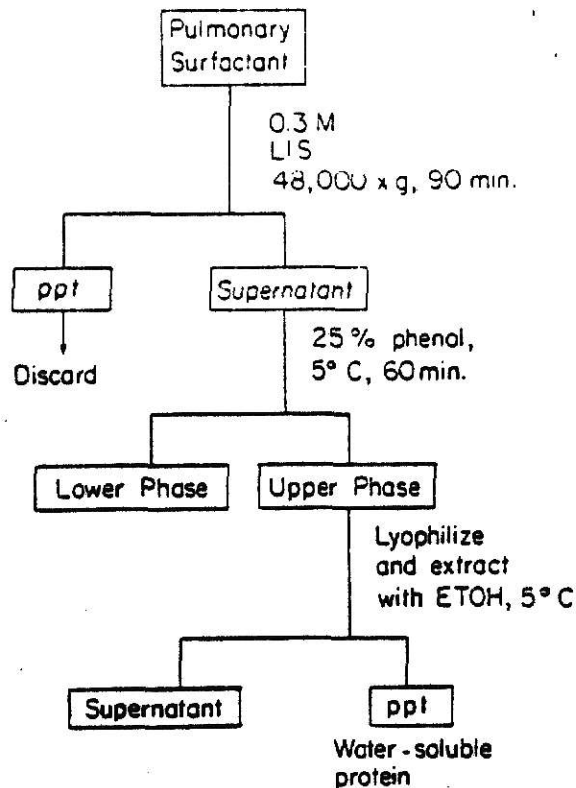


Fig. 1. Procedure for the isolation of the 34,000-dalton apoprotein from surface active material.

As noted earlier, King et al. (1973) found another apoprotein of surfactant which had a MW of 11,000 daltons. This protein shares some immunologic determinants with the 34,000 MW apoprotein as indicated "by their reactions with antisera to whole surfactant" (King et al., 1974). "The 11,000 dalton protein is soluble in ethanol and can be recovered from the ethanol extract of the lyophilized upper-phase material" (King et al., 1974).

Both the 34,000 dalton and 11,000 dalton proteins are also present in human and dog lung surfactant (King et al., 1974). Also, sheep and human apoproteins show some immunologic cross reactivity as do dog and sheep apoproteins (King et al., 1974).

The assay as performed in this study could detect 0.1 μ g to 0.2 μ g of surfactant, and it was said to have a useful range from 0.2 to 1.0 μ g of surfactant (King et al., 1974).

Table 5 demonstrates the specificity of the assay. Investigators have reported the presence of albumin in

Table 5. Specificity for the Apoprotein of Pulmonary Surfactant

Factor	Effect
1) Labeled apoprotein bound to nonimmune serum	Labeled apoprotein bound "nonimmune serum to an extent of less than 6% of that bound to the specific antibody." Therefore, most label bound to Ab is the result of specific interaction.
2) ¹²⁵ I-labeled bovine plasma albumin	No binding to tubes with specific Ab to sheep surfactant or to nonimmune rabbit serum. Therefore, there is no cross reactivity with bovine plasma albumin.
3) Addition of lipids extracted from canine lung and sonication in distilled water	Negligible effects on binding
4) Addition of excess sheep serum	Negligible effects of binding
5) Canine lung lipid containing ¹⁴ C-labeled DPPC added to tubes after any bound surfactant lipid was eluted with 0.1% SDS	No ¹⁴ C-label was detected. Therefore, binding of surfactant protein was not due to lipid moieties of surfactant.
(Conclusions drawn here were made by this author and not by King et al. (1974)).	

surfactant samples, but King et al. (1974) found that bovine plasma albumin was not bound by their surfactant specific Ab (see Table 5).

These authors performed the assay on sheep lungs at different ages and with different methods of preparation (see Table 1 from King et al. (1974)). Animals 1-3 were killed by an overdose of sodium pentobarbital. The rest of the animals were obtained the same day from a slaughter house and had been killed by exsanguination after electrical stunning. All samples were immediately placed in ice for immediate use in the RIA or for storage at -60°C. The concentration of surfactant material in these samples varied from 3.7 mg/g to 9.1 mg/g lung tissue. Their assay was apparently effective in determining surfactant concentrations in these samples. "Surfactant apoprotein comprises less than one percent of the total protein in the lung, indicating that procedures used to isolate surfactant lead to a greater than 100-fold enrichment of the apoprotein in the purified preparations, based on protein analysis" (King et al., 1974).

They have performed a preliminary study on fetal lamb

TABLE 1
AMOUNT OF SURFACE ACTIVE MATERIAL (SAM) IN SHEEP
LUNGS, ESTIMATED BY RADIOIMMUNOASSAY

Animal	Age	Sex	Number of Determinations*	Content of SAM (mg (dry)/ g lung (wet)†)
1	1 month	F	9	5.1 (0.2)
2	1 month	F	22	3.7 (0.2)
3	Adult	F	15	9.1 (0.6)
4	6 months**	Unknown	4	4.9 (0.5)
5	6 months**	Unknown	4	5.4 (0.6)
6	6 months**	Unknown	3	6.2 (0.2)
7	6 months**	Unknown	3	7.8 (0.8)
8	6 months**	Unknown	4	5.3 (0.8)
9	6 months**	Unknown	3	4.7 (0.3)

*Each assay performed in triplicate.

†Mean (standard error).

**Approximate age.

lungs and fetal lamb tracheal fluid (King et al., 1974). They found that antigen was not present in lungs of a 58-day old fetus, and was not detected until 115 days of gestation in tracheal fluid, even though total protein (Lowry et al., 1951 - from King et al., 1974) content could be 800 μ g per ml. Antigen content rose to about 22 μ g per ml by day 146, and this production could be prematurely stimulated by infusion with dexamethasone (King et al., 1974). The authors concluded that their RIA could measure very low concentrations of antigen in lung fluids.

King et al. (1974) determined the amount of surfactant in the lungs of one animal by the RIA combined with determination of DPPC in the same lungs and in surface-active material. They found "that less than 40% of the DPPC in whole lung tissue is contained in pulmonary surfactant." Thus, DPPC does not appear to be a specific marker for pulmonary surfactant in whole lung (King et al., 1974) which indicates the potential usefulness of a specific protein marker.

A different type of immunological technique was used by Sueishi et al. (1977) to trace the apparent path of surfactant protein through granular pneumocytes (type II cells). They obtained antirabbit surface-active material (SAM) sera by immunizing ducks with rabbit SAM containing protein. Fab' fragments of the antirabbit SAM 7S γ -globulin were conjugated with horseradish peroxidase (specific conjugate) for use in immunoelectron microscopy.

Light microscopy revealed that reaction product (RP) coated the surface of the alveolar wall in varied thickness after fixation by vascular perfusion or immersion of lung tissue. Intraalveolar RP was dispersed and irregularly aggregated after fixation by tracheal infusion (which proved

at times to be a less favorable method of fixation). Faint RP was detected in some vacuoles of cuboidal pneumocytes of alveoli no matter what fixation procedure was used.

In general, observation of electron micrographs revealed fixation by vascular perfusion or immersion preserved alveolar lining material (ALM) on the surface of alveolar epithelium better than tracheal infusion. ALM had a diffuse accumulation of granular deposits which indicated a strong positive reaction with specific conjugate (Sueishi et al., 1977). Tubular myelin figures were found to be "in continuity with the surface lining film" (Sueishi et al., 1977), and were sometimes found in the basal layer. The tubular myelin figures appeared in the lattice square pattern occasionally, and were surrounded by "electron-dense reaction product" (Sueishi et al., 1977). The reaction product was often identified at the air-phase (luminal) side of the surface lining film.

Observations grouped according to cell type or tissue structure provided evidence for the generally accepted theory that type II cells synthesize surfactant. These observations also stimulated some new thoughts on metabolism of surfactant and supported other theories concerning its structure. The type I cell contained some pinocytotic vesicles ("opening at or close to the alveolar space") which reacted strongly with specific conjugate, but most of these vesicles reacted with the label "at the inside of the limiting membrane" (Sueishi et al., 1977). These cells had no phagocytic vacuoles containing reaction product. Reaction product was frequently seen in lamellar bodies, multivesicular bodies, Golgi complex, and in the endoplasmic reticulum of type II cells. Most lamellar bodies of the type II cell were vacuolated (probably extracted) and were labeled with specific conjugate at the peripheries. Intercellular junctions between pneumocytes were not labeled by specific conjugate. Likewise, no reaction product was seen in the interstitium, endothelial cells, or in the capillary lumina. Reaction product was occasionally seen on the surface of bronchioles and bronchi, but none was seen in the intracytoplasmic area of these epithelial cells (which include "nonciliated bronchioles and goblet cells") (Sueishi et al., 1977). Some secondary lysosomes of alveolar macrophages were strongly labeled with conjugate. These secondary lysosomes appeared as vacuoles of the alveolar macrophages which contained "irregular, membrane-lined deposits" (Sueishi et al., 1977). ALM labeled with specific conjugate lined the alveolar macrophages, too.

Previous studies have shown that specific protein was found in pulmonary surfactant by immunochemical and immunohistologic investigation (King et al., 1974; Klass, 1973; Scarpelli et al., 1973 - from Sueishi et al., 1977). Sueishi et al. (1977) stated that their results, including "preferential accumulation of immunologic RP" found "on the surface of alveolar epithelium in association with the alveolar lining layer in type II pneumocytes and alveolar macrophages" confirmed these previous studies.

They (Sueishi et al., 1977) also concluded that "phospholipid arranged in the lattice square pattern" may be "interspersed in the compact network of the specific protein to integrate and stabilize the surfactant system." This was based on the fact that ALM was labeled throughout with specific conjugate and tubular myelin figures were surrounded by RP. Electron microscopic (EM) studies of the surfactant system (Gil and Weibel, 1969/1970; Kikkawa, 1970 - from Sueishi et al., 1977) have indicated that surfactant corresponds to alveolar lining layer which has a "basal layer in varied thickness and osmiophilic surface lining film at the air-liquid interface." Finally, the tubular myelin figures were sometimes found in the "homogeneous" basal layer and were often found to be continuous with the surface lining film.

The surface lining layer has been shown to contain phospholipid (Dermer, 1969; Finley et al., 1968 - from Sueishi et al., 1977), protein (possibly lipoprotein) (Gil and Reiss, 1973; Frosolono et al., 1970), and polysaccharide such as in glycoprotein (Frosolono et al., 1970; Bhattacharyya et al., 1975) or mucopolysaccharide (Groniowski and Biczyskova, 1964, 1968). Reaction product of specific conjugate has sometimes been seen at the air-phase side of the surface lining film. This may support Colacicco's (1972 - from Sueishi et al., 1977) theory that the specific SAM protein may be "present in mosaic with phospholipid at the air-liquid interface." Sueishi et al. (1977) also pointed out that their observation may be an artifact of preparation.

Sueishi et al. (1977) suggested that the type II cell synthesized the specific SAM protein and secreted it into the alveolar space to become part of the surfactant system. They based this suggestion on the fact that type II cells showed much accumulation of RP in lamellar inclusion bodies, in Golgi complex, in multivesicular bodies (MVB's), and in endoplasmic reticulum (ER). The accumulation of RP in these organelles demonstrated the "prominent role of type II" cells in metabolism of specific SAM protein (Sueishi et al., 1977). In the following section of surfactant synthesis more evidence for synthesis of surfactant protein in the ER will be given. This study (Sueishi et al., 1977) coupled with the general pattern of protein synthesis suggests that SAM protein is synthesized in rough ER, migrates via Golgi complex and MVB's to lamellar inclusions for storage of surfactant (Sueishi et al., 1977).

Recall that Sueishi et al. (1977) noted the presence of RP in macrophages. They proposed that the RP in secondary lysosomes of alveolar macrophages indicated that these cells may phagocytize SAM, thus participating in SAM clearance.

At this point in the discussion of surfactant protein, the possible presence of MPS and/or glycoprotein will be considered. There is some evidence that mucopolysaccharides (MPS's) make up part of the surfactant matrix or they may be part of a cell coat. Histochemical methods have been used to demonstrate a possible MPS association with surfactant

(Groniowski and Biczyskova, 1964; Groniowski and Biczyskova, 1968; Kikkawa et al., 1970).

It should be kept in mind that some of the results obtained from these staining studies do not rule out glycoprotein uptake of the stain, and some of this evidence is based on histochemistry which is not necessarily diagnostic of MPS. Other investigators (Cledzka-Slotwinska and Maisin, 1970) concluded that MPS was not a component of surfactant itself based on Thorotrast staining.

Kikkawa et al. (1970) suggested that MPS's and phospholipids were present in fetal lung, with MPS's formed early and phospholipids later. The MPS's may be present along the fetal alveolar plasma membrane and the phospholipids may be randomly distributed in the pulmonary fluid. Biochemical investigations have revealed the presence of both lipid and polysaccharides in alveolar wash material (Scarpelli, 1967 - from Kikkawa et al., 1970), and it appears as though MPS's and phospholipids establish the surface film of the alveolar lining layer with the onset of respiration (Kikkawa et al., 1970).

Stein et al. (1969) studied a surfactant fraction from dog lung lavage and concluded that the presence of protein and carbohydrate was probably due to contamination. The amounts of protein and carbohydrate which they found in surfactant were apparently not functionally significant. Even when most of the protein was removed, the physical properties of surfactant were not altered. They emphasized the fact that this study did not employ "harsh" techniques which would denature proteins.

In a paper by Frosolono et al. (1970), biochemical evidence was given for the existence of glycoprotein in surfactant material. (The purpose of their paper was to provide an all around characterization of surfactant, and it included a frequently cited method for isolation of surfactant material from lung homogenate. Their analysis for protein and carbohydrate will be focused on here).

Several lines of evidence for the presence of protein and carbohydrate in surfactant were presented. When the surface-active fraction of dog lung homogenate was centrifuged on a continuous sucrose gradient, radiolabeled palmitic acid and alanine were found in the same band. Absorbance methods at 280 nm also indicated the presence of protein in the same fraction with palmitic acid. The surface-active fraction (SAF) had a lipid:protein ratio (w/w) of 3.86. The SAF contained 0.185 mg/g lung of protein, 0.19 mg/mg glucose, and 0.04 mg/mg sialic acid. After the SAF was delipidated, carbohydrate ("as determined by the anthrone analysis") and sialic acid were found only associated with the protein moiety. The original ratio of labeled palmitic acid to labeled protein (alanine) as found in the SAF, was retained in the SAF material eluted from a Sepharose 2B column. Only after SAF was delipidated did the "protein-containing material"

enter into the gel. The SAF apoprotein gave rise to two peaks on the Sepharose 2B column in 0.005 N sodium borate. Two bands were found in disc gel electrophoresis when SAF apoprotein was used, but native SAF did not enter the gel.

Frosolono et al. (1970) concluded that protein was an integral part of the SAF, and was not an artifact due to nonspecific binding. This was based mainly on the sucrose gradient data which showed both lipid and protein precursors in the same fraction, and on the gel filtration results in which lipid and protein precursors were present in the same ratio in the SAF material eluted in the void volume as in the original SAF applied to the Sepharose 2B columns.

Additionally, the fact that anthrone-positive material and sialic acid remained with the protein after alcohol-ether delipidation, indicated that the protein was a glycoprotein. However, they pointed out that this did not prove the existence of a "covalent linkage between the carbohydrate and protein moieties" (Frosolono et al., 1970).

A possible role of protein in surfactant may be to alter the surface properties of phospholipid in the alveolar lining layer (Frosolono et al., 1970). They noted a study showing that dipalmitoyl PC is solid at body temperature, and stated that phospholipids are usually bound by noncovalent linkages to protein rather than free in tissues (Frosolono et al., 1970).

Finally, Frosolono et al. (1970) speculated that the alveolar lining may resemble a "structured gel." They proposed that FA chains of phospholipids in the lining layer are oriented toward the air-phase. A "protein network of the alveolar lining would be attached to the plasma membranes of type I and type II alveolar cells through interactions with carbohydrate residues." The carbohydrate may be a "glycocalyx." The phospholipid "would be interspersed in the protein network because of noncovalent bonding." The area between the air-phase and protein network may consist of lipids in the liquid crystalline state. Water would probably be less concentrated in the molecular layer at the air interface. "Thermodynamically, this should give rise to an increased surface pressure in the alveolar lining relative to other surrounding tissue components." The arrangement "of fatty acid residues would force water out of the ultimate molecular layer either into the air or into the underlying strata of the structure," which would give rise to variations in surface pressure in response to cyclic compression-expansion of the alveolar surface (if this behavior occurs). Gels and some liquid crystalline states can "tolerate wide variations in water content" (Ferguson and Brown, 1968 - from Frosolono et al., 1970).

As was mentioned earlier, Bhattacharyya et al. (1975) identified a glycoprotein of MW 36,000. Recall that Sawada et al. (1977) and Sawada and Kashiwamata (1977) also identified a protein having this MW from bovine lung wash material, but they did not characterize it as to carbohydrate content.

King et al. (1973, 1974 - from Bhattacharyya et al., 1975) also reported a 34,000 MW protein in lavage material from different animal lungs which Bhattacharyya et al. (1975) suggested as possibly the same protein as the 36,000 glycopeptide because their methods of isolation were similar to those of King et al. (1973). Bhattacharyya and co-workers (1975) worked with rabbit lung lavage and lamellar body material. They found six major bands of protein on SDS-acrylamide gels on which samples of delipidated lavage material were run. The protein bands had apparent molecular weights of 75,000, 69,000, 62,000, 50,000, 36,000, and 25,000, respectively. PAS staining revealed that the bands of 62,000 and 36,000 molecular weight were glycopeptides. This stain also revealed the presence of several other glycopeptides in this material. The same six major bands with MW's identical to the six listed above were found in delipidated lamellar bodies. Here again, the 62,000 and 36,000 MW peptides were PAS positive as well as other bands.

Bhattacharyya et al. (1975) concluded that the two glycopeptides which they isolated, were homogeneous based on "SDS-acrylamide gel patterns, isoelectric focusing, and qualitative NH_2 -terminal amino acid analyses." Carbohydrates of these glycopeptides were also analyzed. They suggested that the molecular weights of the glycopeptides were probably overestimated due to the carbohydrate and collagen-like portions of the molecules.

Similarities were found between these two glycopeptides which they isolated and two glycopeptides of 62,000 and 36,000 MW, respectively, which were accumulated in lungs of patients with alveolar proteinosis.

Bhattacharyya et al. (1975) suggested that their glycopeptides were probably "normal secretory products of type II epithelial cells." They felt "that the lamellar bodies may be the source of these glycopeptides." Also, because similar glycopeptides were found to accumulate in humans with alveolar proteinosis without creating an inflammatory response, they are probably "normal components of alveoli" (Bhattacharyya et al., 1975).

The preceding discussion provides some idea of different results that may be obtained in studying surfactant proteins. Discovery of a protein unique to surfactant (if one exists) would be a great benefit. Such a protein would be most helpful in tracing the production of surfactant throughout the life cycle of an organism. The presence of such a protein may be as an important component of surfactant structure, also. However, investigators have not been fortunate enough to discover a protein which is unquestionably unique to surfactant. The following studies deal with this question of specificity.

Maguire et al. (1977) showed that there is no protein unique to surfactant. The only nonserum protein which they found in lung wash protein was secretory immunoglobulin A (sIgA). The main proteins of lung wash were albumin, IgG,

and sIgA. Other serum proteins were also present. The concentration of serum proteins in lung wash differed from the concentration of these proteins in serum. Previous studies have indicated the presence of serum proteins in lung washings (Colacicco et al., 1973; Shelley et al., 1975; Scarpelli et al., 1973; King et al., 1973; Klass, 1973 - from Maguire et al., 1977) and ultrastructural studies have shown that they are "normal components of the alveolar lining layer" (Bignon et al., 1976 - from Maguire et al., 1977).

Maguire et al. (1977) used different methods of electrophoresis to study the lung wash proteins (LWP) from rabbit lungs. They performed two-dimensional immunoelectrophoresis (2D-IEP), tandem 2D-IEP, and polyacrylamide gel electrophoresis (PAGE). They partially delipidated the LWP to allow PAGE and 2D-IEP of the proteins. In general, the LWP fraction contained an average of 47% protein and 25% phospholipid.

A comparison of the PAGE separation patterns of LWP and delipidated serum revealed several differences in banding patterns. For example, there was more protein band II of LWP than band II of delipidated serum, LWP lacked band V of delipidated serum, and band IV was present in a smaller quantity in LWP than in delipidated serum. Albumin was a major component in both LWP and delipidated serum. This contrasts with the results of Sawada and Kashiwamata (1977) who found evidence that little if any albumin exists in bovine lung surfactant using a density gradient isolated fraction of surfactant.

Sodium dodecyl sulfate (SDS)-PAGE of LWP showed "a high concentration of protein with a MW of about 340,000." PAS, stained the 340,000 dalton protein, a band at 160,000 daltons which was presumed to be IgG, and material near the tracking dye. SDS-PAGE of delipidated serum revealed a 96,000 dalton protein not present in LWP.

"Two-dimensional-IEP of LWP protein against anti-LWP serum" showed several precipitant lines, the largest peak being characteristic of albumin. A heavy precipitant arc extended from the sample well. When 2D-IEP of delipidated serum against anti-LWP serum was run, they found more precipitant lines than when LWP was used as the antigen. Maguire et al. (1977) stated that this indicated the presence of low concentrations of many serum components of the LWP. When the anti-LWP serum was absorbed with delipidated serum and used in 2D-IEP with LWP as antigen, they found only the heavy precipitant peak originating from the sample well.

Colacicco et al. (1976 - from Maguire et al., 1977) demonstrated the presence of secretory IgA in rabbit lung surfactant. "Tandem 2D-IEP of LWP and rabbit milk, which contains sIgA as a major component," revealed the "fusion of the LWP precipitant band with a prominent milk antigen" when these samples were run in a second dimension into anti-LWP serum previously absorbed with delipidated serum. This was taken to mean that the only nonserum protein of LWP was sIgA

Maguire et al., 1977).

Protein of the surfactant fractions from discontinuous NaBr density gradients were analyzed by 2D-IEP and SDS-PAGE (Maguire et al., 1977). The surfactant fraction, and other fractions from the gradient (minus surfactant) gave the same pattern of peaks as unfractionated LWP in 2D-IEP using anti-LWP serum. Also, when the surfactant fraction was compared with the other gradient fractions using equal amounts of protein, "no differences in size or positions of any peaks could be seen." SDS-PAGE of gradient fractions showed that all major protein bands were present in each fraction. Therefore, no selective association of these proteins with lung wash surfactant could be found (Maguire et al., 1977).

Maguire and co-workers (1977) concluded that the main components of rabbit lung washings were "albumin, sIgA, and IgG with sIgA being the only nonserum protein detected." LWP was primarily composed of serum proteins, but the distribution of these proteins differed from serum. By 2D-IEP, it was shown that surfactant fraction proteins contained all the LWP's. Therefore, no apparent "specific association of any protein with the surfactant phospholipids" exists according to their data (Maguire et al., 1977).

In addition to the study by Maguire et al. (1977) which indicates the lack of a specific lipid-protein complex in surfactant, another earlier study provided the same conclusion. However, the earlier study determined that such nonspecific associations of lipids and protein are probably due to methods used in isolating surfactant from lung wash material.

Most evidence for the theory that surfactant contains lipoprotein molecules comes from the fact that lipid and protein are often found together in lung wash or lung homogenate fractions (Shelley et al., 1975). In most studies, one or more centrifugation steps are used in order to isolate the surfactant fraction from lung wash material. One of the first steps is usually sedimentation centrifugation of the cell-free lung wash in order to obtain a surfactant pellet for further analysis (Shelley et al., 1975). Sediments contain lipid and protein (Shelley et al., 1975), but great variation "in the relative amounts of phospholipid and protein" in these sediments have been shown (Galdston et al., 1969; Balis et al., 1971 - from Shelley et al., 1975). Shelley and co-workers (1975) found this to be true in their study of rabbit lung cell-free wash (see Table 1 from Shelley et al., 1975). They suggested that this variation could exist due to differences in size of animals and/or differences in handling of the lung wash material, but they maintained that the PL/protein ratio should be relatively constant. They found that the PL/protein ratio of the sediment varied according to the PL/protein ratio of the cell-free washings from which they were obtained (Shelley et al., 1975). Also, neither the speed nor the length of time samples were centrifuged affected the PL/protein ratio of the sediment. To Shelley et al. (1975) the above findings suggested that there was a "nonspecific" association between the protein and

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phospholipid in the sediment.

They (Shelley et al., 1975) also found that some surfactant material is lost during sedimentation steps as evidenced by the fact that surface-active material was isolated from the supernates which remained after centrifugation of lung washings at 40,000 g for one hour. This could lead to quantitative errors in reporting lipid and protein in studies using these methods.

Table 1. (from Shelley et al., 1975)

TABLE 1. Phospholipid and protein content of cell-free rabbit lung washings

	$\mu\text{g PL/ml}$	$\mu\text{g Protein/ml}$	PL/Protein
32 washings	346 $\pm$ 112 ^a (140-624) ^b	234 $\pm$ 72 (119-424)	1.57 $\pm$ 0.61 (0.49-3.42)
14 washings with no RBC visible in pellet	328 $\pm$ 100 (167-551)	212 $\pm$ 66 (119-364)	1.64 $\pm$ 0.64 (0.99-3.42)
8 washings with few RBC visible in pellet	338 $\pm$ 68 (224-471)	222 $\pm$ 26 (165-315)	1.61 $\pm$ 0.46 (0.79-2.07)
10 washings with many RBC visible in pellet	376 $\pm$ 148 (140-624)	274 $\pm$ 78 (165-424)	1.44 $\pm$ 0.57 (0.49-2.99)

^a Standard deviation.

^b Range of values.

32 Washings
Divided
into
these
groups

SDS-PAGE of cell-free lung washings revealed that plasma and nonplasma proteins were present in the lung washings (Shelley et al., 1975). Maguire et al. (1977) found serum and nonserum protein in lung wash protein. The cell-free lung washings also reacted with anti-rabbit albumin, IgG, and IgM. Albumin appeared to be the major protein of cell-free lung wash, and of sediment and supernates of the cell-free lung wash. Recall that Maguire and co-workers (1977) also found albumin and IgG in lung wash samples from rabbits. The "SDS-PAGE patterns of sediments and supernates" of the cell-free lung washings were the same (Shelley et al., 1975).

Shelley and co-workers (1975) studied the analytical ultracentrifugation properties of lung wash lipid-protein complexes, as well as density gradient characteristics. They found that the lipid-protein complexes of lung wash did not conform to those features which characterize serum lipoproteins in analytical centrifugation. Flotation rates for surfactant lipid-protein complexes were quite high compared to those for serum lipoproteins, and rates were in the range of very low density lipoproteins (VLDL) of serum. However, a density of 1.05 was needed to float these complexes which was the density necessary to float low density lipoproteins (LDL) of serum. Flotation rates varied widely, also. Analytical ultracentrifugation of sonicated sediment samples of lung washings resulted in two peaks, whose flotation rates were 87 to 300 for the faster-moving peak, and 27 to 54 for the slower-moving peak.

Based on the above results, Shelley et al. (1975) suggested that the peaks observed during ultracentrifugation "did not represent homogeneous lipoprotein classes of different densities," but were probably particles of varying size.

Sonication of cell-free lung wash sediments caused "solubilization of more protein than phospholipid." The sonication results indicated that "protein nonspecifically absorbed to the phospholipid during sedimentation" was not readily removed. Shelley and co-workers (1975) were quick to point out that even though some protein remained associated with phospholipid, it did not necessarily follow that it was a specific association because the periods of sonication used were too short to achieve complete dissipation of phospholipids into particles of uniform size (Attwood and Saunders, 1965 - Shelley et al., 1975).

Electron microscopy of negatively stained cell-free lung wash material in different states confirmed the earlier hypothesis that peaks found in analytical ultracentrifugation were not composed of homogeneous lipoprotein classes. The following observations were made:

<u>Sample</u>	<u>Observation</u>
1) Cell-free lung washings prior to sedimentation	Numerous small particles of various sizes, from about 20 nm (200 Å) to 100 nm (1000 Å) in diameter. Particles appeared to be liposomes with characteristic concentrically arranged lamellae.
2) Sediments resuspended in RLS (Ringer's lactate solution) by stirring	Liposomes had clumped to form large aggregates from 1000 nm (10,000 Å) or more in diameter which were not dispersed by stirring.
3) Sonication of resuspended sediment	Reduction in particle size; liposomes dispersed.

Suspensions of DPPC studied by analytical ultracentrifugation exhibited similar flotation rates to the cell-free lung wash samples in NaBr solutions. The peaks had rates ranging from 25 to 320. They (Shelley et al., 1975) further stated that analytical ultracentrifugation was not a sufficient method to discern the "presence or absence of specific lipoprotein components."

Shelley and co-workers (1975) proceeded to perform isopycnic density gradient centrifugation (which separated components on the basis of density only). Previous studies have used this step, but only after first sedimenting the surfactant fraction. Shelley et al. (1975) demonstrated that sedimentation resulted in the loss of some surfactant to the supernate, and promoted formation of aggregates which were not

easily dispersed. For this reason, they applied a sample of the cell-free lung wash to a continuous NaBr density gradient without prior sedimentation. They found that the phospholipid was concentrated in a relatively narrow band at a density of 1.045, but protein was spread throughout the gradient. Only a small amount of protein was associated with the phospholipid band. The PL/protein ratio of the phospholipid band was dependent on the PL/protein ratio of the cell-free wash sample used in the gradient. 'Background protein' prevented any determination of the extent to which protein was associated with the phospholipid.

Samples from density gradient fractions were run on SDS-PAGE gels to determine if any protein(s) was specifically associated with the phospholipid band. Albumin, immunoglobulin, and several nonplasma protein bands were found in the lung wash fractions. However, all of the protein bands present in the phospholipid-containing fractions were also present in the other gradient fractions sampled. There appeared to be no specific association between any protein and phospholipid.

As discussed previously, Maguire et al. (1977) did a similar study and obtained results like those above. Cell-free lung washings from rabbits were placed on discontinuous NaBr density gradients (without prior sedimentation according to their description). Fractions were collected, delipidated, then analyzed by 2D-IEP against anit-LWP serum, and SDS-PAGE. They found no differences in peak patterns between surfactant fractions or nonsurfactant containing fractions and unfractionated LWP by 2D-IEP. Also, there did not appear to be any differences between surfactant and nonsurfactant fractions using 2D-IEP. Finally, SDS-PAGE results showed that all major protein bands could be found in each fraction from the gradient. Maguire et al. (1977) came to the same conclusion as Shelley and co-workers (1975) - there did not appear to be a specific association between surfactant and any protein. The 2D-IEP data was perhaps the most convincing of this conclusion. SDS-PAGE showed only that certain MW classes of protein were not specifically associated with surfactant, but antibodies used in the 2D-IEP experiment were supposedly specific only for surfactant (LWP) proteins. Thus, the results demonstrated that specific proteins could be located in every fraction - not just the surfactant fraction.

Centrifugation of the lipids only, after organic solvent extraction of the phospholipid band from the density gradient, produced a phospholipid band at a slightly lighter density than the original band (Shelley et al., 1975). This indicated that a small amount of protein had been removed from the lipids (Shelley et al., 1975).

Shelley and co-workers (1975) tested their conditions of density gradient centrifugation to determine whether or not lipoprotein complexes were fragmented by their procedures. The NaBr was replaced with sucrose, temperature was altered, and the buffer was changed, but these conditions alone, or in various combinations did not reveal the presence of any specific

lipoprotein complexes. They did find that the position of the phospholipid band was changed if the gradient was kept at 4°C instead of 20°C. However, they (Shelley et al., 1975) demonstrated that pure DPPC as well as lipids extracted from lung washings showed the same density change with variation in temperature. Thus, the change in density did not appear to be due to an increased protein-lipid association (Shelley et al., 1975).

A worthwhile experiment might be one in which a nonserum apoprotein (as isolated by King et al. (1974)) were tested for its presence in different fractions of an isopycnic density gradient of lung wash material. Antibodies to each gradient fraction could be generated and each set of antibodies could be tested for apoprotein binding. If apoprotein were only bound by antibodies to fractions characterized a surface-active, then a specific association may be proved. This may be better than IEP methods previously described, and depends on an ability to isolate a nonserum apoprotein.

Shelley et al. (1975) contend that evidence for the existence of lipoprotein molecules in surfactant may be based on artifacts of experimental methods. Investigators usually worked with resuspended sediments for density gradient work. As they (Shelley et al., 1975) demonstrated, sedimentation promoted formation of aggregates of surfactant liposomes and protein which were not easily dissociated during density gradient centrifugation. The amount of protein trapped in these aggregates and therefore the density would be directly dependent on the protein content of lung wash from which the sediment was obtained. Some surfactant was shown to be lost during sedimentation, which would compromise accuracy. Some investigators only analyzed the visible bands in the gradient tube for protein and lipid, which would not allow for the possibility that they were observing 'background' protein and not specific protein associated with surfactant lipid (Shelley et al., 1975).

The hypothesis set forth by Shelley and co-workers (1975) was that when the monomolecular film of phospholipid is disrupted by aqueous lavage, the insoluble lipids form multilamellar, bilayered liposomes which trap other materials - (including protein from the hypophase). The fact that the same proteins were found in the phospholipid and other fractions of lung wash indicate that the phospholipid-protein association may be nonspecific. (Of course this hypothesis is based on the model which describes the structure of surfactant as including "a monomolecular film of phospholipid at the surface of an aqueous layer or hypophase" (Shelley et al., 1975) which may contain proteins and carbohydrates (Weibel, 1973 - from Shelley et al., 1975)).

Their contention was that density gradient analysis without prior sedimentation was the best way to isolate and study lung wash surface-active fractions. This method would allow separation based on density only, not size and density as analytical ultracentrifugation (flotation) and sedimenta-

tion employ. This method would probably not promote aggregation of lipids either which could cause trapping of nonspecific protein molecules. Based on the results of their gradient fraction analysis, there is no specific association between lipid and protein in surfactant.

When confronted with experiments which show an increase in a particular protein in the surfactant fraction, Shelley and co-workers (1975) could not "reconcile" this with their findings. They used the example of King et al. (1973) who found increased concentrations of protein with a MW of 10,000 - 11,000 in purified fractions of dog lung surfactant. They (Shelley et al., 1975) suggested that perhaps this was due to differences between the two species - dog and rabbit.

The results of Shelley and co-workers (1975) cannot resolve the apparent existence of specific protein in surfactant in alveolar epithelial cells as detected by immunoelectron microscopic studies (Sueishi et al., 1977). Although Sueishi et al. (1977) did not prove that the protein, located in the cell by labeled antibody, was synthesized there, or was even the same protein as that labeled at the alveolar surface, the implication was that type II cells synthesize and secrete lung surfactant lipid and protein. One would expect to see a unique protein associated with surfactant if the type II cells actually synthesized one, but according to Shelley et al. (1975) none has been found.

Whether or not proteins found in surfactant are actually lipoproteins, it has been established that proteins, both serum and nonserum, are somehow associated with surfactant material. "Because the alveolar surface is separated from capillary blood by only a very thin layer of tissue over most of the area of the lung," it is very possible that serum proteins are contaminants of lung washings (Shelley et al., 1975). It is also possible that serum proteins form part of the hypophase (Shelley et al., 1975). Hurst, Kilburn and Lynn (1973 - from Shelley et al., 1975) perfused pulmonary circulation with sucrose solutions, or with the nonaqueous liquid FC-80 fluorocarbon to eliminate the possibility of contamination with plasma proteins. They found that lavage material contained very little protein after such treatment. However, Bensch, Dominguez, and Liebow (1967 - from Shelley et al., 1975) demonstrated that albumin and globulins could "migrate from the alveoli into the circulatory system." Thus, the possibility that serum proteins are part of surfactant has not been disproved, because perfusion may cause absorption of some components (i.e. proteins - including serum proteins) of the alveolar lining layer from the alveolar surface (Shelley et al., 1975).

Some roles for protein in surfactant have been proposed, but none have been proven to this author's knowledge. Proteins have been shown to "stabilize PC monolayers against loss of surface activity by repeated compression," in vitro (Hurst et al., 1973 - from Shelley et al., 1975). Thus, proteins may play a role in "providing a solution on which a stable

surface film could be formed" (Shelley et al., 1975). Alternatively, proteins may play a role in "transport of DPPC from the cell through the hypophase to form the monomolecular surface film" (Shelley et al., 1975). In their monograph on surfactant composition, Clements and King (1976) proposed that apoprotein may play a role in accelerating the adsorption or transport of surfactant to the alveolar interface to make enough material available to the surface to meet physiological requirements. This was based on a study which they cited (King and Clements, 1972b) showing that sonicated lipids from surfactant adsorb more slowly from a saline subphase to the air-liquid interface than surfactant containing apoproteins. The protein may be involved in the structural organization of surfactant as will be seen in the structure section to follow.

Several important points should be emphasized from this section on protein composition. Sawada et al. (1977) were successful in isolating a protein with 36,000 MW from bovine lung surfactant. They indicated that the apoprotein may be lipophilic, and that the 36,000 MW protein may be a subunit of the actual apoprotein of 20×10^4 estimated MW. According to references cited by Sawada and Kashiwamata (1977), King et al. (1973) reported a 10,000 - 11,000 MW protein and a 34,000 MW protein (King, 1974) in surfactant. King et al. (1974) successfully demonstrated an RIA for detecting surfactant "specific" antigen in sheep lungs. Especially significant was their ability (King et al., 1974) to determine surfactant "specific" antigen in fetal lamb lungs. Sueishi et al. (1977) used immunohistochemical techniques to locate surfactant "specific" protein in lung. The labeled antibody was found in ER, Golgi complex, MVB's, and lamellar bodies of the type II cell, and in the alveolar lining material. Histochemical studies indicated a possible MPS association with surfactant (Groniowski and Biczyskova, 1964, 1968; Kikkawa et al., 1970). Using Thorotrast stain, Oledzka-Slotwinska and Maisin (1970) concluded that MPS was not a component of surfactant. Steim et al. (1969) used biochemical methods to demonstrate that the protein and carbohydrate of surfactant were not functionally significant. Frosolono et al. (1970) and Bhattacharyya et al. (1975), using biochemical techniques, showed that glycoproteins were a part of surfactant. Frosolono et al. (1970) found that the protein associated with lipid in SAF had glycoprotein character. Bhattacharyya et al. (1975) found two glycopeptides in both lavage and lamellar body material and suggested that the lamellar bodies were the source of the two glycopeptides. One point missing from these glycoprotein studies was the fact that specificity of these glycoproteins for surfactant was not fully demonstrated. Finally, the Maguire et al. (1977) and Shelley et al. (1975) studies provided evidence for the lack of a specific protein associated with surfactant. Maguire et al. (1977) found secretory IgA as the only nonserum protein in lung washings. Shelley et al. (1975) suggested that the lipid-protein associations of surfactant could have been artifactual. They also showed lack of a specific protein-phospholipid association by SDS-PAGE analysis of density gradient fractions.

These studies provide examples of the type of work being conducted on surfactant proteins. As was stated earlier, the role of these proteins is uncertain.

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C. Carbohydrate Composition

The carbohydrate composition of surfactant has received much less attention than the lipid and protein components of this substance. The discussion presented in the section on protein composition suggests that some carbohydrate found associated with surfactant (lavage) may be due largely to glycoproteins found with surfactant material. Clements and King (1976) gave a very brief overview of surfactant carbohydrate content in their monograph. They cited a study showing that carbohydrate was associated with lung lavage fluid, and proceeded to discuss carbohydrate composition in terms of glycoproteins. They indicated that the 11,000-dalton protein which they have isolated from surfactant had lipid and carbohydrate (hexose and sialic acid) associated with it, and discussed their study of this protein in more detail later. Due to the apparent lack of information on this particular surfactant moiety, this author has come to the conclusion that the relationship of carbohydrate to surfactant has not been clearly established. The literature appears to be very sparse on this subject.

Reference for Carbohydrate Composition

Clements, J.A., and King, R.J. (1976) "Composition of the Surface Active Material" In: Lung Biology in Health and Disease, Vol. 2 (Crystal, R.G., ed.) Marcel Dekker, Inc., New York, New York, pp. 363-387.

D. Structure of Surfactant

Now that the composition of surfactant has been presented, the way in which these components may be arranged in the multilamellar body (MLB) and in the extracellular tubular myelin surfactant (TMS) will be discussed. Progress made in methods of study of lung tissue for electron microscopic examination over the past several years has enhanced understanding of these structures.

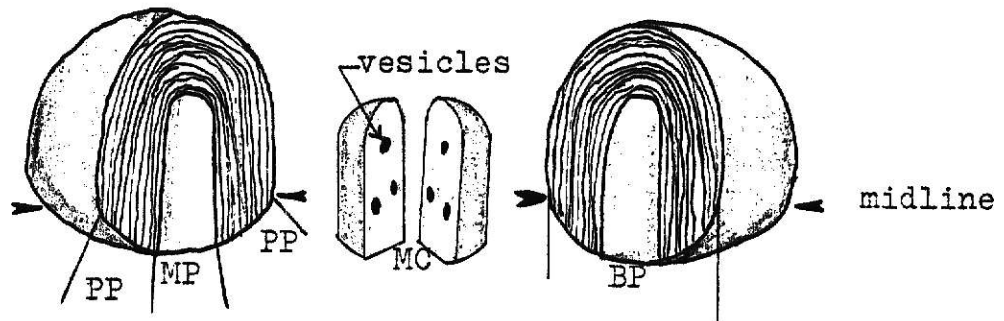
The following discussion will center mainly around the work of C.J. Stratton who has reviewed other studies as well as his own to come up with theories as to the three-dimensional structure of multilamellar bodies and surfactant lining material. He has also discussed mechanisms for MLB formation and proposed models for surfactant formation. The models proposed by Stratton appear to be probable in viewing electron micrographs of others as well as Stratton's work. (However, this author is not an expert on electron microscopy). The papers discussed in this section will hopefully give the reader a framework from which to look at surfactant structure and is not intended to be a fully comprehensive review of this subject.

Only the basics of proposed structures and models will be outlined here. The reader is encouraged to look up the references cited here. Only then can one be exposed to the detailed analysis used in devising the models of surfactant and MLB structure and formation.

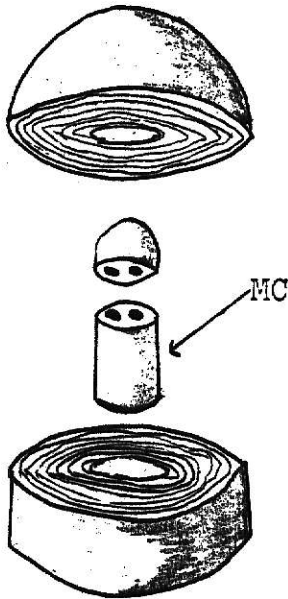
Because MLB's appear to be the storage sites for surfactant, their structure will be discussed separately from the structure of tubular myelin (which can be loosely interpreted as secreted surfactant). Based on thin section and freeze-fracture electron micrographs, Stratton (1976a) came up with a three-dimensional (3-D) model for the structure of MLB's. Figure 4 includes sketches after Stratton's (1976a) paper on this topic. Included in that paper are sketches of what oblique sections may look like, as well as supporting electron micrographs.

The ensuing discussion will include remarks about this model as pictured in Figure 4. Again, this author encourages the reader to look at the paper cited here in order to see how electron micrographs display various features of the model. Figure 4a is a sketch of a longitudinal section through the MLB. The bottom portion of the external limiting membrane has been designated as the "base plate." Two distinct areas lie inside the base plate (BP). They are lamellae and matrix core. The matrix core is described as homogeneous matrix which usually contains 2-25 vesicular bodies. This area projects from the center of the base plate, up through the center of the body about one-third to three-quarters of the way to the top of the body. The matrix core (MC) has the general shape depicted in the sketch, and is slightly convex where it "rests" against the base plate. The top of the MC "is limited by the most internal lamella" (Stratton, 1976a) and is rounded at the top. The lamellae are attached to that

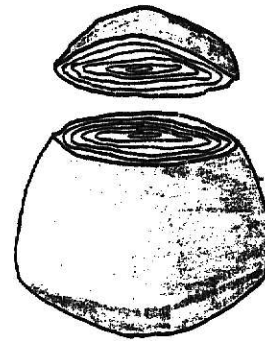
Figure 4. (After Stratton (1976a) Figs. 2-4)



a) Longitudinal Section Through Multilamellar Body



b) Cross section of Multilamellar Body Just above the Midline



c) Cross section of Multilamellar Body above the Matrix Core

BP = Base plate
MC = Matrix core
PP = Projection plate
MP = Matrix plate

Note: This model and Stratton's micrographs refute the position that "parallel lamellae and circular lamellae seen in extracted material are formed by different fixation or embedments or are sectioning-speed artifacts" (Stratton, 1976a)

portion of the base plate labeled projection plate. The lamellae project vertically from the projection plate (PP), and "each lamella is attached in a circular fashion around the entire diameter of the projection plate" (Stratton, 1976a), see Figure 4b. That part of the external limiting membrane designated as the projection plate is also arranged in a circular manner and surrounds that portion of the base plate from which the MC projects. That portion of the external limiting membrane under the MC is designated as the matrix plate (MP). Thus, $MP + PP = BP$. The BP is a lipid bilayer membrane, however, the top of the MLB is not always limited by a membrane. Sometimes the most external lamella of the MLB encloses the top of the structure. At times, the whole body was limited by a distinct biological membrane. Unextracted vesicles were observed in the MC. Vesicles appearing in the MC were always unextracted, not like cytoplasmic vesicles which were mostly extracted. The contents of the vesicular bodies appeared "to be of the same homogeneity as the surrounding matrix" (Stratton, 1976a). Because the matrix core vesicles were always unextracted as opposed to the cytoplasmic vesicles which were generally extracted, Stratton (1976a) suggested that the contents of the vesicle may be altered as it is incorporated into the MC.

In general, most MLB's ranged from 25-70 lamellae. The MC was always present no matter what the size of the MLB was. The MC was present even in large bodies which appeared to be ready for release into the alveolar space. These mature MLB's were from 1.2 - 1.6 μ in diameter and were 1.0 - 1.6 μ high. Another structure not detailed by Stratton's model (1976a), but mentioned by him in a subsequent paper (Stratton, 1976b) and observed by others including Kikkawa and Manabe (1978) is the presence of "particles" on the lamellar surfaces. Kikkawa and Manabe (1978) used freeze-fracture techniques and reported the presence of particles about 15 nm (in diameter) associated with the lining membrane of lamellar inclusion bodies. However, they did not find these particles associated with lamellae as others have (Lauweryns et al., 1973; Smith et al., 1973 - from Kikkawa and Manabe, 1978). Lauweryns and Gombeer-Desmecht (1972 - from Stratton, 1976b) hypothesized that the surface particles might be hydrophilic polar groups or phospholipid heads of phospholipids. Smith et al. (1972 - from Stratton, 1976b) proposed that the surface particles were probably protein-lipid associations. According to Stratton (1976b) this would mean that the surface particles are "peripheral to each lamella (lipid bilayer) or project from it," and "the polar heads and fatty acid tails are incorporated in each lamella."

Apparently, based on his work with biological membranes, Branton (1971 - from Stratton, 1976b) has found that particles found in myelin "indicate areas of physiologic activity." Kikkawa and Manabe (1978) suggested that the particles which they found may be "protein enzymes associated with membranes." Deamer et al. (1970 - from Stratton, 1976b) had found that the freeze-fracture faces of lipid-water preparations were always uniformly smooth. Branton (1971) theorized that the

smooth regions of freeze-fracture faces corresponds to cleavage, through the fatty acid tails. "He (Branton, 1971 - from Stratton, 1976b) proposed that particulate regions corresponded to a cleavage immediately peripheral to the heads, and the particles were protein or lipid-protein associations on the surface of those heads." Branton (1971) also found that the face of the membrane next to the cytoplasm had many fewer particles than the face of the membrane next to the extracellular space. The significance of these particles remains undetermined.

Due to the differences in fixation and embedding procedures, as well as differences in terminology, there has been real variation in the reported measurements for MLB's. Stratton (1976b) defined a "lamella" as a lipid bilayer. His measurements were "from the middle of osmiophilic band to the middle of the next osmiophilic band." He was therefore measuring "from the outside of each phospholipid head to the outside of the other phospholipid head making up that lipid bilayer, and included the fatty acid tails (the electron translucent area)." The lamellar width or width of the lipid bilayer was also defined as the periodicity. Stratton (1976b) determined the periodicity to be 66 Å using glutaraldehyde and urea embedment, and with Epon 812 resin polar dehydrant. Some investigators using freeze-etch techniques (Smith et al., 1972 - from Stratton, 1976b) have reported an interlamellar space or interlipid bilayer space, but Stratton (1976b) did not find this.

Douglas et al. (1975) used the water-soluble plastic, hydroxypropylmethacrylate as the dehydrating agent and en bloc staining of lung tissue with magnesium uranyl acetate in saline to study thin sections of rat lung tissue. Their method showed well preserved MLB's with periodicities of 42 Å (26 Å electron-opaque zone plus 16 Å electron-translucent zone). The external lining membrane of an osmiophilic inclusion body (OIB) was also remarkably clear in one of their electron micrographs. They also observed electron-opaque lamellae with the same periodicity in organelles which resembled MVB's. The lamellae of an OIB in one of the electron micrographs appeared to be arranged parallel or perpendicular to the limiting membrane, but did not appear to contact it. According to Stratton's (1976a) model, this was probably an oblique section through the side of the projection plate.

Stratton (1976a,b) reported several anomalies when looking at thin sections of alveolar tissue. These variations from the norm will not be included in this discussion. For the reader interested in pursuing the electron microscopic study of lung tissue, information on these anomalies would be enlightening.

It is important to note that there was some apparent species diversity when monkey and rat tissues were compared (Stratton, 1976a). The same basic structures were observed in both, but monkey tissue MLB's were larger and generally had 2-4 lamellar foci.

From reading Stratton's (1976a) presentation of the four basic theories on MLB formation, it is apparent that this single concept has been a center of controversy, probably since the time these bodies were linked to surfactant. Due to the already wide scope of this paper, it is not possible to expound on every aspect of surfactant synthesis and secretion. Therefore, in keeping with the goal of restricting this report to our current state of knowledge, no attempt will be made to develop a historical account of those theories.

One theory on MLB formation is that lamellae are formed only from matrix core which originated as a MVB (Stratton, 1976a). This idea comes from the work of Sorokin (1967 - from Stratton, 1976a). Stratton (1976a) finds this the most probable theory in light of his structural model and morphological findings. Stratton (1975 - from Stratton, 1976a) claims to have "observed lamellae forming from the homogeneous matrix of the matrix core." He also said that he has seen that Golgi are always "prolific," and consistently found large membrane enclosed MVB's (Stratton, 1976a). Those MVB's which contained lamellae had a minimum of 15-25 vesicles. Large numbers of single vesicular bodies were also observed in the cytoplasm near the nucleus. (Note: Douglas et al. (1975) found lamellae in MVB's with the same periodicity as those in MLB's). For a mature MLB of 1.3μ in diameter to develop from a growing MVB of 23 vesicles and 4 lamellae (0.3μ in diameter), the MVB would have to increase in size by over four-fold. Stratton (1976a) proposed three ways by which material could be contributed to the MVB for growth. One, those single cytoplasmic vesicles mentioned earlier could become incorporated into the matrix. Two, cytoplasm may be incorporated. Three, microtubules may transport materials to be incorporated into the MC. Stratton (1976a) did observe microtubules (MT's) which appeared to terminate at the base plate, opposite the MC. Stratton (1976a) also stated that the actual addition of material to the MVB may occur by a combination of the above mechanisms or may occur by all of them.

One combination of mechanisms proposed (Stratton, 1976a) included MT's and the single vesicular bodies. Because vesicles in the MC did not appear extracted as opposed to single cytoplasmic vesicles which were extracted, it was thought that there may be a change in content before or after these vesicles were incorporated. Thus, Stratton (1976a) theorized that the "prelamellar material is incorporated into the matrix core via singular vesicular bodies and/or a microtubule system."

Other authors have discussed evidence which may be in line with Sorokin's (1967 - from Stratton, 1976a) hypothesis for MLB formation. From Frosolono's (1977) review came the idea that lamellar bodies are "degenerative lysosomes." This was apparently indicated by the presence of acid phosphatase and other hydrolytic enzymes in lamellar bodies (DiAugustine, 1974 - from Frosolono, 1977). Heath and Jacobson (1976) used a different description. They noted that lysosomal hydrolases have been found in lamellar bodies. They went on

to say that "intracytoplasmic surfactant" may be taken up by lysosomes as part of an autophagic process, then be secreted by exocytosis (Heath et al., 1976 - from Heath and Jacobson, 1976). Heath and Jacobson (1976) also cited two studies which demonstrated the presence of phospholipase A₁ and A₂ activity in lysosomes. Thus, it was suggested that lysosomal phospholipases may be involved in surfactant secretion, or they may destroy surfactant before it is discharged from the cell. Heath and Jacobson (1976) did find lysosomal phospholipase A₁ and A₂ activity in the rabbit lung lamellar bodies, and concluded that this was in agreement with the theory that lysosomes are involved in the development of lamellar bodies.

When considering this theory, a few things should be kept in mind. The MVB is considered to be formed by invagination of lysosome delimiting membrane which pinches off into the interior of the lysosome as a vesicle or tubule (Novikoff and Holtzman, 1976). Looking at Sorokin's (1967) theory, MLB's may start out as lysosomes which incorporate Golgi formed vesicles containing surfactant materials. (Lysosomes are also Golgi derived (Novikoff and Holtzman, 1976)). Massaro and Massaro (1976) cited studies which demonstrated that Golgi complex, lysosomes, and MVB's all contained acid phosphatase and other histochemical similarities.

A second proposal for the formation of MLB's is that the lamellae form peripherally to the body (Stratton, 1976a). In other words, the innermost lamellae are laid down first followed by lamellae being added to the outside of the previous ones. Pease (1975 - from Stratton, 1976a) observed short phospholipid micelles in fixed lung tissue and suggested that these same micelles may occur in vivo to form "secretion bodies." These short phospholipid micelles were not observed in association with Golgi areas of MVB's but were observed in the "great alveolar" cell and in lung epithelial and endothelial cells. He (Pease, 1975) did observe these micelles within what he thought were "developing multilamellar bodies." However, Stratton (1976a) suggested that the phospholipid micelles may have been artifacts formed during the glutaraldehyde and urea embedment. When Stratton (1976a) used this particular embedment, he observed these short (0.02 - 0.08 μ) phospholipid micelles, but did not observe them in Epon 812 resin dehydrated tissues.

Douglas et al. (1975) found areas of smooth endoplasmic reticulum (SER) containing intracisternal electron-opaque lamellae which were 26 $\text{\AA}$ thick using hydroxypropylmethacrylate as a dehydrant. Lamellae of this thickness were also observed in type II cell cytoplasm. Douglas et al. (1975) postulated that the cisternae of SER located near OIB's "may be the site of synthesis of highly saturated phospholipids of the inclusion bodies." In support of this they (Douglas et al., 1975) cited Adamson and Bowden's (1972) autoradiographic studies using tritiated palmitate or acetate precursors which revealed that most of the silver grains were located at the periphery of OIB's over adjacent membranes. Stratton, (1976a) did find large numbers of smooth membranes from 0.03 - 0.84 μ in

length located next to MLB's which lacked a limiting membrane above the midline. He (Stratton, 1976a) also found that some membranes formed a 66A periodicity with the MLB which seemed to support the peripheral growth mechanism. This phenomenon was most evident in the monkey groups. However, Stratton (1976a) did not find lamellae in the SER as Douglas et al. (1975) had reported.

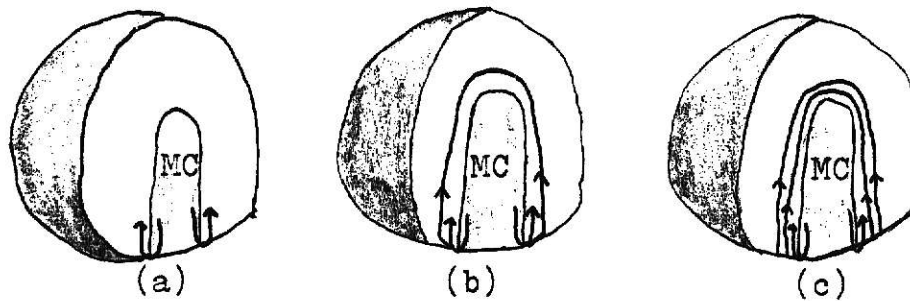
Stratton (1976a) contended that if peripheral growth were to occur, "the preformed lamellae would become attached to the base plate" with increasing numbers of lamellae added peripherally. Two problems which he found with this theory were, 1) there was usually a limiting membrane over peripheral areas of MLB's, and 2) peripheral lamellae were sometimes seen to be attached for 360° around the projection plate (i.e. they were attached to the same side of the base plate as this author perceives it). If these malformed lamellae were the first to have been formed by internal growth, then formation of additional malformed lamellae would inhibit the elongation of the first which could have caused fracturing and movement to the peripheral position.

The third mechanism for MLB formation which has been proposed, involves formation of lamellae by a combination of MC and SER material (Stratton, 1976a). By this method, the MC may provide the matrix for the first lamellae laid down, but subsequent lamellae may be preformed in the SER and laid down peripherally (Stratton, 1976a). Stratton (1976a) implied that support for this comes from the idea that if all lamellae were formed internally from the MC, then growth materials from the MC for elongation of the initial lamellae may have to go through "as many as 106 lamellae." He did not consider the possibility that elongation may occur at the base plate, which may support the internal formation hypothesis (see Figure 5). Of course one would then expect to see fully formed lamellae on the periphery of MLB's with a partially formed lamella most internal to the MC.

The fourth mechanism proposed for MLB formation is that MLB's are formed from mitochondria (Stratton, 1976a). This was the most acceptable hypothesis at one time until investigators found morphological and histochemical evidence against it (Stratton, 1976a). However, Callas and De Groot (1976 - from Stratton, 1976a) used euthyroid, hypothyroid, and hyperthyroid animals and found morphological evidence that MLB's are derived from both mitochondria and MVB's. Stratton (1976a) did not find morphological evidence for this when he used normal rat or monkey tissues.

In addition to these theories of MLB formation brought out by Stratton (1976a), it has been proposed that they are formed by phagocytosis. However, this is an unlikely mechanism. In a review by Massaro and Massaro (1976) they cited Niden (1967) who proposed the phagocytic origin of lamellar bodies. Niden (1967 - from Ryan et al., 1975) proposed that Clara cells produce surfactant and type II cells acquire it by phagocytosis. Massaro and Massaro (1976) also cited

Figure 5.



This figure represents MC material contributing to lamella elongation at the base plate level. In other words, as material is added to the lamella at the base plate level, it gets "taller."

other work which was against this proposal. Three lines of evidence oppose the phagocytic mechanism. First, the type II cell has not been confirmed as an "actively phagocytic cell" (Esterly and Faulkner, 1970 - from Massaro and Massaro, 1976). The Massaro's (1976) stated that "all other studies indicate that they (type II cells) are either nonphagocytic or, at best, weakly phagocytic." Second, Kuhn (1968 - from Massaro and Massaro, 1976) found that the lamellar body limiting membrane did not have the layer of 'fuzz' observed over the type II cell plasma membrane; nor was alkaline phosphatase activity of the plasma membrane present in the limiting membrane of the lamellar body. It was noted that even when the lamellar bodies "communicated with the extracellular space" it did not stain for alkaline phosphatase. They (Massaro and Massaro, 1976) stated that there is a gradual loss of characteristic enzyme activity of the plasma membrane used to form phagocytic vesicles after it has been interiorized. Third, if lamellar bodies were formed via a type of phagocytic action, one would not expect to see labeled surfactant precursors in the MLB before they were found in the alveolar wash. Young and Tierney (1972) demonstrated a delay in labeled DPPC appearance in lavage compared to whole lung.

One final note on this subject which is significant to those investigators interested in secretion and/or microtubular functions. Stratton (1976a) proposed that microtubules (MT's) may be involved in pushing the MLB's toward the alveolar surface as well as transporting material to the MC (Stratton, 1976a). He stated that this may explain the convex shape of the base plate associated with MT's in one of his micrographs. However, he reasoned that if MT's were involved in the pushing action, then they would be found between the MLB and the interior of the cell, not to the side of or between

the MLB and the alveolar space. He found mature MLB's in "random orientation" with respect to MT's throughout the cells, suggesting that perhaps MT's do not push MLB's to the alveolar space (Stratton, 1976a). His reasoning is not complete here because if MT's do indeed transport materials from one part of the cell to the other, then one would expect to find some MT's which were not oriented to MLB's. In other words, some MT's may be performing functions which are not directly related to MLB's.

In 1977, Stratton (1977a,b) published a pair of papers similar to those used primarily in the previous discussion of MLB's. However, he used this format to describe his findings on tubular myelin surfactant (TMS). The first paper dealt with his 3-D model of TMS, and the second paper was mainly a comparison of different embedment procedures and presentation of a model for TMS formation. The following discussion will be based mainly on these two papers in order to present a model for the structure of TMS. As stated at the beginning of this section on structure, Stratton (1977a,b) has set forth probable models which appear to be based on sound research and thought. Other models may exist, but he had not found any models describing transformation of lamellae to form the TMS structure.

From the inferences made in the literature, tubular myelin appears to be the secreted form of surfactant matrix which exhibits a square-like pattern. The term "tubular myelin" is somewhat misleading, especially to the inexperienced morphologist. Figure 6 is a three-dimensional sketch of the tubular myelin figure as envisioned by Stratton (1977a). The face denoted A in the figure is a sketch of what one might see in an exact cross section of the TMS layer. Of course, electron micrographs do not show such a neatly depicted representation. One thing left out of the drawing is the lipid bilayers which often traverse one or more of the membranous corners in TMS (Stratton, 1977a) (see Figure 9d). Also, depending on the type of fixation and embedment methods used, the interior of the squares often appear more extracted. What one generally sees in a cross section or oblique cross section (such as face E) would be more of a fence-like network (see Figure 7a). The same holds true for longitudinal sections, the matrix is extracted in the center so one sees something similar to Figure 7b. Figures 8 and 9 depict what goes into making up the square pattern which is visualized in thin section electron micrographs. Figures 9b and 9c are representations of two mechanisms by which tubular myelin may be formed. This will be discussed later.

Basically, as shown in Figures 6-9, the TMS appears to be simply lipid bilayers which have been transformed from a parallel arrangement to form a square tubule. Figure 6, as stated earlier, is Stratton's (1977a) 3-D model for the structure of tubular myelin. The paper in which this model appears (Stratton, 1977a) has several electron micrographs in which he determined the angle of sectioning based on his model (such as cross section, oblique section through face A,

Figure 6. (After Stratton (1977a) Fig. 1)

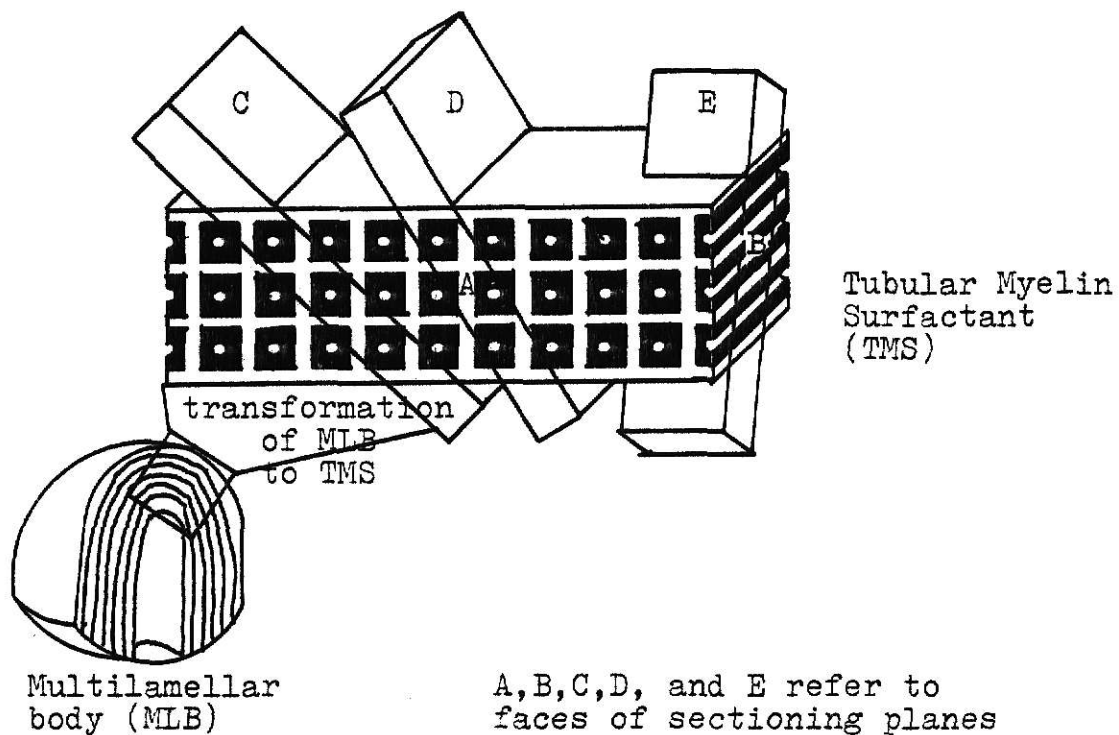
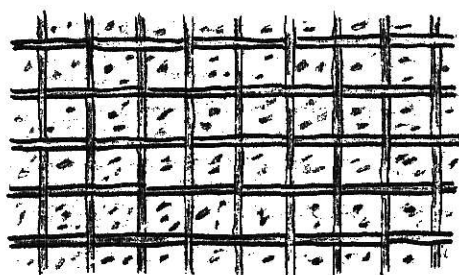
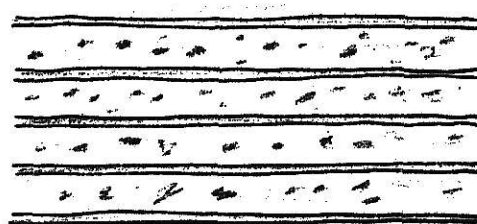


Figure 7.

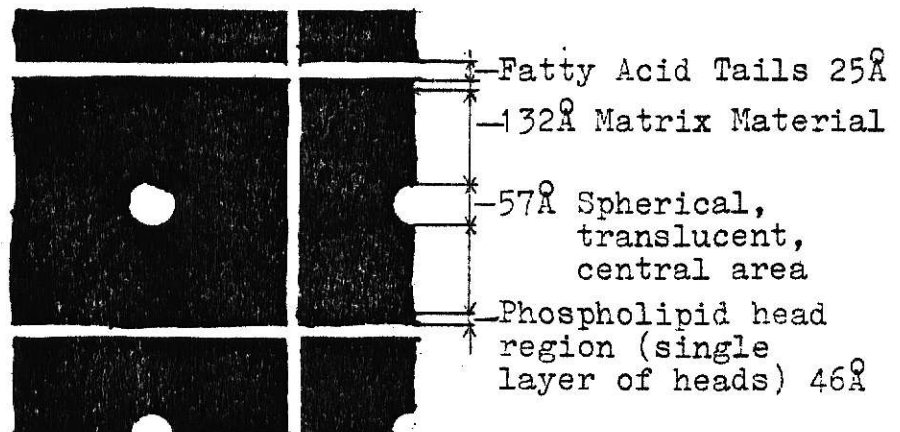


a) Sketch of cross section of TMS as would appear in some electron micrographs



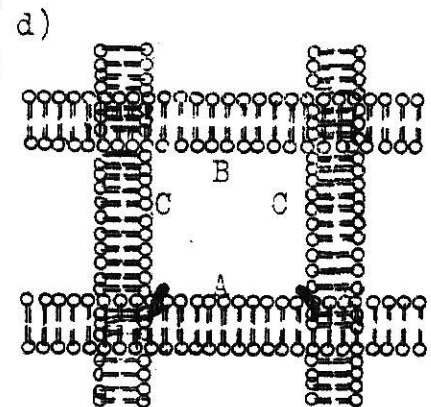
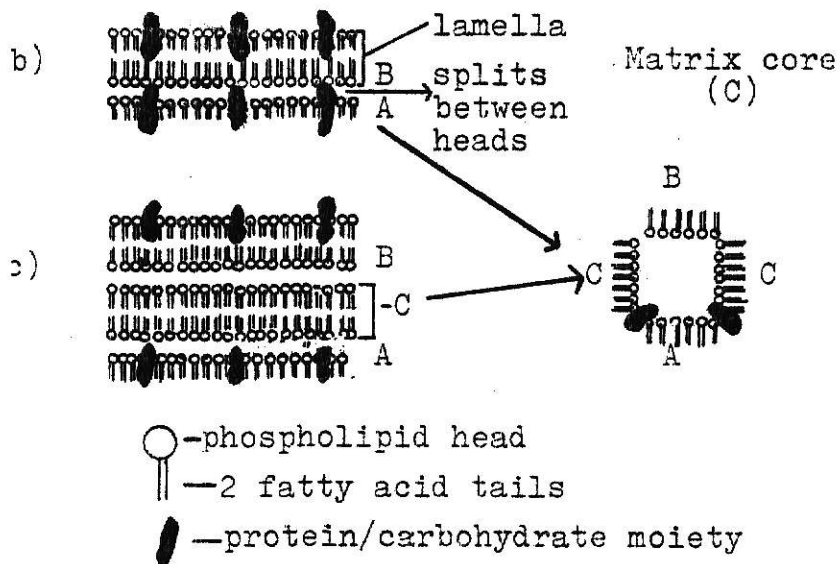
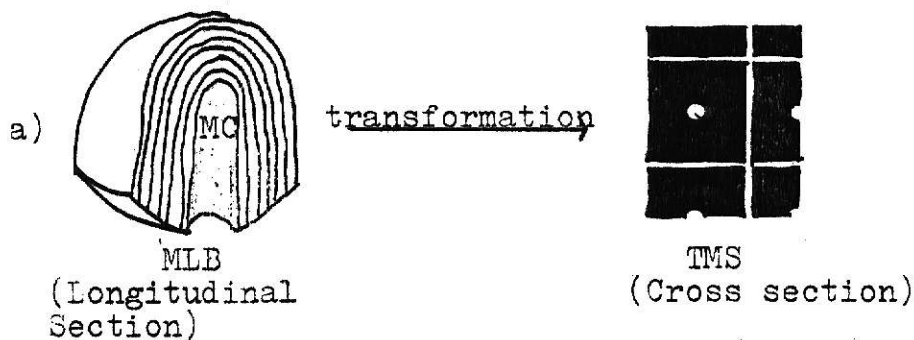
b) Longitudunal section

Figure 8. Sketch of Tubular Myelin Cross Section
(After Stratton (1977b) Fig. 1)



Face of tubular myelin measures $46 + 46 + 132 + 132 + 57 = 413\text{\AA}$
 Intertubular space = 25Å
 Periodicity = 438Å

Figure 9. Models for Tubular Myelin Formation
(After Stratton (1977b) Fig. 2)



Note: Often intertubular area at corners are "traversed by lipid monolayers in an X, as a horizontal or perpendicular pair, or in both directions making a small square" (Stratton, 1977b).

etc.). His contention is that the plane of sectioning of most thin section electron micrographs can be determined using this model. He also cited examples where sections have apparently shown this TMS layer to be homogeneous, but he explained that an oblique section itself could have caused such an appearance based on his model. In a electron micrograph of TMS "longitudinally sectioned at an angle of 60° from the horizontal," there appeared to be homogeneous surfactant. However, Stratton (1977a) concluded that his was not actually homogeneous surfactant or MLB matrix, but rather an oblique section through the TMS. He also stated that previous accounts in the literature of "hypophase or matrix" designations were in reality "oblique cross or longitudinal sections through highly ordered tubular myelin figures."

There appear to be stages in the formation of TMS from MLB's according to Stratton's (1977a,b) observations. First, MLB's are secreted. Once MLB's are secreted, they may remain unexpanded (the lamellae have not increased their periodicity) for a period of time. The matrix core of secreted MLB's is sometimes visible and sometimes not (Stratton, 1977a). Also, no vesicular bodies are observed in the MC of secreted MLB's (Stratton, 1977a). Next comes expansion and rearrangement. Expansion appears to occur by pairs of lipid bilayers. The lamellae appear to be "pushed apart by the absorption of an extracellular material." Lamellae also rearranged to form the cross membranes of TMS (Stratton, 1977a).

Stratton (1977a) observed what he called five distinct layers which characterized TMS in one section which he observed. The elements included here were not in the restricted concept of what constitutes TMS given earlier. These layers and descriptions are given in Table 6. The layers discussed there follow the stages of TMS formation for the most part, and include the degraded surfactant layer which was not listed as a stage in the above discussion.

Stratton (1977a) noted that within the transformed MLB's there were 2-13 groups of tubular myelin oriented at diverse angles within the transformed body. There were from 1-14 cross membranes and 1-12 horizontal membranes in each group of myelin tubules (Stratton, 1977a). The groups of tubular myelin ranged from 0.16 to 1.8 μ long (Stratton, 1977a). Stratton (1977a) found that the horizontal lamellae were not disturbed by the diverse orientations. Presumably, even though the MLB's were expanded and rearranged, their basic 3-D form with "concentric laminations was entirely retained."

Based on his models, Stratton (1977a) proceeded to demonstrate how he could quantitate the number of lamellar bodies in a surfactant area. Each MLB identified in a thin section electron micrograph was described in detail. Although his descriptions will not be covered in this paper, some observations are worth noting because they provide clues as to changes taking place in MLB's upon secretion.

In an oblique longitudinal section through an expanded

Table 6. Five Layers Characterizing Lung Surfactant
Tubular Myelin

LAYER	CHARACTERISTICS
1. Unexpanded surfactant	Sometimes the dominant layer. Could include as many as 63 recently secreted MLB's in random orientation.
2. Formation layer	Incorporation of a homogeneous material and rearrangement of lamellae to form cross membranes as they "fan out."
3. Mature tubular myelin surfactant layer	Square-like arrangement similar to that depicted in Figure 7a.
4. Air-surfactant interface layer	This layer was usually comprised of a single lipid bilayer membrane at the interface. (Sometimes "it was 2 to 8 unexpanded membranes thick"). This membrane was the outermost membrane of from 1-12 expanded MLB's, and "often was either the earlier formed cross membrane or the original horizontal membrane," depending on the orientation of the TMS.
5. Degraded surfactant layer	Loose spheres in the alveolar space which often contained parts which had lost portions or all of their lipid bilayer structure and appeared degraded. Parts of the cross membranes and matrix material were observed.

Note: Stratton (1977a) proposed that spherical lipid bilayers may be formed in TMS during the transformation process, thus the disintegrating spheres may not always be due to a second rearrangement at the air-surfactant interface.

- and rearranged MLB no base plate was present. Stratton (1977a) suggested that the absence of this membrane would expose the contents of the MLB to the external environment. He thought that this would be necessary for incorporation of external materials as matrix of the tubular myelin. The matrix core, which Stratton (1976a - from Stratton, 1977a) had observed as released along with the lamellae into the alveolar space, was noticeably absent. Whether the material was used in forming cross membranes of the TMS (see Figure 9b) or used in some other way is not known. The MC was also absent in TMS and its (the MC) space was filled with TMS as observed in a cross section of an expanded and rearranged MLB. The orientation of tubular myelin membranes was sometimes disrupted which was attributed to the displacement effect

of MLB's on each other and their environment (Stratton, 1977a). "TMS areas varied from 1-72 MLB's per area." The degraded forms of TMS were spheres, usually limited by one membrane and no more than four. Stratton (1977a) suggested that in the degraded state TMS phospholipid, protein, and carbohydrate find "greatest molecular stability in spheres."

The 3-D model of TMS proposed and substantiated by Stratton (1977a) was in direct conflict with the structures proposed by other investigators. Stratton (1977a) cited observations from earlier investigations, which were presumably based on extracted tissues, that supported a homogeneous area with few inclusions as part of surfactant. Stratton (1977a) found that his observations of lipid and carbohydrate retained material indicated that surfactant was TMS. The homogeneous areas he found were determined to be due to the orientation of the section through TMS in many cases. Thus, he called the "epiphase-hypophase" model into question.

Cross membranes of TMS appeared to be less stable than the horizontal membranes (Stratton, 1977a). Cross membranes seemed to be more easily extracted and mechanically disturbed than their un rearranged horizontal counterparts. Stratton (1977a) suggested that this indicates the importance of the TMS orientation in a given surfactant area. Apparently, if the horizontal membranes are oriented perpendicular to the air-surfactant interface, then this is a stronger arrangement than having them oriented parallel to the interface.

Stratton (1977a) also spoke of the possibility that surfactant is recycled, and of the need for adding surfactant to the alveolar surface. He observed lipid bilayer spheres which were apparently derived from the air-surfactant interface, and found such spheres in different stages of disintegration. This indicated to him that there may be absorption of some lamellar components. The surfactant matrix, which was noted as adjacent to both type I and type II cell membranes as well as at the interface, seemed to take on a "globular" appearance (Stratton, 1977a). Presumably this inferred that surfactant was being resorbed into these cells. Also, inferred was that passive or active transport of surfactant components may have been taking place at this site (Stratton, 1977a).

Because most expansion and rearrangement of surfactant took place at the type I cell-surfactant interface and not at the air-surfactant interface, there was apparently no contribution of additional surfactant "peripherally" to the "degrading surfactant-air interface" (Stratton, 1977a). Stratton (1977a) concluded that the surfactant area is totally degraded and then totally replaced by unexpanded surfactant which will be transformed. Stratton (1977a) also concluded that it may be "normal" for different parts of an alveolus to be without surfactant at any given time.

Freeze-fracture studies were also cited to support the stages of development and disintegration Stratton (1977a) suggested for TMS. They were not discussed by him, but the

interested reader is referred to his paper for the references. Kikkawa and Manabe (1978) performed freeze-fracture analysis of lung alveolar tissue and found spherical bodies, tubular cylinders with mostly rectangular fracture faces, and laminated membranous structures in the alveoli (i.e. extracellular environment). They (Kikkawa and Manabe, 1978) believed the tubular cylinders corresponded to TMS, and found stacks of membranes in spherical bodies, as seen in the MLB's. No particles were seen associated with these lamellar membranes (Kikkawa and Manabe, 1978). These observations sounded very similar to what one would expect to find based on the observations of Stratton (1977a,b). This was the first study to show the actual cross sectional appearance of the tubular figures using the freeze-fracture technique (Kikkawa and Manabe, 1978). Kikkawa and Manabe (1978) made further suggestions as to the nature of the tubular cylinders and spherical bodies but this author could not ascertain the basis of their reasoning. They stated that it was "reasonable to assume that the surface layer of the spherical body on which tubules rest is comprised of a polar lipid monolayer with hydrophilic heads pointing outward." "This surface monolayer of lamellar bodies can become a monolayer covering of tubules, apposing other tubules. This would explain the formation of unit membranes between tubules." This author was not sure of what was meant by "monolayer" here. However, the suggestion of formation of a unit membrane between tubules did seem to fit the model visualized in Figure 9d where there appears to be a unit membrane formed. They concluded that the tubular figures may represent a new structure formed "upon interaction between the content of lamellar bodies and the alveolar fluid," because these figures were not observed interior to the stacks of lamellar membranes in the alveolar space or in MLB's (intracellular MLB's).

The preceding discussion of TMS was mostly based on the first of two accompanying articles published by Stratton (1977a) in 1977. The following material will mainly focus on his second paper (Stratton, 1977b), where for the first time a mechanism for MLB transformation was proposed.

In the first portion of his study, Stratton (1977b) compared the appearance and measurements of lung tissues prepared by different embedment techniques. The comparisons will not be covered here. However, Stratton (1977b) found Epon 812 resin embedment to be the best method for preserving lipids and carbohydrates. His measurements of TMS using this technique were given in Figure 8. For purposes of comparison, Stratton (1977b) defined a tubular myelin as "the four 46Å osmiophilic bands (the phospholipid heads of a lipid bilayer and whatever osmiophilic material that had been attracted to them during the transformation process) which limited the square myelin tubules, plus all the material that was included within that square (the surfactant matrix and spherical area)."

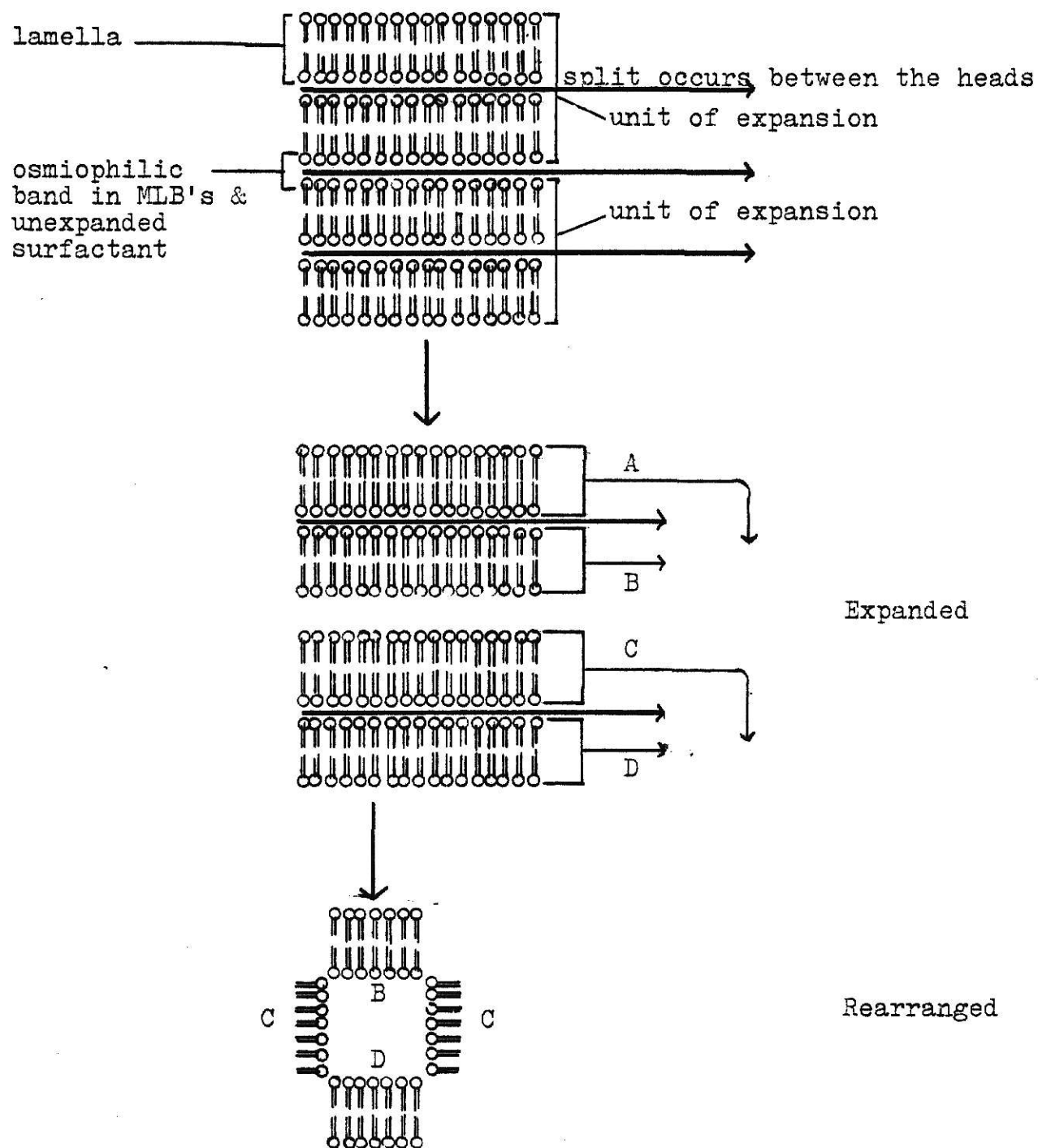
Before getting into a discussion of the proposed mechanisms of MLB transformation, several of Stratton's (1977b) noteworthy observations will be presented. Stratton

(1977b) found evidence which suggested that MLB's were made up of the same phospholipids as unexpanded surfactant. He observed a hexagonal array of membrane subunits in both a primate intracellular MLB and in extracellular unexpanded surfactant of rodents. This hexagonal array may indicate the presence of lysolecithin. In studying a micrograph which showed an area of unexpanded surfactant which was in the process of expanding and rearranging, Stratton (1977b) made three significant assertions. First, the lamellae were found to be splitting between phospholipid heads which provided evidence for the idea that each osmophilic band in MLB lamellae and in unexpanded surfactant represented two layers of heads (Stratton, 1976a,b - from Stratton, 1977b). Second, the unit of expansion appeared to be a pair of lamellae (see Figure 10). He also observed cross membranes finishing their formation "immediately after expansion." Third, it appeared as though the most external lamella of the pair "(most peripheral in the MLB)" formed the cross membranes, and the internal lamella of the pair continued in the horizontal direction (see Figure 10 and 9c). Stratton (1977b) referred to a figure almost identical to 9c as representing this mechanism of transformation, however, in light of his explanation, Figure 10 seems to be a better representation. Stratton (1977b) also regularly observed expanded, but not rearranged lamellae in TMS. It was rare to see groups of lamellae pairs which had not expanded, but pairs were not always rearranged.

Stratton's (1977b) models for MLB transformation to TMS have been touched upon already. Figures 9b and 9c are his representations of two of the proposed mechanisms. Quite simply, Figure 9b represents a method whereby the matrix core of the MLB forms the cross membranes of the TMS. Evidence for this mechanism seems to come from the fact that the MC material "present in mature, rodent MLB's positioned for release into the alveolar space" may contain enough material to form cross membranes (Stratton, 1975; Stratton, 1976a - from Stratton, 1977b). Figure 9c represents the mechanism whereby alternating MLB lamellae form the cross membranes of TMS. As discussed above Stratton (1977b) found evidence for this mechanism in the thin section of an area of expansion and rearrangement. What he saw was more specifically represented in Figure 10. A third mechanism, which Stratton (1977b) did not elaborate on, involved an en masse rearrangement of all MLB lamellae to form the TMS. This mechanism is argued against on the premise that the "physical integrity of alternating lamellae is different," and paired expansion appears to exist (Stratton, 1977b).

Stratton (1977b) contended that the second mechanism, the one pictured in Figure 9c, was the most probable one for TMS formation from unexpanded surfactant. He stated that basic membrane ultrastructure and biochemistry as is currently known as well as morphological evidence supports this theory. He gave eight points upon which he based the argument for this mechanism. These points are given in Table 7 because they can be stated more concisely in tabular form than in

Figure 10. Expanding Lamellae



Unit of expansion = "Two lipid bilayers with adjacent phospholipid heads in immediate opposition" (Stratton, 1977b).

Expansion and rearrangement may occur simultaneously.

Table 7. Morphological Evidence for Transformation of Unexpanded Surfactant to TMS via Expansion and Cross Membrane Formation by Alternate Lipid Bilayers (Stratton, 1977b)

Type of Evidence	Observation
1. Split between phospholipid heads	Separation of pairs of lipid bilayers between phospholipid heads was observed in an expanding, rearranging area of TMS.
2. Pairs of horizontal membranes	Areas of paired horizontal membranes were observed in mature TMS which had no cross membranes. (Expansion without rearrangement)
3. Membrane Fusion	Stratton observed possible lysolecithin presence in MLB's and in surfactant. Presence lysolecithin supports rearrangement hypothesis because it is thought to induce membrane fusion
4. Alternating lamellae contain different components	Slight extraction of MLB's revealed extraction of alternating fatty acid tail layers, denoting different physical properties. In the model, Fig. 9c, alternating layers of lamellae are shown to have different amounts of protein and carbohydrate.
5. Parallel and angled series of ribs (in freeze-etch electron micrographs)	Angled series of TMS groups (observed in thin section) or ribs (observed in freeze-etch micrographs) indicates that there may be some pattern of protein and/or carbohydrate in every other lipid bilayer which influences the rearrangement of surfactant to form TMS. The rearrangement of lamellae of an MLB to form TMS when exposed to the extracellular environment is probably due to "the chemical nature of the lamellae and their interaction with the new, carbohydrate-rich alveolar environment" (Stratton, 1977b). The areas of protein and/or carbohydrate may be the points of rearrangement (as pictured

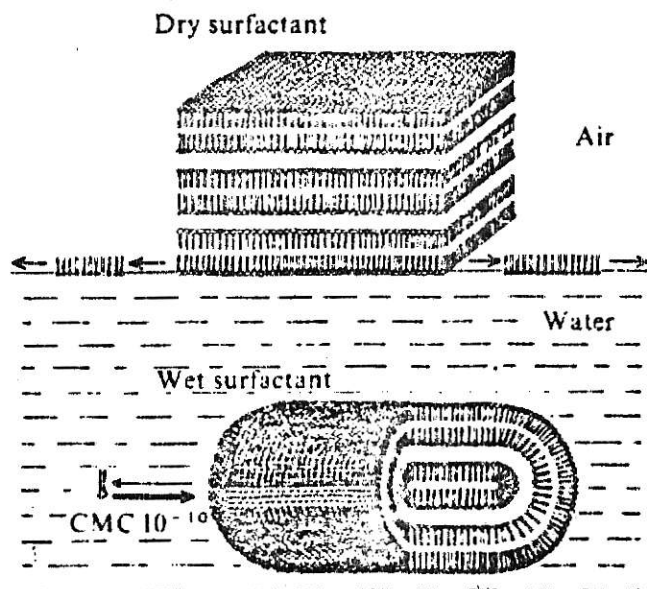
6. Right angle configuration of lamellae

7. Incomplete cross membranes

8. Quantitation

Figure 1 (from Morley et al., 1978)

Fig. 1 The molecular configuration of dry and wet surfactant. b, The dry surfactant has open ended layers from which molecules can spread freely to an air/water interface. a, In water the surfactant aggregates as a smectic mesophase and few molecules are free to reach the interface.



in Fig. 9c) or the area between these points may be the rearrangement points.

Lamellae were observed forming right angles to each other in the alveolar environment. Therefore, the rearrangement from straight lamellae to the right angle configuration seems probable. (See Fig. 10 - this is presumably what Stratton is referring to).

It was observed and expected that excess lamellar material would form incomplete (partial) cross membranes if this model is correct. (This point does not appear to be strong evidence for this particular model).

If alternating lamellae of MLB's form cross membranes in the TMS, then the expanded and rearranged MLB's of surfactant should show half as many membranes as horizontal membranes. For example, a MLB with 26 lamellae would form a TMS area with 13 horizontal lamellae. (Unless one could trace a MLB from its intracellular to its extracellular environment, and through rearrangement to TMS, this is a difficult concept to prove). Stratton claimed to have observed this, but it was not clear how he made this determination. It may have been based on an average number of MLB membranes.

the narrative. As is noted in the table, not all of his arguments appear to be strong - points 1, 2, and 4 seem to speak most directly to the model and appear to be more convincing than the rest.

In the preceding pages, Stratton's models for MLB structure, and TMS structure and formation have been covered in detail. However, one final observation may be significant when thinking of these models in the future. Morley and co-workers (1978) came up with a proposal recently which, to this author's knowledge, has not been considered in the literature. They suggested that surfactant may exist in a relatively dehydrated state (Morley et al., 1978) in the MLB of the type II alveolar cell. They demonstrated that fully hydrated surfactant was less effective than dehydrated surfactant in lowering the surface tension of saline. They also found that less pressure was required to expand lungs treated with dry lung surfactant than to expand untreated lungs. Their contention is that dehydrated surfactant may exist in bimolecular sheets which could donate lipid molecules to the surface of the alveoli faster than a hydrated liposomal structure (see Figure 1 from Morley et al., 1978). The hydrated surfactant would presumably have to overcome an "unfavorable hydration barrier" before it could donate molecules to the alveolar surface. They suggested that treatment with dry lung surfactant may be more effective than treatment of infants with RDS using the ineffective surfactant component/water mist. The idea of surfactant existing in the dehydrated state is possible, especially because there is evidence for a limiting membrane surrounding the MLB (Stratton, 1976a).

At this point, a few general comments on extracellular surfactant models will be given. This will be done in order to give one an idea of how Stratton's ideas fit in or differ from other points of view. One idea referred to in the protein section was that specific SAM protein may be "present in mosaic with phospholipid at the air-liquid interface" (Colacicco, 1972 - from Sueishi et al., 1977). Sueishi et al. (1977) found that specific conjugate labeled the alveolar lining material throughout and TMS was surrounded by reaction product. Thus, they concluded that specific protein may be present in a "compact network" in which phospholipid (in the lattice square pattern) was interspersed. Another brief concept was noted in the protein section by Kikkawa et al. (1970) who proposed that MPS and lipid may form the alveolar lining layer with MPS distributed along the alveolar plasma membrane and phospholipids randomly present in the pulmonary fluid in the fetus, but both components establish the surface film with the onset of breathing. Finally, from the protein section, recall the proposal of Frosolono et al. (1970) which involved a protein network attached to the plasma membranes of type I and II cells of the alveoli by interactions with carbohydrate residues. At the air-phase side, phospholipids (of the lining layer) would have their fatty acid chains oriented toward the air-phase. Phospholipid was thought to be interspersed in the protein network, and lipid was thought to exist in

a liquid crystalline state between the air-phase and protein network.

In a couple of reviews, some models have been schematically drawn to depict the synthesis and secretion of surfactant. Figure 11 (after King, 1974) shows the presence of tubular myelin surfactant supposedly suspended in a fluid subphase. The suspended aggregates of surface-active material may act as "a reserve of surfactant that adsorbs to the air-liquid interface and spreads there as an insoluble monomolecular film of lipid (or duplex film of lipid and protein) when this surface becomes partially depleted and requires replenishment" (King, 1974). Goerke (1974) presented a somewhat similar diagram in his review which suggested essentially the same idea, that lamellar bodies are secreted into a fluid subphase and become TMS which will somehow "spread as a monolayer at the air/water interface." He also offered the idea that tubular myelin figures may be surfactant which has been used and "irreversibly desorbed from the air/water interface." Farrell and Avery (1975) provided a diagram depicting a possible arrangement of lipid and protein at the air-water interface (see Figure 5 from Farrell and Avery (1975)). The preceding diagrams and discussion should give one an idea of the types of lipid-protein arrangements proposed for surfactant.

Stratton's articles did not speak directly to the surface chemistry and eventual contribution (if any) of material from TMS to the surface of the air-fluid interface as proposed above. They were more involved with structure as well as mechanisms of MLB formation and the eventual rearrangement of MLB's to form TMS in the extracellular layer. However, he did suggest that the surfactant material was made up of TMS and that the homogeneous appearing "hypophase" may be due largely to sectioning artifact. From Table 6 under the description of the air-surfactant interface layer it appears that membranes from the expanded and rearranged MLB's made up this layer.

Several different interpretations of how the surfactant may be "structured" to carry out its proposed surface tension lowering function have been proposed as seen in the examples above. It seems probable that surfactant exists in different states in the extracellular layer as pointed out by Stratton (1977b) (see Table 6). The actual molecular arrangement of surfactant in its various states and the contribution of these arrangements to the surface chemistry of extracellular alveolar material remains a matter of speculation.

Figure 11.
(after King (1974) Fig. 5)

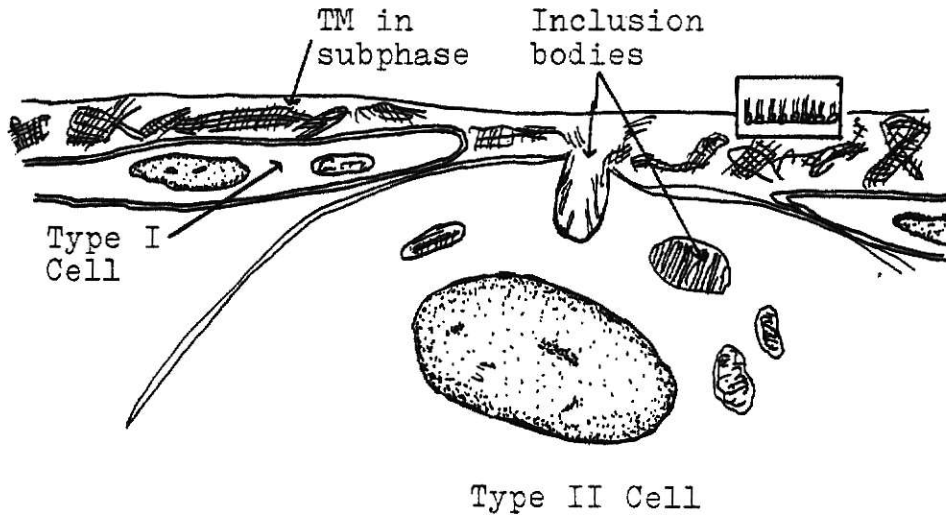


Figure 5. (from Farrell and Avery, 1975)

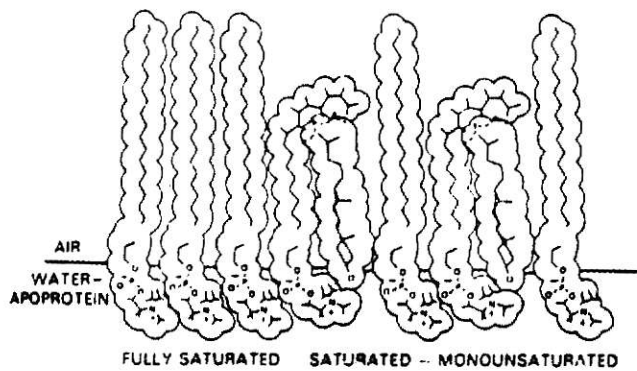


Fig. 5. Presumed interfacial arrangement of saturated (S) and unsaturated (U) phosphatidylcholine (PC) and cholesterol (C) molecules in the pulmonary surfactant. Complex shown is adapted from that described and constructed in stereomodel form by Vandenhoevel (165). Molecules sequentially depicted are: S-PC, S-PC, S-PC, S-PC, U-PC, C, S-PC, U-PC, C, and S-PC.

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E. Surfactant Synthesis

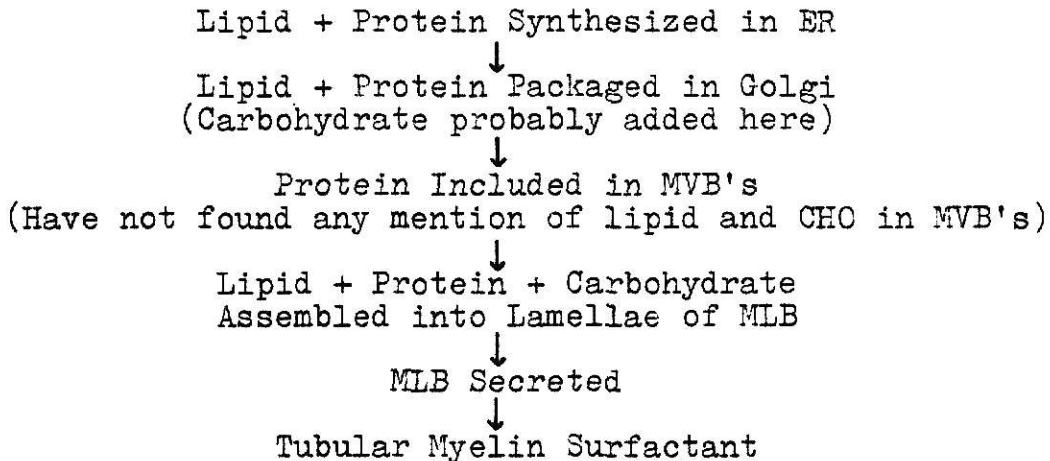
Now that the composition and structure of surfactant have been presented, this section will deal with synthesis of this material. This will mainly include a discussion of phospholipid profiles and enzyme activities during development. Material on the possible mechanisms of DPPC synthesis will also be covered. Finally, the possible role of glucose and glycogen in phospholipid synthesis will be discussed.

Before getting into the specifics of phospholipid synthesis, it would be helpful to review some of the evidence for the pathway of surfactant through type II cells. A few brief comments will be made about a possible role of lamellar bodies in surfactant synthesis, and about secretion of these bodies.

The pathway of surfactant synthesis and secretion is outlined in Figure 12. The type II cell, sometimes called the granular pneumocyte or great alveolar cell (Stratton, 1976) is believed to be the site of surfactant synthesis. Several lines of evidence point this out. Barańska and Van Golde (1977) cited experiments which revealed that DPPC is the predominant surface-active component of surfactant and the main lipid of isolated lamellar bodies. Autoradiographic studies have traced choline to the lamellar bodies of this cell (Chevalier and Collet, 1972 - from Massaro and Massaro, 1976). This may mean that choline is incorporated into PC of lamellar bodies. Goerke (1974) also cited references in his review which show that preparations of isolated lamellar bodies have surface activity. Additional morphological evidence comes from the study of Sueishi et al. (1977) who demonstrated the presence of a surfactant protein in organelles of type II cells and covering the alveolar surface (see protein section). Morgan and Morgan (1973) cited studies which showed that the appearance of type II cells was accompanied by an increase in lung phospholipid and PC. They used some of their own observations in the developing monkey lung to show the presence of surface-active lipoprotein after the appearance of type II cells. They cited additional evidence for the production of surfactant by type II cells, and outlined one of their studies which showed, among other things, apparent pilocarpine stimulation of DSPC secretion. Pilocarpine treatment has been associated with a decrease in the number of lamellar bodies observed microscopically (Goldenberg et al., 1967 - from Morgan and Morgan, 1973). From the above evidence it appears as though type II cells are indeed sites of surfactant production. The following discussion will attempt to show the involvement of the different cellular organelles in this process.

It is apparent from several studies that both lipid and protein of the surfactant complex are synthesized in the endoplasmic reticulum. Additional details of lipid synthesis will be discussed later. Massaro and Massaro (1972 - from Massaro and Massaro, 1976) found evidence to show that protein is initially synthesized in the rough endoplasmic reticulum (RER) and then transported to lamellar bodies. They pulse

Figure 12. Pathway of Surfactant Through Type II Cell



labeled rat lung slices with $[4,5-^3\text{H}]$ leucine. Immediately after pulse labeling, most grains in the type II cell appeared over the RER. After 40 minutes, fewer grains were found over the RER and more in lamellar bodies. The percentage of grains over mitochondria and nuclei was essentially unchanged, and the amount of radioactive protein in the lung tissue was unchanged over this time period. They concluded that the change in distribution of grains from RER to lamellar bodies, without a change in the specific activity of the protein of the homogenate, was probably due to protein transport rather than different synthetic rates at both sites.

Sueishi et al. (1977) (see protein section) also found evidence for protein synthesis and transport. They labeled Fab' fragments of γ -globulin molecules with horse radish peroxidase. These labeled antibody fragments were specific for surfactant protein. They found the specific label over the endoplasmic reticulum, Golgi complex, MVB's, and lamellar bodies of type II cells in electron micrographs.

Chevalier and Collet (1972 - from Massaro and Massaro, 1976) demonstrated that lipid was apparently synthesized in the RER before being transported to lamellar bodies. They found that grains from radioactive choline were first found over RER, then apparently "transferred through the Golgi apparatus into lamellar bodies."

Two lines of evidence have been introduced to support the hypothesis that protein and lipid pass through the Golgi on their way from the ER to the lamellar bodies. Sueishi et al. (1977) found that labeled antibody fragments to surfactant protein were found over the Golgi complex, as well as the ER, MVB's, and lamellar bodies of type II cells. Chevalier and Collet (1972 - from Massaro and Massaro, 1976) demonstrated the apparent transfer of lipid from RER, to the Golgi apparatus, and to the lamellar bodies. Chevalier and Collet (1972) also demonstrated the involvement of the Golgi apparatus in sugar, and protein transport. They traced the path of

tritiated leucine through the Golgi "apparatus" and MVB's to lamellar bodies. They also found that radioactive galactose appeared first over the Golgi apparatus, and then over the lamellar bodies.

The involvement of MVB's in surfactant production has been indicated. Sueishi et al. (1977) and Chevalier and Collet (1972) noted their involvement in protein transport. Sorokin (1967 - from Stratton, 1976) hypothesized that protein material is synthesized on chains of ribosomes attached to membranes of the ER, then the protein material is released into the cisternae of the ER. This material is thought to migrate to the Golgi apparatus where it becomes packaged into a vesicle. The pinched off vesicles then are incorporated into MVB's which may contribute to or become MLB's. Stratton's (1976) work and some work of Douglas et al. (1975) supports the theory that MVB's may become MLB's (see discussion on theories of MLB formation in structure section).

The accepted function of the multilamellar body is that of the storage site for surfactant material in the type II alveolar cell. Bensch et al. (1964 - from Massaro and Massaro, 1976) discovered morphological evidence to support this theory. They obtained electron micrographs showing the apparent release of lamellar contents onto the alveolar surface. Askin and Kuhn (1971 - from Massaro and Massaro, 1976) found that radioactive palmitate became incorporated into lamellar lipids as indicated by the appearance of silver grains concentrated over the MLB's. Recall that Chevalier and Collet (1972) found apparent transport of radioactive choline labeled lipid from RER, to Golgi apparatus, and to the lamella bodies.

As stated previously, protein also appears in the lamellar bodies. Sueishi et al. (1977) found labeled antibody fragments to surfactant protein in the lamellar bodies in electron micrographs. Massaro and Massaro (1972) found an increase in radioactive leucine label in lamellar bodies 40 minutes after pulse labeling. Chevalier and Collet (1972) found apparent lipid and carbohydrate moieties in lamellar bodies as stated earlier. The presence of labeled sugar in lamellar bodies indicates the possible presence of glycoproteins (Massaro and Massaro, 1976). Kikkawa et al. (1970) found possible evidence for MPS in lamellar bodies. Bhattacharyya et al. (1975) isolated 36,000 and 62,000 molecular weight glycoproteins in both lamellar bodies and lung wash material. However, Cledzka-Slotwinska and Maisin (1970) used Thorotrast stain and showed no staining of inclusion bodies of type II cells (see protein section). From the above studies it appears as though protein and carbohydrate may be found in lamellar bodies as well as lipid. (See evidence for type II cell as site of surfactant synthesis for further references to lamellar bodies and indications that they are surfactant storage sites).

A number of investigators have obtained results which indicate a possible role for lamellar bodies in surfactant

synthesis. This is based on the presence of various phospholipid synthesizing enzymes associated with lamellar bodies. However, there does not seem to be agreement between investigators as to the presence or absence of phospholipid synthesis in lamellar bodies. It is difficult, at the present level of understanding, to make definite conclusions from the data on this subject. Table 8 lists various enzymes which may be involved in surfactant lipid synthesis along with references for papers which have looked for their activity in lamellar bodies.

Table 8.

Enzyme	Reference
Phospholipase A	Heath and Jacobson, 1976 Longmore et al., 1979 Garcia et al., 1976
Lyso-PC:lyso-PC acyltransferase	Barańska and Van Golde, 1977
Lyso-PC acyltransferase	Barańska and Van Golde, 1977; Engle and Longmore, 1975 - from Frosolono, 1977
PAPase	Spitzer et al., 1975 - from Mavis et al., 1978; Johnston et al., 1978 Mavis et al., 1978
Phosphatidylglycerophosphate phosphohydrolase	Johnston et al., 1978
Cholinephosphotransferase	Rooney et al., 1975 Barańska and Van Golde, 1977
Glycerolphosphate phosphatidyltransferase	Rooney et al., 1975

Massaro and Massaro (1976) mentioned in their chapter on synthesis, transport, and secretion of macromolecules that some investigators suggested lamellar bodies were actually phagocytic vesicles. (This was also discussed in the structure section with regard to theories on MLB formation). They cited studies which disputed this theory. The limiting membrane of the MLB's and apical plasma membrane of type II cells appears to be different. Kuhn (1968 - from Massaro and Massaro, 1976) found that the limiting membrane of lamellar bodies lacked the layer of 'fuzz' seen over the plasma membrane of type II cells, and he found that the plasma membrane of the type II cells contained alkaline phosphatase activity, but the MLB limiting membrane did not. The lack of 'fuzz' and alkaline phosphatase activity in limiting membranes of the intracellular lamellar bodies as well as in lamellar bodies which "communicated with the extracellular space," was noted. However, membranes

of phagocytic vesicles would probably express enzyme activity before becoming entirely "interiorized" (Massaro and Massaro, 1976). Massaro and Massaro (1976) also stated that the Golgi apparatus, MVB's and lamellar bodies all exhibit similar staining characteristics (i.e. all three organelles display acid phosphatase presence). This suggests an intracellular derivation (from Golgi apparatus) of MLB's (Massaro and Massaro, 1976).

As noted in Figure 12, MLB's appear to be secreted from type II cells. Both Ryan et al. (1975) and Stratton (1977) have discussed lamellar body secretion. The work of Ryan et al. (1975) supports the theory that lamellar bodies are secreted by the process of merocrine secretion. Investigators have found electron micrographs in which the lamellar body limiting membrane appears to be fused with the plasma membrane of the type II cell, and the contents of the lamellar body appear to be extruded into the extracellular space. The article by Massaro and Massaro (1976) cited above and an article by Massaro (1975) discuss various factors which may affect the secretory process. A study by Young and Tierney (1972) also approached the subject of surfactant secretion. These articles will not be covered here.

The biological half-life data of phospholipids in lung wash (see phospholipid composition section) and the degrading forms of surfactant seen by Stratton (1977) indicate that surfactant is degraded and replenished. Goerke (1974) in his review, cited means by which surfactant may leave adult alveoli. They will be noted here, omitting references which he cited for some of these ideas: 1) surfactant may flow up the respiratory tree, as it apparently does in the fetus; 2) alveolar macrophages may remove it (recall Sueishi et al. (1977) brought up this idea); 3) it may be removed by trans-epithelial absorption; and/or 4) it may be degraded in situ by enzymes. He cited other studies indicating that "desorption of lung surfactant from the surface is an irreversible process," and that "degradation products of surfactant components" may re-enter lung tissue lipid and protein pools.

The following discussion will center on phospholipid synthesis as related to surfactant. As has been shown, lipid is the primary component of surfactant. The involvement of protein and carbohydrate as surfactant components will not be covered in any more detail than presented already.

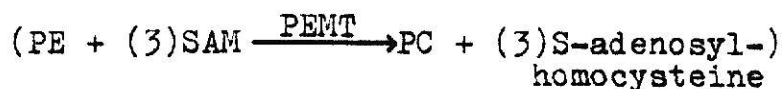
Two of the major phospholipid classes found in surfactant will be discussed at length in terms of their synthesis and less attention will be given to the others. The synthesis of PC and DPPC will be the first topic, followed by the synthesis of PG. Brief attention will be given other phospholipids.

Figure 13 shows two pathways by which PC can be synthesized, and the paths by which glycogen may contribute to PC formation through phosphatidic acid (PA). PC appears to be the most thoroughly studied phospholipid of surfactant, probably

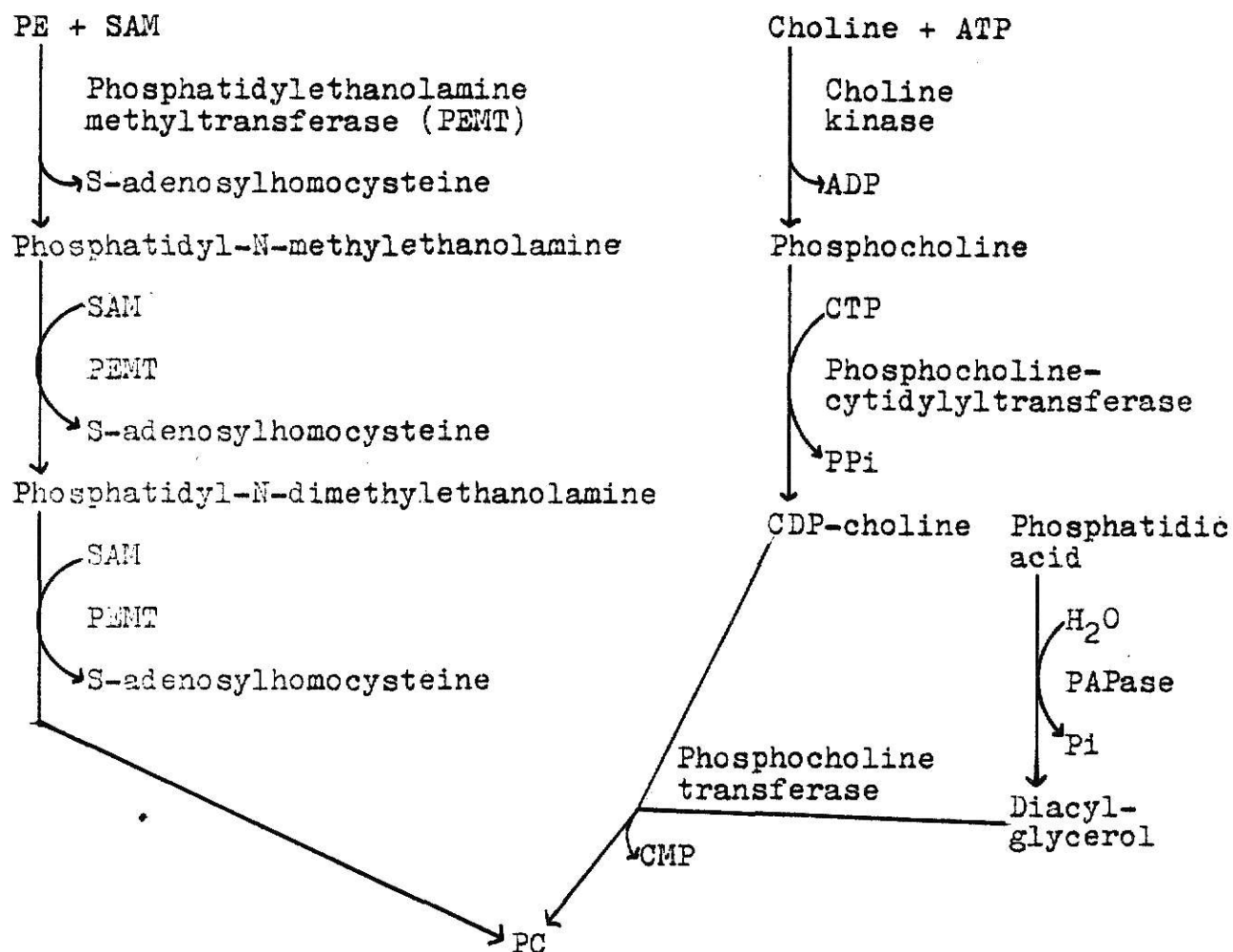
Figure 13.

2 Pathways of PC Synthesis
(Lehninger, 1976, pp. 672-673)

PE = phosphatidylethanolamine
SAM = S-adenosylmethionine
PAPase = phosphatidate phosphatase

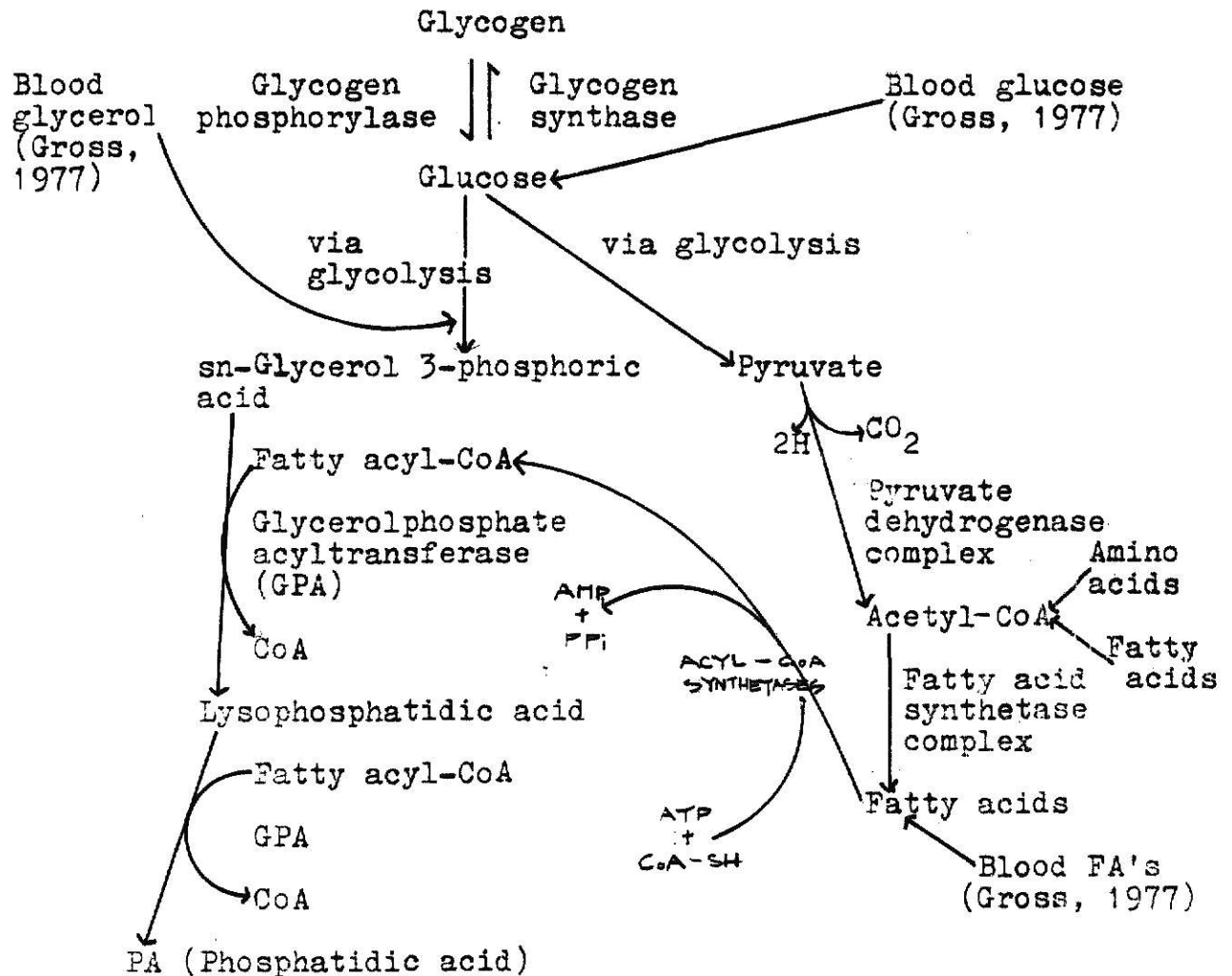


MAJOR PATHWAY OF
DE NOVO PC SYNTHESIS
IN ADULT AND FETAL
LUNG



The CDP-choline pathway is the major pathway of PC synthesis according to refs. cited by Farrell (1977) and Oldenborg and Van Golde (1976). Weinhold (1968) demonstrated the predominance of the CDP-choline pathway in adult and fetal lung using labeled precursors.

Figure 13. (Continued)



Documentation of Steps Outlined on this Page:

- 1) Sequence from sn-glycerol 3-phosphoric acid to diacylglycerol (Lehninger, 1976, pg. 671 - on triacylglycerol formation).
- 2) L-glycerol 3-phosphate derived from dihydroxyacetone phosphate from glycolysis or from free glycerol formed by degradation of triacylglycerols (Lehninger, 1976, pg. 670 - on triacylglycerol formation).
- 3) Acetyl-CoA is the "ultimate precursor" of the fatty acid carbon atoms (Lehninger, 1976, pg. 660).
- 4) Fatty acid synthesis is catalyzed by the fatty acid synthetase complex which uses malonyl-CoA derived from acetyl-CoA (Lehninger, 1976, pp. 660-661).
- 5) Activation of fatty acids to form fatty acyl-CoA (Lehninger, 1976, pg. 546 - on fatty acid oxidation).
- 6) Glucose to acetyl-CoA plus contribution of amino acids and fatty acids to acetyl-CoA (Lehninger, 1976, pg. 445 - on respiration flow sheet).
- 7) Pyruvate dehydrogenase complex catalyzes conversion to acetyl-CoA (Lehninger, 1976, pg. 450 - on TCA cycle).
- 8) Glycogen synthetase and phosphorylase roles in synthesis and breakdown of glycogen, respectively (Lehninger, 1976, pp. 433-436).

due to its relatively high abundance in surfactant. The following discussion will include a comparison of the PC profiles during development and of the profiles for enzymes involved in PC synthesis. This will be broken down into the different animal systems used.

Rooney and co-workers (1976) found marked changes in the PC content of lung wash as compared to lung tissue in the developing rabbit. They followed the PC quantity in two different ways - change in amount and change in percent of total phospholipid (%phospholipid phosphorus). Using the percent of total phospholipid is not a good indicator of changes in synthesis because such a change would be relative to changes in other phospholipid amounts. As shown by the percent of total phospholipid, PC jumped almost 40% from 27 to 31 days' gestation and approximately 13% between 31 days' gestation to 2 days after birth in lung wash. At 2 days after birth, PC comprised 81.5% of total lung wash phospholipid. The amount of PC went from 2.6 to 25.4 μg phospholipid phosphorus/g lung dry wt. from 27 to 31 days' gestation, then jumped to 389 μg phospholipid phosphorus/g lung dry wt. at 2 days after birth. This is a good indication that PC synthesis was indeed increasing. In lung tissue, there was only about a 12% increase in percent composition of PC from 27 days' gestation to 31 days' gestation. PC comprised about 50% of total lung tissue phospholipid at term which was also the adult level of PC according to one of their references. The amount of phospholipid in lung tissue was much greater than that in lung wash, but the level of PC changed very little in lung tissue compared to lung wash. There was only a 2-fold increase in amount of lung tissue PC from 27 days' gestation to 2 days after birth (went from 1.3 - 2.7 mg phospholipid phosphorus/g lung dry wt. during this time - 2.5 mg phospholipid phosphorus/g lung dry wt. = adult level).

Enzyme activities in the rabbit lung do not seem to account for the prenatal rise in lung PC of lung wash or lung tissue, but may account for postnatal increases in PC. Choline kinase (CK) (measured in homogenate) does not appear to play a role in increased PC synthesis because it showed no significant change in activity from 21 days' gestation to adult stage (Rooney et al., 1976). Cholinephosphate cytidyltransferase (CPCT) (measured in soluble homogenate fraction) may have been partially responsible for postnatal increases in PC. CPCT activity decreased between 21 days' gestation and term, but after birth it showed a sharp increase (Rooney et al., 1976). This author could not find a profile on phosphatidate phosphatase (PAPase) for rabbit lung. Cholinephosphotransferase (CPT) (assayed in homogenate) activity may have also contributed to the postnatal increase in PC. CPT activity decreased between 19-31 days' gestation, then increased significantly after birth (Rooney et al., 1976).

The rat lung system displayed some characteristics which were similar to the rabbit lung production of PC. During the latter stages of prenatal and early postnatal development the

rat lung also increased in PC content. Between 19 days' gestation and the first postnatal day the rat lung PC showed a large increase (Maniscalco et al., 1978; Okano and Akino, 1978). Choline incorporation verified the apparent rise in PC synthesis found by these authors who were looking at amount and not percent of PC. The rate of choline incorporation into lung lipids increased between 19 - 22 days' gestation (last day measured) according to Weinhold (1968), and the rate of choline incorporation into PC increased sharply from 20 days' gestation to the first postnatal day according to Maniscalco et al. (1978). Okano and Akino (1978) found that the rate of glycerol incorporation into rat lung PC increased from 19 days' gestation to 2 days after birth. The rate of palmitoyl lyso-PC incorporation increased from 19 days' gestation to 1 day before birth (Okano and Akino, 1978). Weinhold (1968) in trying to discern the major pathway of lung PC synthesis found that incorporation of methionine into PC dropped during late fetal development and levels were very low compared to choline incorporation at all stages of development studied. Thus, the PE methylation pathway of PC synthesis appeared to be minor compared to the CDP-choline pathway.

The increase in amount of rat lung PC seen by Okano and Akino (1978) was apparently due to increased PC synthesis as indicated by the choline, glycerol, and palmitoyl lyso-PC incorporation studies. Here again as in the rabbit studies, there was apparently no enzyme activity which could account for the prenatal rise in PC synthesis, but postnatal increases could be accounted for. As was found in the rabbit, there was little change in CK activity during late fetal and early postnatal development (Maniscalco et al., 1978). The data of Maniscalco et al. (1978) seen in Table IV from that paper indicate that some significant peaks and drops do occur in the last 5 fetal days. CPCT activity changed little in late fetal development, but increased 5-fold after birth (Maniscalco et al., 1978). This agrees with the increase in CPCT activity seen in the rabbit, which may partially accelerate PC production seen by Okano and Akino (1978). PAPase activity may also contribute to the postnatal rise in rat lung PC. Maniscalco et al. (1978) and Ravinuthala et al. (1978) found little change in PAPase activity during late fetal development, but obtained sharp increases in activity after birth. Contrary to activity profiles in rabbit lung, CPT showed few developmental changes in activity (Maniscalco et al., 1978) with no statistically significant rise in activity after birth.

This author has not found a phospholipid profile for mouse lung PC during development. However, Oldenberg and Van Golde (1977) have come up with some enzyme profiles which were similar to those in the rat and rabbit. They found that there was no significant change in activity during the last 4 days of gestation and only a very gradual drop in activity during the first 3 postnatal days for CK. This agrees with the results in rabbit and with the Maniscalco et al. (1978) interpretation of their results in rat. CPCT in the cytosolic fraction of mouse lung increased sharply during the last 2 fetal days and reached a maximum at 1 day prior to birth, and

Table IV (from Maniscalco et al., 1978)

TABLE IV

ACTIVITIES OF ENZYMES OF PHOSPHOLIPID SYNTHESIS IN FETAL, NEWBORN AND ADULT RAT LUNG

The data are the means $\pm$ S.E. from the number of experiments indicated in parentheses.

Age (days)	pmol/min per mg protein			nmol/min per mg protein
	Choline kinase	Cholinephosphate cytidyltransferase	Choline- phosphotransferase	Phosphatidate phosphatase
Fetal				
18	734 $\pm$ 93 (3)	264 $\pm$ 43 (3)	469 $\pm$ 53 (3)	6.5 $\pm$ 0.3 (3)
19	901 $\pm$ 30 (3)	227 $\pm$ 27 (2)	627 $\pm$ 40 (3)	6.5 $\pm$ 0.4 (3)
20	614 $\pm$ 27 (3)	276 $\pm$ 19 (3)	585 $\pm$ 32 (3)	6.9 $\pm$ 1.0 (3)
21	760 $\pm$ 24 (3)	433 $\pm$ 40 (3)	719 $\pm$ 99 (3)	7.0 $\pm$ 0.4 (3)
22	519 $\pm$ 73 (3)	301 $\pm$ 13 (3)	679 $\pm$ 78 (3)	6.6 $\pm$ 0.6 (3)
Postnatal				
1	525 $\pm$ 50 (4)	1017 $\pm$ 45 (3)	692 $\pm$ 32 (4)	10.4 $\pm$ 0.6 (4)
2	597 $\pm$ 22 (4)	1506 $\pm$ 123 (3)	738 $\pm$ 55 (4)	9.9 $\pm$ 0.2 (4)
3	610 $\pm$ 21 (4)	1512 $\pm$ 70 (3)	601 $\pm$ 53 (4)	10.2 $\pm$ 0.8 (4)
Adult	660 $\pm$ 31 (3)	684 $\pm$ 45 (3)	981 $\pm$ 126 (3)	3.6 $\pm$ 0.3 (3)

remained at that level until 3 days after birth (Oldenberg and Van Golde, 1977). Microsomal CPCT gave a similar profile to the cytosolic enzyme but the increase did not occur until one day before birth (Oldenberg and Van Golde, 1977). In the rat and rabbit studies the increase in CPCT activity did not occur until after birth. This discrepancy may be due to differences in developmental states of the lungs at birth in different species. No PAPase profile was available. CPT activity increased between 2 and 3 days before birth, and peaked at 2 days before birth. After that CPT activity dropped to a low at 1 day before birth, then gradually increased (Oldenberg and Van Golde, 1977). This increase in CPT may correspond to the same increase in rabbit lung development which occurred after birth.

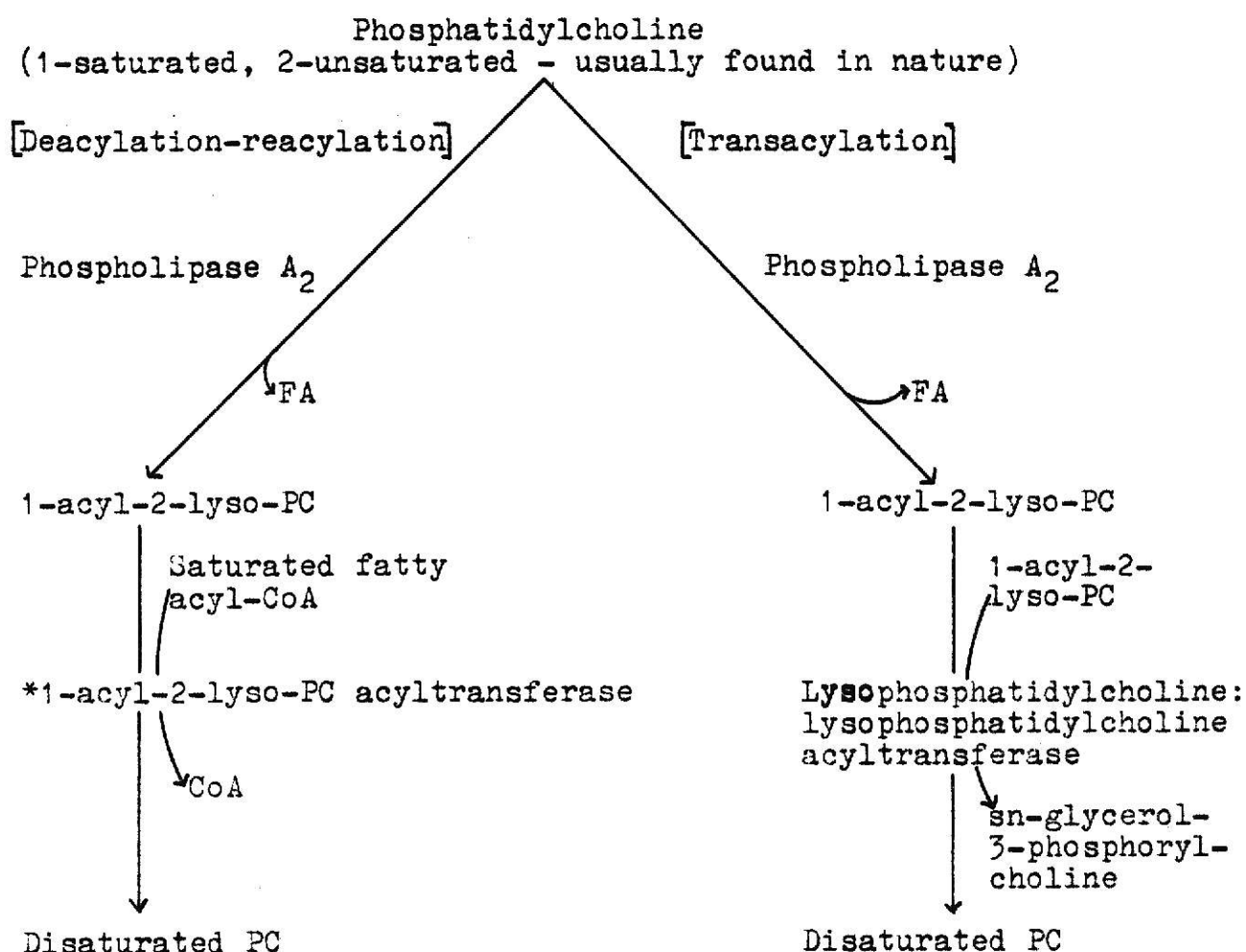
From the above studies on the rat, rabbit, and mouse, it is possible that 3 different enzymes of the CDP-choline pathway of PC synthesis may be responsible for developmental increases in PC content. In all 3 animal systems CPCT showed a significant increase in activity during late fetal or early postnatal development. This enzyme may be a regulatory enzyme of this pathway. Although PAPase was not studied in all three animal systems, it appeared that this enzyme may also play a role in the regulation and/or synthesis of PC. CPT activity has been a source of controversy in the literature because apparently, some investigators used less than optimal substrates to determine its activity. According to the rabbit and mouse studies this enzyme may be involved in increases in PC synthesis during late fetal and/or early postnatal development. Maniscalco et al. (1978) had such a high standard error of the mean for several of their data points that it was difficult to make solid conclusions unless one assumed there was little statistically significant difference between activity at different time points which was apparently what Maniscalco et al. (1978) did.

Phosphatidylcholine is a term which describes a multitude of choline phosphoglycerides having various fatty acids attached to the 1- and 2-positions of the glycerol backbone. In general, the 1-position contains a saturated fatty acid while the 2-position contains an unsaturated fatty acid. However, in the lung there is a unique amplification of the disaturated species of PC - particularly the dipalmitoyl PC species. Figure 14 depicts two different pathways by which a disaturated molecule of PC may be synthesized. Other pathways or mechanisms which may account for the selective production of dipalmitoyl PC will be presented later.

Before discussing the activity of different enzymes in the production of disaturated PC (DSPC), evidence for an increase in DSPC during development will be covered. Maniscalco et al. (1978), using the rat, demonstrated a 100% increase in pulmonary DSPC from days 19 to 22 of gestation with an additional 41% increase from term to the first postnatal day. DSPC went from 37% to 46% of total PC between the 19th day of gestation to the first postnatal day. Additional support for the apparent increase in DSPC synthesis came from choline incorporation studies. The pattern of choline incorporation into DSPC followed the same pattern as incorporation into total PC only it occurred at a much lower level. The rate of choline incorporation into DSPC increased sharply beginning at 20 days' gestation, then peaked on the first postnatal day (Maniscalco et al., 1978). Funkhouser and Hughes (1978) studied DSPC in two different lung states - lung "homogenate" and lung organ culture. The rat lung tissue was "disrupted in distilled water" with an ultrasonic device, not truly homogenized in the sense of using a "pestle." Lung "homogenate" from 15 to 20 day old fetal lungs showed a gradual drop in DSPC expressed per mg DNA. From 20 to 22 days' gestation, there was a sharp amplification in DSPC content (Funkhouser and Hughes, 1978). This is in agreement with the findings of Maniscalco et al. (1978) who followed DSPC content and choline incorporation. The only difference is that Maniscalco et al. (1978) noted an increase in DSPC content by 19 days' gestation rather than by day 20 (Funkhouser and Hughes, 1978). However, Maniscalco et al. (1978) saw only an approximately 0.1 μ g phosphorus/mg protein jump from days 19 to 20 and larger jumps (about 0.3 or more) later. The pattern of DSPC accumulation in organ culture lungs was identical to the in vivo lungs, but it took 1-2 days longer to see a marked increase in vitro (Funkhouser and Hughes, 1978).

As can be seen in Figure 14, there are two generally cited mechanisms for DSPC synthesis. Phospholipase A₂ is common to both mechanisms. Unfortunately, this author was unable to find a developmental profile for this enzyme. However, developmental profiles were available for the two distinct enzymes of the two different pathways. Oldenburg and Van Golde (1976) found that lyso-PC:lyso-PC acyltransferase activity increased in the mouse between 1 and 2 days before birth, with a peak at 1 day before birth. Thus, there is evidence for action of the transacylation pathway in

Figure 14. Formation of Disaturated PC



*Equivalent name: lysolecithin acyltransferase

developmental increases of DSPC. Lyso-PC acyltransferase of the rabbit showed an overall increase in activity between 21 and 31 days' gestation and a continued increase in activity after birth. There were two drops in activity at 28 and 31 days' gestation (Rooney et al., 1976). Therefore, in the rabbit there was support for the deacylation-reacylation pathway of increased DSPC synthesis. It is possible that both pathways may contribute to the "remodelling" of PC to form DSPC. On the other hand, Oldenborg and Van Golde (1976) found that this enzyme did not show significant changes in activity during development in mouse lung microsomes, and its activity remained lower than lyso-PC:lyso-PC acyltransferase throughout development.

Rooney et al. (1976) proposed that lysophosphatidic acid acyltransferase may be involved in DSPC synthesis. They found that its activity profile was similar to that of lyso-PC acyltransferase - with an overall increase in activity from 21-31 days' gestation including a drop in activity at 28 and 31 days' gestation, and continued increase after birth.

Looking at Figure 13, one can see that such an enzyme may attach a saturated fatty acid at the 2-position of lysophosphatidic acid, thereby creating a disaturated phosphatidic acid for use in the CDP-choline pathway. Such a mechanism would negate the absolute dependence on a remodeling process of PC to form DSPC. (However, there appears to be evidence that the de novo CDP-choline pathway of PC synthesis mainly produces 1-saturated, 2-unsaturated PC's (Vereyken et al., 1972; Moriya and Kanoh, 1974; Akino et al., 1971; Sarzala and Van Golde, 1976 - from Longmore et al., 1979)).

Because the discussion at this point has led into DSPC synthesis, the possible mechanisms for DPPC synthesis will be covered. General discussion of surfactant lipid synthesis will continue after this digression. To this author's knowledge, no other species of phospholipid or class of phospholipids in surfactant has been characterized to as great an extent as PC or DPPC in the lung. The mechanisms of PC and DPPC synthesis have been studied in greater detail than other surfactant phospholipids, too.

The uniqueness of the DPPC molecule to the lung and its apparent importance in conferring surface-active properties to lung surfactant have led many investigators to probe for a mechanism(s) of DPPC synthesis. Study of PC fatty acids during development has revealed some selectivity in the type and placement of fatty acids on PC. Palmitate appeared to be a preferred fatty acid of lung PC during late development which was consistent with increases in DPPC synthesis during this time. Oldenburg and Van Golde (1976) found that palmitic acid of PC in mouse lung homogenate showed a greater increase than any other fatty acid from 4 days before birth to one day after birth. During this time period there was a small increase in palmitoleic acid and a decrease in oleic and arachidonic acids (Oldenburg and Van Golde, 1976). Okano and Akino (1978) did extensive characterization of rat lung homogenate PC. Similar to the findings of Oldenburg and Van Golde (1976) palmitate made up the largest percentage of PC fatty acid. There was a rapid rise in the level of palmitate from 3 days before birth to the first postnatal day. Palmitic acid was mainly found at the 1-position of PC throughout the latter stages of development. There was a rise in palmitate at the 1-position from 4 days before birth to the first postnatal day. Palmitic acid was the main fatty acid at the 2-position of PC 2 days before birth through the first postnatal day. There was an increase in palmitate at position 2 over that time period. Oleic acid was just below palmitic acid in percentage of PC fatty acid during the latter stages of development. Oleate peaked 3 days before birth, then rapidly decreased through the first postnatal day. This pattern was similar to that of oleate at position 2 of PC which peaked at 3 days before birth then dropped rapidly through the first postnatal day. This appears to indicate possible hydrolysis of unsaturated FA (oleate) from position 2 with replacement by a saturated fatty acid (palmitate). Coincident with the drop in oleate after 3 days prior to term was a sharp rise in the saturated class of PC after the third day before birth.

Okano and Akino (1978) found a drop in the tetraenoic class of PC's from 4 days prior to birth until the final day before birth, similar to the drop in arachidonic acid seen by Oldenborg and Van Golde (1976). In general, Okano and Akino (1978) found a drop in the unsaturated class of PC's and a rise in the saturated class, during the latter stages of development. The palmitoyl-oleoyl, dipalmitoyl, and palmitoyl-palmitoleoyl species comprised most PC during late development. The dipalmitoyl species of PC began to rise after the second day prior to birth and peaked on the first postnatal day. Incorporation studies using $[2-^3\text{H}]$ glycerol and $1-[1-^{14}\text{C}]$ -palmitoyl lyso-PC into different PC classes (Okano and Akino, 1978) showed that incorporation into the saturated class occurred at a rate below the diene and tetraene classes, respectively, for the different precursors. However, the incorporation of palmitoyl lyso-PC into the saturated class eventually obtained a higher rate than incorporation into tetraenes. This may indicate a higher activity of either the transacylation and/or deacylation-reacylation pathways compared to the de novo pathway in DPPC synthesis. Okano and Akino (1978) showed that glycerol incorporation into the saturated class was highest at 4 days prior to birth (first day sampled) then dropped to a level at 2 days prior to birth which was maintained with little variation until the second postnatal day (developmental sampling ended). Incorporation of palmitoyl lyso-PC into the saturated class increased greatly between 2 and 1 days before birth then dropped less rapidly after birth. Here again, developmental increases in DPPC may be due more to remodeling of PC rather than de novo synthesis.

The selectivity of lung PC synthesis for the dipalmitoyl species may be accounted for in several different ways. No mechanism for DPPC synthesis has been thoroughly tested or accepted. The two main mechanisms of interest include the deacylation-reacylation and deacylation-transacylation theories mentioned earlier in the discussion on enzymes of DPPC synthesis. This author will point out three additional "mechanisms" which may play a role in DPPC synthesis. The breakdown of these theories into a total of five different types is admittedly artificial. Such a division is unlikely in actual cell processes. Other hypotheses may have been made, but the information to follow should cover the significant and some less important mechanisms of DPPC synthesis.

Rather than discussing each of the proposed mechanisms of DPPC synthesis in any detail, a table (Table 9) has been constructed which lists these five possible mechanisms. A brief description of each mechanism is also provided along with references from which additional information may be derived.

After that presentation of mechanisms for DPPC synthesis, attention will now return to one other surfactant phospholipid and its developmental profile. From the information in the lipid composition section it appears

Table 9. Proposed Mechanisms of DPPC Synthesis

Mechanism	References
<u>De novo</u> synthesis of DPPC - <u>De novo</u> PC synthesis is pictured in Figure 13. By this mechanism, the PC's formed via the CDP-choline pathway would be dipalmitoyl species, not requiring any remodeling.	Frosolono, 1977 Oldenborg and Van Golde, 1976 Ravinuthala et al., 1978 Mavis et al., 1978
Deacylation-reacylation - (See Figure 14). By this mechanism, a PC molecule having a palmitic acid at the 1-position and unsaturated FA at the 2-position may have the unsaturated FA replaced by a palmitoyl-CoA.	Rooney et al., 1976 Oldenborg and Van Golde, 1976 Tansey and Frosolono, 1975 Frosolono, 1977
Transacylation - (See Figure 14). Using this mechanism, DPPC may be formed by the transfer of palmitic acid from one lyso-PC molecule to another lyso-PC molecule having palmitic acid at the 1-position.	Oldenborg and Van Golde, 1976 Frosolono, 1977 Okano and Akino, 1978
Direct exchange - Direct exchange of the unsaturated fatty acid at position 2 of 1-palmitoyl-2-unsaturated acyl-PC with palmitate.	Longmore et al., 1979 Kyei-Aboagye et al., 1973
Bihydrogenation - Palmitoyl-palmitoleoyl PC may serve as a precursor to DPPC by bihydrogenation with the onset of breathing.	Okano and Akino, 1978
Note: Multiple fatty acid pools may affect either the transacylation and/or deacylation-reacylation mechanisms (Longmore et al., 1979; Frosolono, 1977; Jobe, 1979).	

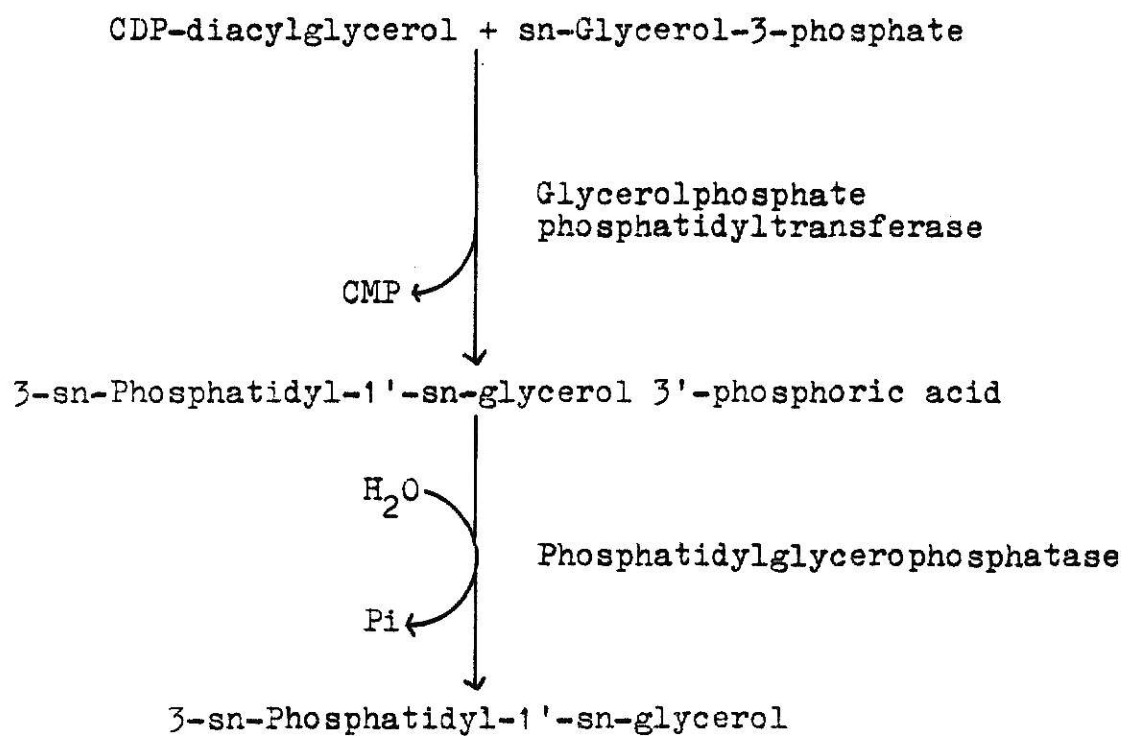
as though PG comprised the second highest quantity of phospholipid in lung wash and lamellar bodies, below that of PC. The percent lipid composition was considerably lower than that of PC, but significantly higher than the other phospholipids of surfactant. Investigators have come to regard this lipid as a good indicator of surfactant lipid synthesis and have assigned it a prominent role in the surface-active properties of surfactant. PE and PI are next highest in percent of total surfactant phospholipid according to the information in the phospholipid section. However, these lipid profiles will not be discussed because this author has not

found a corresponding enzyme profile for them. A comparison of where they fit in to the overall hierarchy of surfactant phospholipids during development will be given later.

The developmental profile of PG for rabbit shows a decrease in percent of total phospholipid in both lung wash and tissue (see Figure 15 for outline of PG synthesis). Lung wash showed a decrease from 29 days' gestation to the second postnatal day. Lung tissue showed a decrease from 28 days' gestation to the first postnatal day, then increased by the second postnatal day (Rooney et al., 1976). It should be kept in mind that these decreases represented a relative percent decrease, not necessarily a decrease in quantity. Also in the rabbit, Hallman (1977) showed that both labeled glycerol and palmitate were rapidly incorporated into PG from 30 days' gestation to the first postnatal day. Thus, in the last 2 days of fetal development there appeared to be an increase in PG synthesis. This correlated well with the results of Okano and Akino (1978) who studied PG synthesis in the rat lung. They found little change in PG from 4 - 2 days before birth, then it gradually increased after that, up to adult levels by the second postnatal day. However according to the results of Rooney et al. (1976), the glycerolphosphate phosphatidyltransferase (see Figure 15) enzyme of the PG synthesis pathway actually decreased in activity throughout the last third of fetal development and first two days of postnatal development in the rabbit. In the rat then, there is an apparent prenatal and early postnatal increase in synthesis of PG (Okano and Akino, 1978). Also, there is apparent increased synthesis in the rabbit PG fraction (Hallman, 1977). However, the enzyme activity as demonstrated in the rabbit (Rooney et al., 1976) cannot account for this. Hallman and Gluck (1977a - from Rooney et al., 1976) found little change in this enzyme's activity during fetal rabbit development, but the activity of the microsomal enzyme increased significantly after birth along with a rise in PG of alveolar wash and lamellar bodies after birth.

It is possible that rat lungs require an earlier onset of PG amplification than rabbits, due to species differences in lung development. Rabbit PG synthesis may remain relatively constant or decrease during late fetal development, then get "turned on" after birth when surfactant begins to be used up and replaced. Rats may simply turn the process on at an early stage in preparation for early postnatal requirements for PG. Hallman and Gluck (1975b) and Kiyasu et al. (1963) (both references from Hallman, 1977) contend that the glycerolphosphate phosphatidyltransferase is the rate limiting enzyme in PG synthesis. It may therefore be helpful to look at the developmental profile of CDP-diacylglycerol and 3-sn-phosphatidyl-1'-sn-glycerol 3'-phosphoric acid as well as this enzyme's activity (see Figure 15) in order to characterize PG synthesis controls more thoroughly. (Presumably sn-glycerol-3-phosphate would be used greatly in PC synthesis as well as PG synthesis and would thus be more difficult to interpret as an indicator of PG synthesis

Figure 15. Phosphatidylglycerol Synthesis
(Lehninger, 1976, pg. 675)



only).

Frosolono (1977) discussed the subject of PG metabolism in his review. His material will not be covered here. However, his review may contain helpful information for the interested reader.

Hallman and Gluck (1975b), concluded that lung PG appears to be synthesized in the endoplasmic reticulum. They calculated total activity for PG synthesis in the endoplasmic reticulum fraction, and its density "closely corresponded to the peak of PG synthesis" from lung homogenate. They concluded that "most extramitochondrial PG synthesis is associated with the ER." In the lung, extramitochondrial PG synthesis was as active as mitochondrial which has not been found in other organ studies according to references cited by Hallman and Gluck (1975b). Additional evidence for their conclusions are as follows: 1) high PG synthetic activity was found in the microsomal fraction, 2) PG synthesizing activity in microsomal fractions obtained in density gradient correlated well with choline phosphotransferase and CDP-diglyceride-inositol phosphatidyltransferase phospholipid synthesizing activities considered to be located in microsomes (which rules out "contamination" with any activity released by mitochondria during isolation), 3) the FA pattern of PG from microsomes resembled that of lamellar bodies but differed from mitochondrial PG, 4) there was a lack of cardiolipin synthesis in microsomes and lack of cardiolipin in surfactant, and 5) they cited a developmental study which has shown a correlation between microsomal activity for PG synthesis and its concentration in surfactant; no correlation between PG synthesis in mitochondria and surfactant was found. Therefore, most if not all surfactant PG is synthesized in the ER and mitochondrial PG synthesis may serve as a cardiolipin precursor (Hallman and Gluck, 1975b).

Hallman and Gluck (1975b) cited several references which demonstrated the significance of PG to surfactant. The points which were made included that PG appears to be tightly bound to surfactant apoprotein, and it appears to be highly surface-active. Also, there is a lack of PG in surfactant phospholipids from newborns with RDS.

Up to this point in the discussion of surfactant synthesis several points have been demonstrated. The relationship between PC, DPPC, and PG synthesis and their respective enzyme activities have been covered. Several mechanisms for DPPC synthesis have been presented. It appears as though most, if not all surfactant phospholipid synthesis occurs in the lung microsomal fraction. More work needs to be done in order to firmly establish type II cell microsomes as the site of surfactant phospholipid synthesis. Thus, the origin and characteristics of the significant surfactant phospholipids has been covered.

Developmental profiles which include some of the less abundant surfactant phospholipids should be considered.

Weinhold and Villee (1965) performed studies on rat lung slices and came up with the relative order of phospholipid synthesis to follow, over a period of 19 to 22 days' gestation. Just looking at the amount of phospholipid, they found: PC>PE>PI>PS>SP, but by day 22 of gestation PI>PS. They also followed ^{32}P incorporation into lung phospholipids over the same prenatal period. They followed the hierarchy relative to the rate of ^{32}P incorporation and found: PC>>PI>PE>SP>PS, but by 22 days' gestation PS>SP>PE. It should be noted that ^{32}P incorporation into an unknown phospholipid was seen at 22 days' gestation, and the unknown was second highest in ^{32}P incorporation at day 22 of gestation and in the adult. This unknown phospholipid may be PG due to the fact that it had the second highest rate of ^{32}P incorporation and the time at which it appeared.

To end this discussion on phospholipid synthesis, the possible role of carbohydrate in lipid synthesis will be presented. According to a review-type article by Gross (1977) "glycogen is probably a major source of carbohydrate (CHO) substrate for phosphatidylcholine (PC) synthesis in the alveolar type II cell." There is no biochemical proof for this, but much evidence to indicate this claim. Glycogen of the terminal airway cells increases during early fetal life as seen in morphological studies. As the type II cells mature, there appears to be a decrease in glycogen content as the production of lamellar bodies begins (Kikkawa et al., 1971 - from Gross, 1977). The glycogen also tended to be "located in close proximity to the lamellar bodies." Gross (1977) cited references which showed that administration of cortisol or thyroxin resulted in an increase in lamellar bodies which was associated with a decrease in type II cell glycogen. From this he (Gross, 1977) proposed that the breakdown of glycogen provides substrate for phospholipid synthesis in the developing type II cell.

Glucose may be derived from glycogen breakdown or from blood circulation and used in type II cell PC synthesis (Gross, 1977). It may form acetyl-CoA for use in FA synthesis (see Figure 13). It may be converted into glycerol-3-phosphate (see Figure 13). Mims and co-workers (1975 - from Gross, 1977) demonstrated that circulating glycerol is rapidly incorporated into lung PC and both the glucose and glycerol pools appeared to be "interchangeable." Felts (1965 - from Gross, 1977) showed that the lack of glucose in the medium somehow caused lung slices to oxidize acetate to CO_2 , but if glucose was present, the acetate was used in phospholipid synthesis. Other nutritional and hormonal effects noted by Gross (1977) were covered in the section on hormones and other agents. Gross (1977) noted in his review that in the presence of insulin, lung explants contained more glycogen and fewer lamellar bodies than controls. Also, insulin "antagonizes" the "glucocorticoid-induced stimulation" of fetal lung PC synthesis (Smith et al., 1975 - from Gross, 1977). Thus, processes which cause storage of glycogen reveal its apparent importance in surfactant synthesis (i.e. lack of glucose decreases lipid synthesis).

Maniscalco et al. (1978) studied possible correlations between lung glycogen content and lipid synthesis. From days 17 to 20 of rat lung gestation, there was a rise in glycogen content. After the peak on the 20th prenatal day, there was a drop until 1 day after birth after which time the glycogen content appeared to level out. Glycogen synthase I (the active form of glycogen synthase) increased in activity by 2-fold from day 17-18 of gestation until day 21 of gestation. Following that, its activity was relatively constant. They found an increase in both the inactive and active forms of glycogen synthase during prenatal lung glycogen accumulation. Glycogen phosphorylase a (the active form of the enzyme) activity began to rise on day 20 of gestation and peaked at 22 days' gestation. After birth, this enzyme's activity dropped. There appeared to be a correlation between the prenatal increase in activity of glycogen phosphorylase a and the prenatal drop in lung glycogen content.

Total phospholipid, including PC and DPFC increased from day 19 of gestation through the first postnatal day (Maniscalco et al., 1978). Thus, the increase in phospholipid content shortly preceded the prenatal drop in glycogen content and increase in glycogen phosphorylase a activity. Glycogen content continued to drop until the first postnatal day which was when phospholipid synthesis began slowing down. By day 21 of gestation, continuing through the first postnatal day there was a sharp rise in choline incorporation into PC and DPFC. Here again it is shown that when glycogen content began to decrease, phospholipid synthesis increased.

Maniscalco and co-workers (1978) suggested three different ways in which glucose may contribute to phospholipid synthesis. (1) It may be incorporated mainly into PC of lung phospholipid as a precursor of the glyceride-glycerol moiety and FA components of phospholipids. They cited references showing that most incorporation is through the glyceride-glycerol moiety, and cited another reference which indicates that triose phosphate intermediates of glycolysis are probably the precursors of the glyceride-glycerol component of PC. (2) Oxidation of glucose 6-phosphate by the hexose mono-phosphate (pentose phosphate) pathway may be a source of NADPH for use in de novo fatty acid synthesis. (Note that they found a sharp postnatal decline in this pathway in rat lung, but could not determine the significance of its developmental pattern). (3) Oxidation of glucose 6-phosphate from fetal stores of lung glycogen may provide energy for phospholipid synthesis and cell differentiation. They stated that glucose plays an important role in PC synthesis of the adult lung and is likely to do so in the fetus.

It appears as though glucose may be a vital component in the synthesis of surfactant phospholipid. The review of Gross (1977) and evidence from the Maniscalco et al. (1978) study supports this view.

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IV. CELLULAR ASPECTS

Throughout most of this report, a rather obvious need for detailed studies using isolated populations of type II cells has been revealed. Much of the information obtained on the surfactant system of the lung has come from studies on lung homogenate and lung wash, with the exception of microscopic studies on intact lung tissue. Morphological and autoradiographic studies point to the alveolar type II cell as the site of surfactant synthesis (see synthesis section). Biochemical studies using hormones or other neurohumoral agents combined with microscopic observation contributes to this theory, also (see section on hormones and other agents). Isolated fractions of lung lamellar bodies (presumably from type II cells) have similarities between their phospholipid composition and that of surfactant (see synthesis section). Thus, the evidence for the type II cell as the site of pulmonary surfactant production has been established, but relatively few studies have been performed on isolated populations of these cells until recently.

In 1974, Kikkawa and Yoneda (from Mason et al., 1977a) published the first method for isolating type II alveolar epithelial cells from the rest of lung cells. According to data presented by Weibel et al. (1976) the type II epithelial cells comprise 14.1% of lung cells. Type I cells make up 8.3% of the lung cell population, and endothelial and interstitial cells make up 39.0% and 38.6%, respectively. Sorokin (1970 - from Mason et al., 1977a) has reported that the lung is comprised of over 40 different cell types. Recognition of lung cell diversity has stimulated a proliferation of studies on isolated type II cells. Investigators have employed the principles of Kikkawa and Yoneda (1974) with some modifications (Mason et al., 1977a).

Because the type II cell appears to be the site of surfactant production, most of the discussion in this section will deal with studies on this cell. However, the presence of other cells in the alveoli (and in other areas of the lung) should not be ignored. In an attempt to gain some perspective on where type II cells "fit" into the alveoli, the development of alveoli will be briefly covered. The presence and significance of one other alveolar cell type in the lung will also be included.

Light and electron microscopic examination reveals that the lung is perfectly designed for its task - that of oxygen uptake and CO₂ release by the blood. Exquisite electron micrographs using thin section, scanning, and freeze-fracture techniques (Weibel et al., 1976) reveal the very thin epithelial barrier between the alveolar space and capillary lumen (see Figure 1 in Weibel et al. (1976)).

In the next few pages, alveolar development as reviewed by Williams (1977) will be outlined. Some discussion on these as well as other lung cells will be covered.

Williams (1977) reviewed the development of rat lung alveoli from 15 days' gestation to 21 days (22 days = term). Only the main points of her paper will be repeated here.

On day 15 of gestation, the "undifferentiated epithelial cell" was observed. According to the review (Williams, 1977), this cell is thought to be a stem cell whose daughter cells "differentiate into at least 12 different epithelial cell types prior to birth." This stem cell "is an elongated columnar cell" with "a large oval or pleomorphic nucleus and a prominent nucleolus." The lateral membranes of the cell are flattened with "few interdigitating processes." The apical surface has few microvilli with fine filaments, and the basal surface is smoothly curved. The primitive epithelium sits on a continuous basement membrane which generally adheres closely to the basal surface. Unbound ribosomes and glycogen granules filled much of the cytoplasm. "RER containing a dense homogeneous material" are occasionally found throughout the cell, and small amounts of SER are found throughout the cell. The Golgi apparatus is apically located and is made up of three to four flattened cisternae, often with "large and small coated vesicles, and associated small smooth-surfaced vesicles." Membrane-bound granules which were originally found in the basolateral margins of some cells contained dense material believed to be protein, are found to contain lysosomal enzymes at later stages of development. Golgi cisternae and RER are also shown to have lysosomal enzymes at later gestational stages. Microtubules, apically-located microfilaments, and mitochondria are also present in this cell.

The presumptive alveolar cell underwent few changes in its organelles from 15-19 days. From days 15-19 of gestation, there was a significant accumulation of glycogen in the epithelial cells which underwent a twofold increase per cell from 17-19 days' gestation. Enough glycogen was packed into dense pools located above and below the nucleus to "displace other cell organelles toward the cell margins." On day 16, ciliogenesis had apparently begun due to the presence of fibrogranular deposits in airway cells. Adjacent cells still lacked characteristic paracrystalline granules and abundant SER of the Clara cells, but had domed apices.

By day 19 of gestation, significant changes occur in the alveolar cell. The appearance of MLB's apparently signals the onset of type II cell differentiation. There is some evidence that type II cell enzymes are present prior to day 19, but type II cells cannot be positively identified until lamellar bodies appear. Figure 16 outlines the proposed events leading up to mature MLB formation as noted by Williams (1977). These agree in some ways with the MVB precursor theory given in the structure section. As phospholipid-rich lamellae increased, glycogen deposits decreased. By day 21, the level of glycogen per cell approached the value at day 17, and continued to drop post-natally. There was a marked increase in saturated PC per cell concomitant with glycogen decrease and formation of lamellar

bodies. Earlier differentiation stimulated by glucocorticoids and repair of alveolar epithelium after damage by N-nitroso-N-methylurethane are associated with glycogen loss and rapid accumulation of phospholipid.

According to one reference cited by Williams (1977), lamellar bodies were secreted and may have an acid-phosphatase reactive core delimited by innermost lamellae, within 24 hours after the onset of MLB assembly. Studies show that many secreted MLB's were converted to TMS. The presence of particles may play a role in TMS assembly as indicated by information from Williams (1977). Particle-free lamellar body membranes were continuous with TMS membranes by means of transition membranes. Both the transition and TMS membranes were coated with particles.

By day 21 of gestation, many type II cells have acquired structural characteristics like those of the adult. The mature type II cell has a cell volume of about $300 \mu\text{m}^3$ (Weibel et al., 1976) as well as readily observed lamellar bodies. Other characteristics of mature type II cells include 18-24% of the cytoplasmic volume occupied by lamellar bodies, and a diameter of $0.73 - 1.09 \mu\text{m}$ in mice and an average of $1.1 \mu\text{m}$ in rats (according to references cited by Williams (1977)).

Finally, Williams (1977) made comparisons between keratinocyte and type II cell secretory bodies, and discussed the transition of type II cells to type I (squamous) alveolar cells. Her (Williams, 1977) brief discussion on the keratinocyte will not be covered in this report. However, some points which were made on the transition of type II to type I cells will be included with other studies later on. On days 20-21 type II cells differentiate. According to Weibel et al. (1976), there are fewer type I cells in the rat lung cell population than type II, endothelial, or interstitial cells, but they occupy more volume than each of these other cell types.

Figure 16. Formation of MLB (Williams, 1977)

Membrane-bound dense basal granules present at day 15 shown to contain lysosomal enzymes later in gestation

↓
Trilaminar lamellae (phospholipid bilayers) appear in dense basal granules

↓
MVB's (may have lysosomal enzymes + phospholipid vesicles) appear to fuse with the granules, possibly adding phospholipid to the enlarging granule. (Origin of MVB's in lung and other tissues is not known for certain, but MVB's have similarities to Golgi membranes).

Williams (1977) also discussed the development of the epithelial and endothelial diffusion barriers. However,

this will be left to the reader to pick up from her paper if interested. It is not idrectly pertinent to this report on the surfactant system.

Now that the development of alveolar structure has been covered, the following discussion will concentrate on two epithelial cell types found in alveoli. The type I and type II alveolar epithelial cells are unique to the lung (Sorokin, 1970).

Type I and type II cells form the epithelial lining of alveoli. In the comparison of the abundance of type I, type II, endothelial, and interstitial cells, recall that type I cells were least plentiful (Weibel et al., 1976). However, the average volume of this cell was around 2-3 times that of the other cell types compared (Weibel et al., 1976). Perhaps the most significant feature of the type I cell was its mean luminal surface area. It was $4003 \mu\text{m}^2$ compared to $69 \mu\text{m}^2$ and $995 \mu\text{m}^2$ for the type II and endothelial cells, respectively (Weibel et al., 1976). Thus, the appearance of the alveolar epithelium was that of a layer of flattened type I cells with type II cells interspersed (Evans et al., 1975).

The type I cell does not appear to be a simple squamous cell. It is a complex branched cell, and each branch can form an 'apical' "cytoplasmic plate on either side of the alveolar septum" (Weibel, 1971 - from Weibel et al., 1976). Weibel et al. (1976) stated that their morphometric data indicated that as many as five or more cytoplasmic plates may be commonly formed by one type I cell. They also suggested that species differences undoubtedly exist.

Weibel and co-workers (1976) brought up the main advantages and disadvantages of the unique branched structure of the type I cell. One plus was that the distance between the metabolic region (nuclear region) of the cell and the "peripheral cytoplasmic flaps becomes shorter," which may be important because the flaps have very few organelles. New branches of existing type I cells may be able to repair small defects in the epithelial lining. A disadvantage of this structure may be that it seems to limit the cell's ability to divide (Weibel, 1974 - from Weibel et al., 1976). Evans et al. (1975) and Adamson and Bowden (1975) have demonstrated the inability of this cell to undergo mitosis. Type I cells appear to originate from type II cells during normal development (Adamson and Bowden, 1975) and after damage to the cells (Evans et al., 1975). Bachofen and Weibel (1974 - from Weibel et al., 1976) observed that damage to the type I cell flaps was generally followed by "proliferation of type II cells in form of a cuboidal lining of alveoli." These type II cells presumably form type I cells which will again form attenuated branches of the air-blood barrier. Williams (1977) noted that the type I cell contained little RER and Golgi apparatus, few mitochondria, and lysosomes in the perinuclear region, but many pinocytic vesicles in the thin cytoplasmic plates. She (Williams, 1977) also noted

that the mature type I cell is not believed to be able to form lamellar bodies or produce surfactant, but an article was cited which suggested it may perform "transepithelial movement of substrates" in its pinocytic vesicles.

Evans and co-workers (1975) used NO_2 to damage adult rat lungs in order to observe if there was transformation of type II to type I cells after type II cell proliferation. In this study, rats were exposed to NO_2 for 48 hours, then injected with ^3H -TdR, and sacrificed at various time intervals. Autoradiography was then performed with fixed lung tissue slices to determine which cells were dividing and what cell types resulted from the division.

In general, they found "areas of cuboidal epithelium near the peripheral openings of the terminal bronchioles." After three days of recovery, tissues had returned to normal and remained so up to 14 days after exposure to NO_2 . Labeled cells were found throughout the sections, but more were positioned near openings of the terminal bronchioles.

One hour after label injection, about 87% of the labeled cells were type II cells, around 11% undetermined cells, and 0.8% type I cells. By the first day after injection, about 60% of the labeled cells were type II cells (a 27% decrease), about 36% were undetermined cells (a 25% increase), and around 4% were type I cells (an approximately 3% increase). By the third day after injection, about 30% of the labeled cells were type I cells (a marked increase). The undetermined cells had dropped off to about 15%, and the type II cells had dropped to 55%. The percentages of labeled cell types changed little over the period from 3-14 days. Undetermined cells could not be identified as definitely type I or type II cells.

Labeled type II cells were similar in appearance at all time intervals. Labeled type I cells had a few inclusion bodies and irregular cell surfaces at 2 and 3 days after exposure, but appeared to be normal after that time. The undetermined cells tended to be cuboidal and rested on the basement membrane. These cells did have some inclusions (not typical of lamellar bodies) and microvilli, similar to the type I cell observations at two and three days. Most undetermined cells were similar to type II cells, and a small number appeared to be spreading over the basement membrane like type I cells. Thus, whether the undetermined cell was a type I, type II, or intermediate between these cells was not known.

The labeling pattern and morphological observations indicated that type I cells were derived from type II cells. Along with the decrease in labeled type II cells was an increase in labeled undetermined cells. As the percentage of labeled undetermined cells dropped, there was an increase in labeled type I cells. Thus, the label progressed from the type II cells, through the undetermined population, and into the type I cell. They (Evans et al., 1975) proposed that the

process of transformation from type II cell to type I cell began before the end of the first day after exposure because the percentage of type II cells decreased then remained about the same from 1-14 days.

Not all type II "sister cells" were transformed into type I cells. "If each dividing type II cell contributed one sister cell to the type I cell population and the other remained as a type II cell, the percentage of labeled type II cells after three days (when labeled type I cells appear) would be about one-half of the one-hour value, and the type I cells would increase by a like amount." The decrease in labeled type II cells was less than 50%, resulting in a net increase in the type II cell population following NO₂ exposure. This also showed that not all type II "sister cells" were transformed into type I cells.

Evans and co-workers (1975) suggested that the undetermined cells were intermediate forms in the transformation of type II to type I cells. The passage of label through this population and decrease in label concomitant with an increase in labeled type I cells indicates an intermediate role. Also, the presence of electron-dense inclusions similar to those observed in labeled type I cells at two and three days after NO₂ exposure points to this conclusion. Other articles backing up this point were also cited.

Finally, Evans et al. (1975) concluded that the time required for type II to type I cell transformation was about two days under their conditions. There was a rapid increase in the percentage of labeled type I cells between one and two days after exposure to NO₂, and little change in percentage after that.

Adamson and Bowden (1975) also concluded that the type I epithelial cell was derived from the type II alveolar epithelial cell. Their study was done with developing rat lungs rather than adult lungs. Pregnant rats were injected with ³H-thymidine two hours before being sacrificed. They were sacrificed on each day from 18 days' gestation to term (22 days). Thus, they were following the normal proliferation of lung cells during development.

On day 18 of gestation, the lung showed tubular structure, and the alveoli were lined by only cuboidal cells. These cells contained large amounts of glycogen, few organelles, and no lamellar bodies. This was similar to the observations of Williams (1977) on the 15 day old lung cells. By day 19 there were no significant changes in morphology of the cells, and it appeared as though epithelial cells were predominantly labeled. Endothelial and interstitial cells were also labeled, but bronchi and bronchiole lining cells rarely incorporated the label. At 20 days of gestation, the glycogen content had decreased and lamellar bodies were seen in alveolar epithelium. By day 21, there was some evidence of surfactant secretion, and almost all of the cuboidal epithelial cells were classed as type II based on the presence of lamellar

bodies. Electron microscopy also revealed the presence of "intermediate cells" in the epithelium which contained glycogen and microvilli, plus those cells "were attenuated as they stretched over the capillaries." (Note that Williams (1977) found type I cells containing "residual dense and lamellar bodies" in 21 day fetal rat lungs). The labeling pattern had changed by days 21 and 22. The interstitial cells were predominantly labeled, and label incorporation into epithelial cells was markedly reduced. At day 22, alveoli appeared to be larger, and type I cells were often observed covering new capillaries. No cell divisions were seen in the type I cells, nor was there much thymidine label found in these cells. After birth, there was little incorporation of label into epithelium and much more incorporation into the endothelial and interstitial cells. The epithelial composition after birth resembled that of the adult lung.

The percentage of labeled pulmonary cells (excluding the bronchial epithelium) was highest at day 18 of gestation (the first time point observed), then continued to fall until day 22 at which time it leveled out somewhat. Twelve percent of the cells were labeled on day 18 of gestation and around three percent were labeled at term. From day 18 of gestation until term, the percentage of labeled cells which were cuboidal epithelium went from 39.2% to 9.9%. The squamous (type I) epithelium made up only about one percent of the labeled cells from day 21 of gestation through 10 days after birth. The percentage of labeled cells which were endothelial and interstitial increased overall from day 18 of gestation through term. They also found that only 2.8% of the type I cells incorporated label in the 22 day fetal lung and one day newborn even though the number of these cells was rapidly increasing at that time.

One of the conclusions which Adamson and Bowden (1975) made was that mitosis seemed to drop off as differentiation began. The greatest amount of mitotic activity occurred in the cuboidal lining cells of the alveoli, but when labeling of the epithelial cells dropped, the type II and type I cells began to be recognized.

They also concluded that the type I cell was derived from the type II cell. This was based on the finding that these cells appeared at a time when maximal mitosis had ceased in the epithelium. The low percentage of label taken up by these cells was further evidence. Also, the presence of intermediate cells between type II and type I at the time of type I cell initial appearance added to support of this theory. Some of these same and similar observations were made by Evans et al. (1975). The observation that intermediate cell types were present when no type I cells were present indicates that the direction of transformation is from II to I.

Adamson and Bowden (1975) suggested that the type I cell incorporation of label may not have been due to mitosis of these cells. They did not observe any mitotic figures in

this cell type. Also, "not all cells that incorporate thymidine actually divide." A differentiated cell may produce 'working copies' "of its genes as metabolic DNA" after its last division (Pelc, 1972 - from Adamson and Bowden, 1975). Therefore, thymidine incorporation may not have been due to mitosis in these cells.

It was also proposed that type I cell formation was "influenced by the development of the capillary system." The appearance of type I cells from 21 days gestation on was coincident with increased labeling of capillary endothelium. "Bulging" of new capillaries into the alveoli may play a role in the transformation of type II cells to the attenuated type I cells covering the capillaries.

In summary, the type I cell appears to have a structure suited to its apparent role as an air-blood barrier. It has thin cytoplasmic branches covering capillaries in the alveoli. These branches seem to be devoid of most organelles, except for pinocytotic vesicles. The mature type I cell does not appear to play a role in surfactant secretion because it lacks lamellar bodies. The type I cell is apparently formed by transformation of a type II cell during late gestational development and following injury to lung tissue. Normal development of this cell may be under the influence of capillary growth in some way. These cells do not appear to have any mitotic activity of their own. Intermediate forms of the transforming type II-type I cell have been tentatively identified.

In a review by Mason et al. (1977a) different pieces of evidence for the type II cell as the site of surfactant synthesis and secretion were given. References given by them will not be cited here. The evidence includes: 1) rapid incorporation of lipid precursors by type II cells in vivo and in vitro; 2) DSPC and PG is synthesized by isolated type II cells; 3) apoproteins of surfactant are found in isolated type II cells; 4) DSPC is secreted by isolated type II cells in vitro; 5) lamellar bodies contain surface-active material and MLB's appear in fetal type II cells just before increases of SAM occur in fetal alveolar fluid.

The type II cell of alveolar epithelium is the primary cell of interest in this report. It is the main site of pulmonary surfactant production and secretion as shown earlier in this paper. Not only does this cell secrete surfactant which keeps the alveoli from collapsing, but it appears to be the stem cell from which type I cells are formed. Three other possible functions of this cell are presented by Mason et al. (1977a) in their short review. Type II cells probably become more resistant to some lung injuries after an initial injury. They (Mason et al. (1977a) cited studies in which hyperplasia of type II cells and a change in several enzyme activities in lung homogenate accompanied resistance of some animals to further injury after an initial exposure to sublethal doses of oxygen or

ozone. Secondly, the secretion of enzymes by type II cells was mentioned. Studies were cited in which lysosomal enzymes in lamellar bodies and possibly PAPase were secreted along with MLB lipids. It was postulated that these enzymes played a role in clearing substances from the alveolar surface or possibly alter other lung cell functions. Finally, they suggested that type II cells may regulate the electrolyte composition of the alveolar subphase (the thin aqueous layer between the epithelial cells and the surface film). A study was cited in which frog lung alveolar epithelial cells similar to mammalian type II cells were believed to "secrete chloride against an electrochemical gradient." Possibly mammalian type II cells "secrete or selectively resorb different electrolytes" (Mason et al., 1977a).

This discussion will focus on isolation of type II cells and studies on these isolated populations (with emphasis on their lipid synthetic capabilities). A number of different isolation techniques have been developed as well as a number of different culture methods. Mason et al. (1977a) described the main elements of their isolation procedures pictured in Figure 17. In an earlier paper, Mason et al. (1975) described the isolation of type II cells using the basic technique seen in Figure 17. At that time they (Mason et al., 1975) reported that the fluorescent compound phosphine 3R would cause the "lamellar inclusions of unfixed type II cells in suspension to fluoresce intensely" thus allowing them to be differentiated from other cell types. However, macrophages from newborn lambs and dogs could not be distinguished from type II cells using this stain or the commonly used Papanicolaou stain for light microscopy (Mason et al., 1975). Electron microscopy was required for further identification.

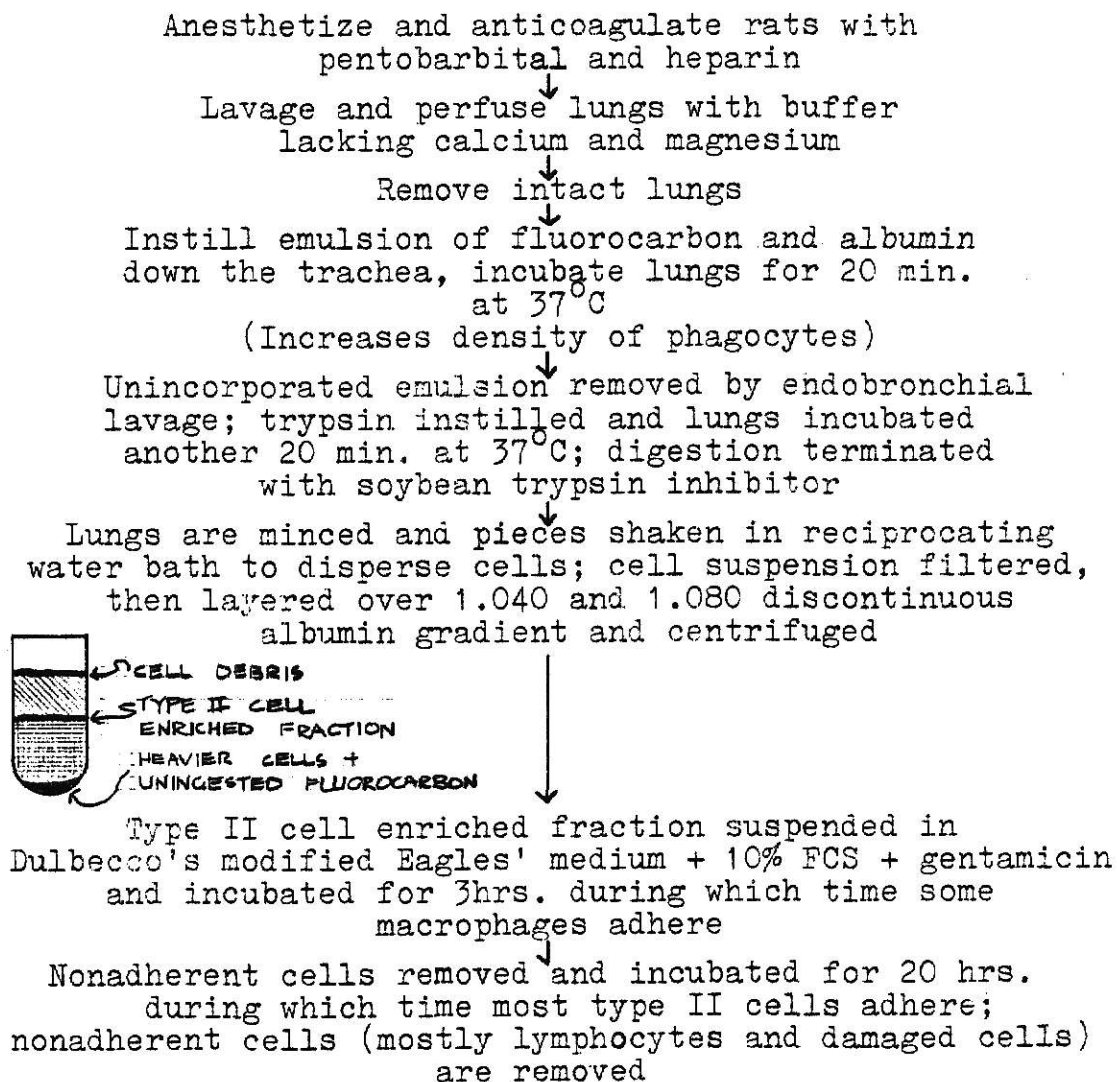
In a later paper, Mason et al. (1977b) performed basically the same isolation procedure as previously described (Mason et al., 1975), only they went into more detail. They obtained the same basic results with their phosphine 3R stain and modified Papanicolaou stain. Electron micrographs of isolated type II cells revealed that lamellar bodies were well preserved but there were areas of dilated endoplasmic reticulum and cytoplasmic vacuoles. Also, most subcellular organelles were well preserved. Mason and co-workers (1977b) determined that yield and purity of type II cell fractions varied between rats which were raised under different conditions. The yield from pathogen-free rats was significantly greater than the yield from other rats. Purity between the three types of rats compared, varied from 60-67% type II cells in isolated fractions. Unit gravity velocity sedimentation (separation based on size) of their isolated fractions was successful in purifying the type II cell fraction. The purest fraction obtained using this method was 89% type II cells. However, these purified fractions were not used for biochemical measurements due to the low yield. They did not use differential adherence in primary culture in this experiment which probably would have increased their purity, but the experiment was on isolated, not cultured cells.

Figure 17. Method of Type II Cell Isolation
(Mason et al., 1977a)

Common elements to all isolation methods:

- 1) Limited digestion of lung with crude or crystalline trypsin
- 2) Increasing density of phagocytic cells with particles such as barium sulfate or emulsions of fluorocarbon or mineral oil
- 3) Purification of type II cells from the mixed population of dispersed cells by density, size, or adherence as examples.

Their method:



Metabolism and composition of isolated type II cells and isolated macrophages were compared by Mason et al. (1977b). Oxygen consumption, glucose utilization, and lactate production were much less in the type II cell when results were expressed in nmole/10⁶ cells/hour. Addition of succinate stimulated O₂ consumption, indicating that mitochondria of the type II cells had "ready access to substrates in the

medium" due to cellular damage (possibly increased permeability of the plasma membrane). They suggested that decreased glucose utilization compared to O_2 consumption by type II cells may have been due to the use of "endogenous substrates of fatty acids in the medium, rather than glucose," and/or trypsin may have impaired glucose oxidation. Type II cells (70% pure) contained significantly greater amounts of phospholipid and DSPC than did macrophages. The macrophages contained a little over twice as much protein as the type II cells. They (Mason et al., 1977b) also found that type II cells could synthesize DSPC from choline, palmitate, glycerol, and acetate. Table 1 from the review by Mason et al. (1977b) provides an overall comparison between the phospholipid composition of isolated type II cells, surfactant, and lung. This table shows that type II cells, freshly isolated or in culture, contain relatively large amounts of PC, PG, and PE. Table 2 from the same review shows the incorporation of acetate and glycerol into the various phospholipids of cultured type II cells. The highest level of acetate and glycerol incorporation was into PC, followed by PG. Over 85% of the incorporated glycerol was found in the "backbone" of the phospholipids and over 85% of the incorporated acetate was found in the acyl portion of the lipids. They (Mason et al., 1977a) cited a study in which a decrease in the percentage of palmitate incorporated in DSPC and PC was associated with a "decrease in the number of inclusion bodies in the cells with time in culture." Batenburg et al. (1978) also studied precursor incorporation into PC of primary type II epithelial cultures. Labeled acetate, palmitate, choline, glycerol, and glucose were all shown to be incorporated into PC containing a high percentage of DSPC by 97% pure type II cells in monolayer culture. The studies performed using these precursors were useful in establishing the normal PC synthetic pattern in these isolated cells for comparisons with other studies, and in deciphering the "normal" as opposed to altered activity of these cells.

In additional studies cited by Mason et al. (1977a), secretion of DSPC by type II cells in primary culture were discussed. According to their results, phorbol myristate acetate and the calcium ionophore A23187 were found to increase DSPC secretion over basal levels. The effect of phorbol myristate acetate was blocked completely at 4°C, and inhibited by 25% with colchicine and vinblastine. This indicated the need for intact microtubules for secretion by these cells and possibly energy (due to low temp. inhibition). (However, secretion in type II cells prepared with trypsin had not been stimulated by any physiologic compounds at that time).

In 1977, Diglio and Kikkawa published what they claimed was the "first report to document accurately the fate of isolated type II cells under monolayer culture conditions." One of the main findings of their morphological study was that primary type II cell cultures could be maintained from 3 to 5 days. After that time the cells deteriorated and lost phenotypic properties. They also found that fetal calf serum was necessary to supplement the media for optimal attachment

Table 1. (from Mason et al., 1977a)

TABLE 1. Percent composition of phospholipids

Lipid	Rat S.A.M.*	Type II Cells		Rat lung ^d	Rat alveolar macrophages ^e
		Acute ^b	Culture ^c		
Origin ^f	0.7	0.3	1.0	1.0	1.2
Phosphatidylserine					
lysophosphatidylcholine	0.2	0.3	1.4	4.8	6.0
Phosphatidylinositol	1.8	0.5	2.0	8.5	3.4
Sphingomyelin	0.3	1.2	1.6	9.8	16.3
Phosphatidylcholine	83.8	71.8	75.0	45.9	49.2
Phosphatidylglycerol	9.3	10.3	6.1	3.3	1.5
Phosphatidylethanolamine	1.9	12.6	10.7	19.1	17.0
Diphosphatidylglycerol					
bis(monooacylglyceryl)phosphate	1.8	1.8	1.4	4.0	3.7
Other	0.2	1.2	0.3	3.1	1.6

* The mean of duplicate analysis of surface-active material (S.A.M.) purified by isopycnic centrifugation.

^b Mean of four preparations of freshly isolated type II cells purified by centrifugal elutriation (purity 90 ± 5%).^c The mean of three preparations of type II cells cultured for 25 hr. ^d Mean of analyses of three differentadult rat lungs. ^e Mean of three different preparations of rat alveolar macrophages obtained by broncho-pulmonary lavage. ^f The lipids were separated by two-dimensional thin-layer chromatography with boric

acid impregnated thin-layer plates (40). This table is taken in part from ref 35.

Table 2. (from Mason et al., 1977a)

TABLE 2. Lipid synthesis in cultured type II cells

Lipid	Acetate	Glycerol
Origin	0.5 ± 0.1	1.1 ± 0.2
Phosphatidylserine		
lysophosphatidylcholine	1.1 ± 0.7	2.4 ± 0.7
Phosphatidylinositol	1.9 ± 0.1	3.6 ± 0.1
Sphingomyelin	2.4 ± 0.3	0.5 ± 0.2
Phosphatidylcholine	76.4 ± 1.4	60.8 ± 1.4
Phosphatidylglycerol	6.0 ± 0.5	17.3 ± 2.3
Phosphatidylethanolamine	4.4 ± 0.5	6.7 ± 0.6
Diphosphatidylglycerol		
bis(monooacylglyceryl)phosphate	0.5 ± 0.1	2.9 ± 0.2
Other	0.4 ± 0.3	0.5 ± 0.8
Neutral lipids	6.4 ± 0.8	4.2 ± 0.9

Type II cells were cultured for 20 hr with added [¹⁴C]acetate (0.1 mM) or [¹⁴C]glycerol (0.1 mM). The cells were washed, and the lipids were extracted and separated by two-dimensional chromatography with boric acid impregnated thin-layer plates (40). The results are the mean ± standard deviation of three experiments (for additional details, see ref 35).

of the type II cells. They hypothesized that the loss in phenotypic character of cultured type II cells may have been due to their transformation into type I cells. It was interesting to note that macrophages could not spread out after adhering to the plastic culture dish, and eventually lost their ability to adhere and never divided under the culture conditions used. Conditioned medium was necessary to obtain "actively dividing macrophages."

The three stages of morphological transition which

Diglio and Kikkawa (1977) described for these cultured cells will be briefly described here. First, the viable type II cells adhered to the culture vessel and had a similar appearance to type II cells of the intact lung during the first 48 hours. Second, from 48 hours to five days the "type II cells actively divided to achieve confluence." A number of morphological changes took place during this stage which included an increase in the number of MVB's and MLB's with vesicles, an increase in "smooth small vesicles" and Golgi complex, and a decrease in the number of lamellar bodies. Third, from 5 to 10 days in culture the type II cells lost their phenotypic character. There was a decrease in Golgi apparatus, MVB's, and MLB's, and an increase in cell volume.

These studies on freshly isolated and cultured type II cell populations have established that type II cells in vitro retain many of the same characteristics of type II cells in vivo. Many of these studies have served to establish the normal behavior of these cells in vitro. With the ground work laid, future studies may employ different hormonal agents as well as other agents in order to derive an in vitro model of surfactant synthesis and secretory regulation.

Two other types of studies on type II cells will be discussed here. Other methods not covered in this section may be called for and briefly described in the proposed research part of this report. Douglas and Teel (1976) have reported an "organotypic" in vitro model system for studying type II cell surfactant production. Cell lines derived from adenomas of the lung and lung adenomas themselves have served as models for in vitro type II cell study, also.

The organotypic in vitro model system used by Douglas and Teel (1976) did not involve a purified population of type II cells, but the structures formed were primarily composed of type II cells. In their experiment, 19 day fetal rat lungs were minced, then treated with a mixture of collagenase, trypsin, and chicken serum. The cell suspension was supplemented with "fetal bovine serum," filtered, pelleted and resuspended twice, then 50 μ l of the cell suspension were placed on a gelatin sponge disc. After attachment of the cells to the sponge, F12K medium was added to the culture disc. The F12K medium was supplemented with fetal bovine serum, penicillin G, kanamycin, and amphotericin. Light and electron microscopy were then used to follow the changes in these cell cultures.

During the first hour after being placed on the discs, the cells attached to the gelatin matrix, "but remained in a monodisperse state." By two days of incubation, the cells had reorganized into alveolar-like structures. They were able to maintain these alveolar-like structures for up to six weeks in culture. Cells which made up these structures contained inclusions which had the appearance of osmiophilic lamellar bodies in type II cells of the intact lung. The osmiophilic lamellar bodies also appeared in the lumen of the

alveolar-like structure, presumably released from the cells making up that structure. Microvilli were present on the apical surface of these cells and intercellular junctional complexes were formed, mainly in the apical regions. Interdigitating folds were also found between cells in the alveolar-like structures. Tubular myelin figures were also seen in the lumen of the alveolar-like structures. The morphological observations above indicate that the cells making up the in vitro alveolar-structure are type II cells. Additional evidence for this conclusion is that cells of the 19 day fetal rat lung do not contain osmiophilic lamellar bodies. Thus, the lamellar bodies observed in their study must have developed in vitro (Douglas and Teel, 1976).

Two additional experimental conditions were tested with this system. During the preparation of cells for addition to the sponge matrix, the cell pellet was incubated for one hour with the supernatant after the second centrifugation. Following this step, the pellet was resuspended and added to the gelatin sponge. Cycloheximide or actinomycin D added to the incubating pellet supernatant material blocked formation of the alveolar-like structures when the cells were added to the matrix. Douglas and Teel (1976) suggested that these protein synthesis inhibitors blocked the resynthesis of membrane receptors which were disrupted during enzyme treatment. They cited a study by Hausman and Moscone (1973) showing that embryonic "cells use plasma membrane receptors" in reaggregation.

Another study with this system showed an 80-fold increase in [³H]choline incorporation into PC with the sponge matrix cultures compared to WI-38 cells plated at the same initial concentration. Therefore, they had preliminary evidence that the cells of the alveolar-like structures were very active in PC synthesis.

Due to the relatively long life of these cultures, they may be extremely valuable in studying the factors regulating surfactant production and secretion (Douglas and Teel, 1976). Comparing the results of this system with observations made by Williams (1977) on the normal rat lung alveolar development reveal some similarities, especially in the 19 day fetal lung. However, her observations place the onset of lamellar body appearance at this day, but Douglas and Teel (1976) suggest it occurs later. One thing which puzzles this author is the lack of type I cells in the sponge matrix system after prolonged incubation. Perhaps the stimulus for type II cell surfactant synthesis is different from that causing these cells to become type I cells, thus type II cells produced surfactant but were not transformed. The agent causing type II cells to differentiate further by producing surfactant may have entered the cell before it was removed from its in vivo environment, but the signal for transformation into a type I cell may not have been received by that time. This is all highly speculative and it assumes that an external stimulus is necessary for these cellular changes. It also assumes that type II cells are the source

of type I cells. If such an external stimulus for type II cell surfactant production is required, it is quite possible that the fetal calf serum may have contained the hormone(s) or other agent providing the stimulus. Another possibility is that the type II cell becomes "committed" at a certain point in development to continue through a certain pattern of development implying a directly genetic control rather than hormonal or a combination of hormonal and genetic control.

In addition to the organotypic culture of lung cells, cell lines from adenomas, adenomas themselves, and a clonal line of cells have been used as model systems for the study of type II cells. Cell line A549 is a continuous, transformed cell line "derived from a human adenocarcinoma of the lung" (Shapiro et al., 1978; Smith, 1977). It retains the morphological character of the type II alveolar cell, and has some characteristics which suggest it may retain some of the same functions as the type II cell (Shapiro et al., 1978; Smith, 1977). Urethane induced adenomas as a whole tissue also retain some characteristic functions of the type II cell (Wykle et al., 1977). A clonal cell line (L-2) derived from type II cells is useful in that it retains morphological features of the type II cell for as many as six passages (Douglas and Kaighn, 1974 - from Smith, 1977; Douglas et al., 1976). It also has some biochemical characteristics of the type II cell.

A few additional characteristics of the clonal (L-2) cell line will be covered, then the A549 cell line and urethane induced adenoma will be discussed. The L-2 cells appear to be able to actively produce PC in vitro (Douglas et al., 1976). According to Douglas et al. (1976), monolayer cultures derived from the L-2 clones "retain the following differentiated characteristics of type II pneumocytes of intact lung: the presence of osmiophilic lamellar bodies, the presence of surfactant-associated esterase patterns, the biosynthesis of PC via the same pathway utilized in whole lung, the presence of glucocorticoid receptor sites and the presence of peroxisomes."

Smith (1977) has studied a variety of characteristics of the cell line A549. His main goal was to observe saturated PC synthesis and secretion in this cell line. Shapiro et al. (1978) performed a study with similar aims only using some different conditions. Briefly, Smith (1977) observed that when cell growth ceased, the percent of [3 H]choline incorporated into saturated PC was the highest. (FCS was removed from the culture 48 hours prior to cessation of cellular growth). A similar result was obtained for the percent of saturated unlabeled (endogenous) PC when the rate of cell growth ceased. Smith (1977) suggested that during the period of cell proliferation, unsaturated PC was greater than saturated PC synthesis due to a need for structural components. As cell growth slowed down, the PC synthesized was "diverted" to the highly disaturated pool(s) of surfactant phospholipid. Using [3 H]choline and [14 C]S-adenosyl-L-methionine, Smith (1977) found that PC was synthesized mainly by the CDP-choline

pathway in these cells because no ^{14}C was found in either the total or saturated PC fractions. The half-life of labeled, disaturated PC in this cell culture was around 100 hours, which was about 10-fold longer than the in vivo half-life. The corticosteroids, cortisol and cortisone, were tested for their effect on saturated PC synthesis. Cortisol stimulated saturated PC synthesis to a "modest" extent. This effect was inhibited by 11-deoxycortisol ("an antigluco-corticoid"). Cortisone did not stimulate saturated PC synthesis. It was demonstrated that these cells were not able to convert the inactive cortisone to its active, cortisol form. Finally, Smith studied secretory activity of these cells. Acetylcholine, carbachol, and isoproterenol each stimulated secretion of labeled, saturated PC. Atropine inhibited the effects of acetylcholine and carbachol. Propanolol blocked the effect of isoproterenol. Thus, "cholinergic (acetylcholine and carbachol) and adrenergic (isoproterenol) agents directly stimulate release of saturated PC" by the cell line. In cortisol pretreated cells, the effects of acetylcholine and isoproterenol on secretion were enhanced. Atropine still blocked the effects of acetylcholine, and propanolol blocked the effects of isoproterenol in the cortisol "primed" cells.

Shapiro and co-workers (1978) used high cell density cultures ("considered to be the most differentiated or mature" cultures) of A549 cells in most of their studies. At this stage, the number of lamellar bodies in the cells was maximal. They cited a previous study which showed that type II cells isolated from adult rabbit lungs contained a much greater percentage of disaturated PC than the A549 cell line. Lamellar bodies were removed from these cells, and it was determined that they were significantly enriched for DSPC compared to cellular phospholipids (see Table 10 for a comparison). This evidence may infer that the A549 cell line has retained the mechanisms for surfactant production. Shapiro et al. (1978) found that the phase of culture development had an effect on the DSPC content as did Smith (1977). They observed an increase in the percentage of DSPC in the cell culture as it increased in density. They found 45% of the PC was disaturated in the "mature culture."

Shapiro et al. (1978) used the calcium ionophore (A23187) to study secretion in this cell system. Recall that Mason et al. (1977a) reported an increase in DSPC secretion by type II primary cultures treated with this same ionophore and phorbol myristate acetate. Shapiro and associates (1978) found that the calcium ionophore stimulated a 9-fold increase in PC (46% of which was disaturated) secretion. EDTA blocked the stimulation by the ionophore indicating calcium dependence.

To determine whether or not the PC and DSPC synthesis by the A549 cells was unique to that cell system, Shapiro and co-workers (1978) used the mouse fibroblast cell line (B82). The B82 cell line was grown under identical culture conditions to the A549 cells. The mouse cell line produced more PC than the A549 cell line, and both cell types synthesized approximately equal percentages of DSPC. This indicated that

Table 10. (Shapiro et al., 1978)

Lipid Origin	% PC of total phospholipid	% DSPC of total PC
Type II cells (rabbit)	48%	50%
A549 cells	41%	16%
Lamellar bodies of A549 cells	56%	39%

the lipid content and composition of the A549 cell line may not greatly differ from other cell types grown under the same or similar culture conditions. However, even though these cells (A549) did not contain large amounts of DSPC, the amount of DSPC is higher than in other tissues (Mason, 1973 - from Shapiro et al., 1978). Two things which appeared to distinguish these cells from other cells in terms of lipids were the enrichment of lamellar bodies for surfactant phospholipids and the inducement of the A549 cells to secrete large amounts of PC which was 46% disaturated.

Both of the above studies on the A549 cell line indicate that this may be a good model system for studying surfactant production and secretion. These investigations demonstrated active PC and especially DSPC synthesis at maturity, the presence of lamellar bodies enriched for PC, and response to some of the stimuli of secretion used in lung type II cell studies. However, Smith (1977) and Shapiro et al. (1978) emphasized the need for a comparison of their results with other systems such as freshly isolated type II cells and other lung cells cultured under similar conditions to determine the usefulness of this genetically different cell line for type II cell study.

Wykle et al. (1977) studied the synthesis of DPPC synthesis in whole adenomas from the lungs of mice which appeared to be almost entirely made up of type II cells. They cited studies which have already demonstrated that whole adenomas synthesize DPPC, contain lamellar bodies, have active lipid metabolism, have levels of FA synthase and acetyl-CoA carboxylase much higher than in normal lung, and have "key enzymes of lipid synthesis" which appear to be regulated by diet in the same way as their normal counterparts.

Wykle and associates (1977) followed the incorporation of labeled glycerol, palmitate, and acetate into PC of lung type II cell adenomas. Over a four hour time period the incorporation of glycerol into the saturated and monoene molecular species of PC increased around 10% and decreased in the more highly unsaturated species. Fifty-five percent of the total lipid activity was in the PC fraction and 24% of this activity was DSPC.

A multi-labeled glycerol precursor was used to determine the role of glycerol and dihydroxyacetone phosphate (DHAP)

in selection towards saturated and unsaturated species of PC. They found that DPPC was not selectively formed by the DHAP or sn-glycero-3-phosphate pathways.

Labeled acetate and palmitate were traced into the PC fraction after incorporation. Seventy-one percent of the total acetate activity was found in PC, which was 65-73% disaturated. Ninety-four percent of the acetate label in FA's was found in the disaturated species and 90% of the FA's labeled with acetate were in palmitate. Of the total PC labeled with palmitate, 63% was disaturated.

In order to determine which pathway of DSPC synthesis was used (i.e. transacylation, deacylation-reacylation, etc.) the positional distribution of labeled acetate and palmitate was followed in the adenoma and lung minces. In the adenomas and minced lung preparations, the majority of acetate and palmitate labels were found in the 2-position of DSPC. They concluded that these results were best explained by the deacylation-reacylation mechanism. With transacylation or de novo mechanisms one would presumably expect equal labeling of the 1- and 2-positions.

This type of culture (whole adenoma) may be a valid model for type II cell study, but the same cautions mentioned earlier should be exercised in drawing conclusions from this genetically dissimilar (from the type II cell) tissue. Some labeling similarities were found between the adenomas and minced lung.

This section has served to focus on the type II cell and its relationship to other cells in the alveoli. Model systems for study of this cell have also been discussed. The proposed research section of this paper will focus on some areas of in vitro study involving some of these same model systems.

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V. INFLUENCE OF HORMONES AND OTHER FACTORS ON THE SURFACTANT SYSTEM

The previous material presented in this report has outlined the basic components of the surfactant system, its structure, synthesis, and relation to the type II cell. The discussion given here provides examples of attempts to manipulate this system in order to determine how it may be regulated. Corticosteroids have been the most thoroughly tested class of agents, and discussion of this hormone class will comprise the bulk of the material in this section. This class of agents, above all others, appears to have the most dramatic overall effect on lung development and particularly on alveolar maturation and surfactant secretion. A parasympathomimetic agent (Merck, 1968), pilocarpine, has also been used alone and in combination with hydrocortisone (a synthetic corticosteroid), to test its effect on surfactant secretion and alveolar development. Thyroxine influences on surfactant secretion and alveolar development have been studied. Conflicting results have been obtained from thyroxine studies, and in some corticosteroid studies. The effects of insulin, a protein hormone, have been tested. Also, aminophylline, an agent which inhibits cAMP phosphodiesterase, and dibutyryl cAMP itself have been studied for their influence on lung development. Prostaglandin metabolism in the lung has been investigated, and scientists have made attempts to correlate events in prostaglandin biosynthesis or catabolism with events in lung development. Finally, nutritional influences on lung development have been studied. This approach most nearly follows the in vivo state of development, but it is less susceptible to finely controlled biochemical analysis.

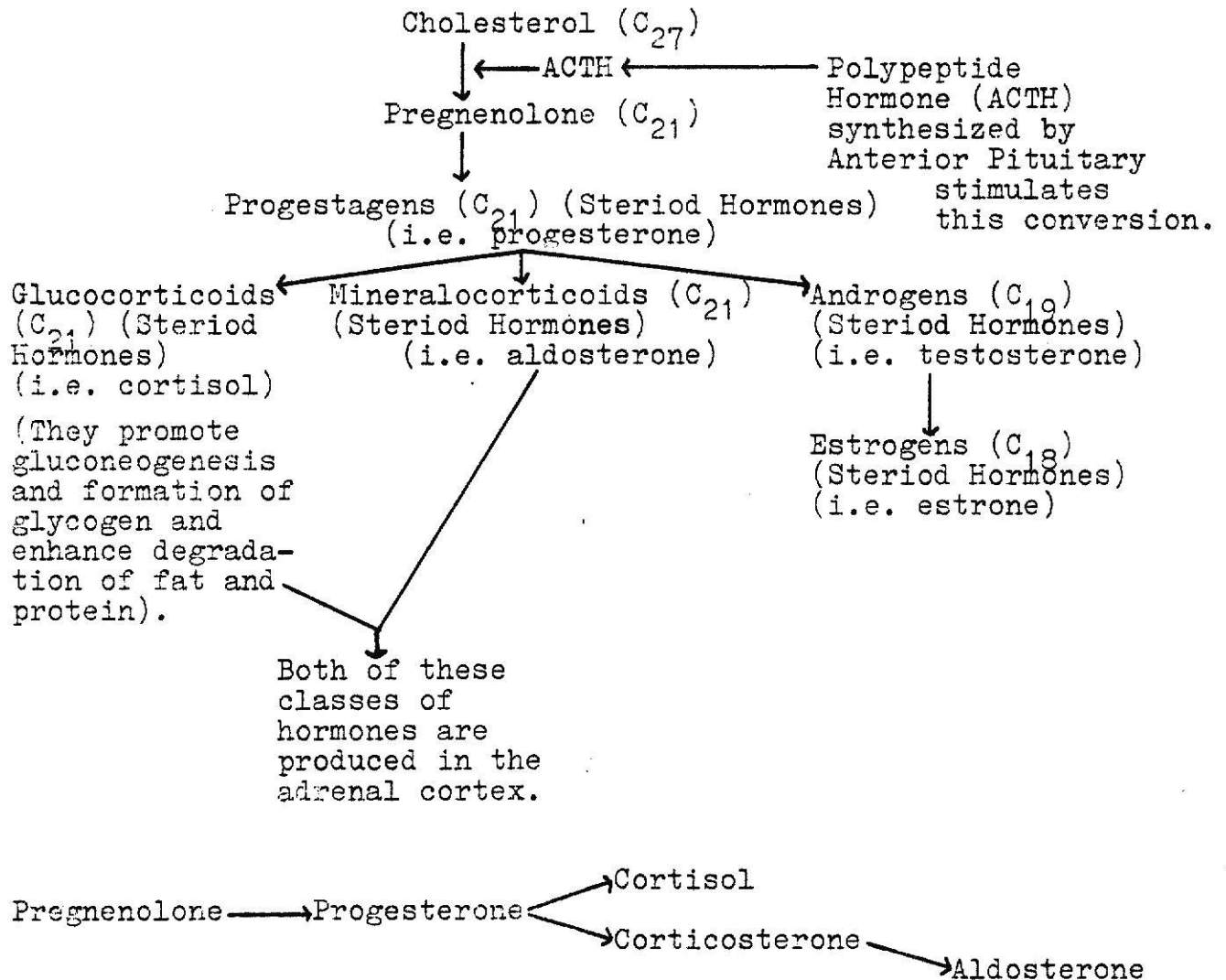
In this section, each of the agents mentioned above will be discussed mainly with reference to their role in alveolar maturation and surfactant secretion. Only brief attention will be given to the biochemistry of these agents themselves.

Corticosteroids have been the most thoroughly tested agent with respect to their role or effects on alveolar development and surfactant secretion. These hormones are derived from cholesterol as are the other major classes of steroid hormones (see Figure 18 after Stryer, 1975).

It was not until the late 1960's and early 1970's that investigators began to show that the fetal lung was a possible target tissue for corticosteroids (Liggins, 1969; Kotas et al., 1971 - from Farrell, 1977). Recent research in this area has led to clinical trials in the antenatal treatment of premature infants with positive results. However, the studies utilizing this hormone have been restricted mainly to smaller mammals such as mice, rats, rabbits, sheep, and monkeys.

In his review, Farrell (1977) stated that there were three basic approaches to the study of glucocorticoid effects

Figure 18. (after Stryer, 1975)



on lung development - anatomical, physiological, and biochemical. The anatomical effects of glucocorticoids are: 1) increased potential air space (Farrell, 1977; Rooney et al., 1975; Kauffman, 1977b; Farrell and Avery, 1975) by attenuation of alveolar cells, narrowing of septae, and greater alveolarization (Farrell, 1977); 2) increased numbers of type II alveolar cells (Farrell, 1977); 3) inhibition of lung cell proliferation (Adamson and Bowden, 1975; Kauffman, 1975; O'Hare and Townes, 1970; Carson et al., 1973 - from Kauffman, 1977b) including a decrease in the number of pulmonary epithelial cells (Kauffman, 1977b); and 4) increase in the proportion of epithelial cells containing osmiophilic inclusion bodies (OIB's) (presumptive type II cells) (Kauffman, 1977b). These effects are of course age dependent (Smith et al., 1974 - from Kauffman, 1977b) and dose dependent (Kauffman, 1977b).

There are three basic physiologic effects of glucocorticoid administration on lung maturation. They are: 1) greater distensibility (Farrell, 1977); 2) greater deflation stability

(Kotas et al., 1971; Kotas and Avery, 1971 - from Farrell, 1977); and 3) "an earlier appearance of surface active material (SAM)" (Farrell, 1977). Kotas et al. (1971; Kotas and Avery, 1971) have demonstrated that rabbit fetal lungs which are treated with glucocorticoid at 24 days of development show greater deflation stability at day 27 than control lungs at the same day. The percent volume remaining at 10 cm pressure ($%V_{10}$) is more than 2-fold greater in the lungs of the 27 day steroid treated animal, as compared to the 27 day control (Farrell, 1977). Kauffman (1977a) has also shown that there was a greater volume density of air space (V_{va}) in fetal mouse lungs exposed transplacentally to dexamethasone. With doses which were not high enough to inhibit body and lung weight, it was shown that dexamethasone accelerated the development of fetal lung air space. She demonstrated a linear increase in V_{va} with increasing doses, from 170% of control with 0.4 $\mu\text{g}/\text{gm}$ to a 530% increase in fetuses exposed to 1.5 $\mu\text{g}/\text{gm}$ of hormone (for fetuses 18 days old). It is important to note that this linear increase was seen for fetuses of greater gestational age. The fetuses sacrificed on day 17 with body weights in the 600 - 650 mg range did not show such a large response to dexamethasone treatment. Kauffman (1977a) also found that there appeared to be a maximum V_{va} at each gestational age tested.

Also, among Kauffman's (1977a) observations is the fact that the mesenchymal component of the lung appears to be more sensitive to hormone treatment than the epithelial component. She found that lower doses of dexamethasone were required to stimulate increases in the V_{va} of 17 and 18 day old mice than to cause an increase in the number of OIB's. She also found that fetuses exposed to 1.6 $\mu\text{g}/\text{gm}$ dexamethasone showed significant increases in V_{va} within 14 hours, but no significant change in the proportion of cells containing OIB's. Thus, the earliest effect of transplacental dexamethasone treatment was an increase in V_{va} . Normally, canal formation and epithelial maturation proceed at the same time (Kauffman, 1977a), but Kikkawa and Motoyama (1973 - from Kauffman, 1977a) showed that development of these two components was not inseparable. Kauffman (1977a) suggested that the faster maturation of canal formation, as compared to epithelial development, may be due to the possibility that mesenchymal cells contain a greater number of corticosteroid receptors. The presence of hormone receptors in lung tissue cells will be discussed later.

Corticosteroids also influence the biochemical development of lung tissues. Rooney et al. (1976) have shown that the total phospholipid from lung lavage in fetal rabbits treated with cortisol was 70% greater than the amount found in control littermates. Following administration of cortisol, the PC concentration more than doubled. The composition of lung wash was also altered by cortisol treatment (see Table I from Rooney et al., 1976). The PC to SP ratio went from 1.2 in control fetuses to 2.1 in treated. However, they found that cortisol administration had no effect on the content or composition of lung tissue phospholipid. This contrasts

Table I (from Rooney et al., 1976)

TABLE I

PHOSPHOLIPID CONTENT AND COMPOSITION OF LUNG LAVAGE FROM CORTISOL-TREATED FETAL RABBITS AND THEIR CONTROL LITTERMATES

The fetuses were injected with cortisol or 0.9% NaCl at 25 days' gestation and delivered at 27 days, as described in Materials and Methods. The data are the means ($\pm$ S.E.) from 4 experiments, in each of which fetuses from 5 litters were pooled. Statistical analysis was by paired *t* test. N.S., not significant.

	Control	Cortisol-treated	Treated/control	P
Phospholipid content (μ g phospholipid phosphorus/ g lung dry wt.)				
Total phospholipid	15.6 $\pm$ 1.7	25.5 $\pm$ 2.5	1.7 $\pm$ 0.1	<0.02
Phosphatidylcholine	5.5 $\pm$ 0.9	12.0 $\pm$ 1.3	2.3 $\pm$ 0.2	<0.05
Composition (% phospholipid phosphorus)				
Phosphatidylcholine	34.6 $\pm$ 2.1	47.0 $\pm$ 1.9	1.4 $\pm$ 0.0	<0.005
Phosphatidylethanolamine	9.0 $\pm$ 0.7	7.2 $\pm$ 0.5	0.8 $\pm$ 0.1	N.S.
Sphingomyelin	30.0 $\pm$ 1.1	22.4 $\pm$ 0.9	0.7 $\pm$ 0.0	<0.005
Phosphatidylinositol + phosphatidylserine	10.7 $\pm$ 1.0	10.5 $\pm$ 1.4	1.0 $\pm$ 0.1	N.S.
Phosphatidylglycerol	2.7 $\pm$ 0.2	1.9 $\pm$ 0.1	0.7 $\pm$ 0.0	<0.005
Lyso-bisphosphatidic acid	3.4 $\pm$ 0.7	2.8 $\pm$ 0.5	0.8 $\pm$ 0.0	<0.05
Lyso-phosphatidylcholine	9.7 $\pm$ 0.3	8.2 $\pm$ 0.3	0.8 $\pm$ 0.0	<0.001
Phosphatidylcholine/ sphingomyelin ratio	1.2 $\pm$ 0.1	2.1 $\pm$ 0.1	1.8 $\pm$ 0.1	<0.005

with results obtained by Farrell and Zachman (1973 - from Farrell, 1977) which showed a much higher value for PC concentration in lung parenchyma from corticosteroid treated fetal rabbits over control levels. Results from Brehier et al. (1977) demonstrate that betamethasone treatment increases the rate of choline incorporation by fetal lungs. This supports the findings mentioned above on the increase in PC synthesis after cortisol administration.

The PC to SP ratio (lecithin to sphingomyelin = L/S) has been shown to increase with increasing lung maturity (Rooney et al., 1976). This ratio has been studied in samples derived from human fetal amniotic fluid. Fencel et al. (1975 - from Peltonen et al., 1977) and Tan et al. (1976 - from Peltonen et al., 1977) have found a positive correlation between the amniotic L/S ratio and cortisol level. However, in a study by Peltonen et al. (1977) they could find no increase in the mean cortisol level in samples taken from groups at 33 - 38 weeks of gestation. This same group showed a sharp increase in the proportion of cases with mature L/S (>2) ratios and cases with raised levels of lecithin bound palmitic acid (LBP) (Peltonen et al., 1977). Thus, there is some controversy over clinical prediction of fetal lung susceptibility to RDS based on cortisol levels in amniotic fluid. The metabolism of amniotic fluid cortisol and the age of the fetus at the time of amniocentesis also play important roles in studies such as those above (Peltonen

et al., 1977).

Because PC is the primary component of pulmonary surfactant, investigators have studied which of the two pathways used in its synthesis is stimulated by corticosteroids. The main pathway by which PC is synthesized appears to be the choline incorporation pathway (Epstein and Farrell, 1975; Farrell et al., 1976; Farrell et al., 1974 - from Farrell, 1977). Farrell and Zachman (1973 - from Farrell, 1977) followed the rate of incorporation of [^{14}C]-choline into PC and the conversion of [^{14}C -methionine] to the final product in lung slices from corticosteroid treated rabbit fetuses. Lungs from the hormone treated fetuses showed about 50% more activity in the choline pathway rates than those of controls. The second pathway of PC synthesis, PE methylation, did not appear to be affected by hormone administration. These results have been supported in experiments with fetal rats (Russell et al., 1974 - from Farrell, 1977), human fetal lung explants (Eklund et al., 1975 - from Farrell, 1977), and in lung cells in tissue culture (Smith and Torday, 1974 - from Farrell, 1977).

Having established that certain end-products of phospholipid biosynthesis show an increase in production in response to corticosteroid treatment, investigators have turned their attention to the enzymes involved in phospholipid biosynthesis. Their studies have demonstrated the fact that corticosteroid treatment does alter the activity of these enzymes. However, investigators do not yet agree on whether or not given enzymes are influenced by corticosteroid treatment.

In Table 11, several enzymes are listed along with their role in phospholipid biosynthesis, and the results of studies on these enzymes are given. Each enzyme will be briefly discussed and comparisons made where possible.

The first enzyme listed in Table 11, phosphatidic acid phosphatase, is an important enzyme in phospholipid synthesis. It is involved in diacylglycerol synthesis which is used in the last step of phosphatidylcholine production and in the synthesis of other phospholipids. A regulatory role for this enzyme has been suggested by Brehier et al. (1977). This enzyme showed an increase in activity in response to hormone treatment. The increase was closely correlated with stimulation of choline incorporation due to corticosteroid treatment. Thus, Brehier et al. (1977) have suggested that PAPase may play a regulatory role by controlling the number of diglycerides available for phospholipid synthesis.

The three enzymes following PAPase in the table are all enzymes of the choline incorporation pathway. As mentioned earlier, this is the major pathway by which PC is synthesized. The first enzyme in the pathway, choline kinase, does not appear to be affected by corticosteroid treatment. In untreated controls the level of this enzyme's activity is about 2.5 times higher, during the final stages of development, than adult levels (Oldenborg and Van Golde, 1977). Choline-

Table 11. Corticosteriod Effects on Enzymes

Enzyme	Role in Lipid Synthesis	Experimental Description	Effects of Hormone Treatment
Phosphatidic acid phosphatase (PAPase) (A)	Catalyzes hydrolysis of phosphatidic acid to diacylglycerol	Celestone Soluspan (betamethasone derivatives) were injected into rabbits at 12, 24, or 48 hrs. prior to sacrifice on day 26 of gestation. Assayed in lung homogenate, lung microsomes, and liver homogenate.	Hormone treatment increased the PAPase activity in lung homogenate and lung microsomes by 12 hrs. and nearly doubled in activity when treated with hormone at 48 + 24 hrs. PAPase activity and choline incorporation closely associated in regard to steroid dose required, time course and extent of increase. PAPase may be regulatory in controlling number of diglycerides.
Choline kinase (B)	Catalyzes the transfer of a phosphate group from ATP to choline to form PC + ADP. A cytosolic enzyme.	Mice were injected on day 14 of pregnancy with dexamethasone. Fetuses ages 15, 16, 17, and 18 were delivered by Caesarean section. Lungs homogenized. Choline kinase assayed in cytosolic fraction.	Control (Normal) - Level of activity remained at a constant level about 2.5 times higher than adult levels of activity during last period of fetal development but dropped after delivery or term of pregnancy. Dexamethasone - No significant change over control levels.

Table 11. (Continued)

Enzyme	Role in Lipid Synthesis	Experimental Description	Effects of Hormone Treatment
(C)		Rabbit fetuses at 25 days gestation were injected with cortisol and amniotic sacs were injected. Fetuses delivered 27 days by C-section. Choline kinase assayed in homogenate.	No change in activity of choline kinase due to hormone treatment. Surgical procedure did cause some decrease in activity when saline injected and non-injected fetuses compared.
Choline-phosphate cytidyl-transferase (B)	Catalyzes formation of CDP-choline from phosphocholine + CTP.	Same procedure as above for ref. (B). This enzyme assayed in cytosol and microsomes.	Control (Normal) - Activity increases sharply about 2 days before term and remains at that level exceeding adult levels until 3 days after birth. Same profile for microsomal enzyme except specific activity increased 1 day before birth. In fetal lung, enzyme about equally distributed between cytosolic and microsomal fractions. Dexamethasone - No significant change over control levels in either microsomal or cytosolic.
(C)		Same procedure as above for ref. (C).	Small but statistically significant increase of activity over control levels. Surgical procedure caused decrease in level of activity when saline injected and non-injected controls were compared.

Table 11. (Continued)

Enzyme	Role in Lipid Synthesis	Experimental Description	Effects of Hormone Treatment
Phospho-choline transferase (A) (C)	Catalyzes formation of PC from CDP-choline + diacylglycerol	Same procedure as for ref. (A). Enzyme studied in lung homogenate. Same procedure as above for ref. (C). Assayed in lung homogenate.	No significant effect of hormone on specific activity of enzyme in lung homogenate. No significant change from control levels. (Changed substrates to see if endogenous or added substrate was being assayed. Did not change results). Surgical effects in this expt. may have masked any increase due to hormone treatment. Procedures cause increase in activity - not decrease as is normal in development.
(B)		Same procedure as above for ref. (B). Measured in microsomal fraction.	Control (Normal) - Sharp increase at 2 days before birth, returned to minimal level at 1 day before term, then began to increase slowly from that level up through adult development. Dexamethasone - Was a significant increase in specific activity on days 17 and 18 of gestation. Saline injection alone caused increase (see ref. (C)). (Agrees with Farrell and Zachman (1973)).

Table 11. (Continued)

Enzyme	Role in Lipid Synthesis	Experimental Description	Effects of Hormone Treatment
(D)		Rabbit fetuses were injected with cortisol at 24 days' gestation and cortisol injected into amniotic sac. At 27 days' gestation fetuses were delivered by C-section and lungs homogenized. This enzyme measured in cell free homogenate.	No significant change from control levels. (used dipalmitin)
Glycerol-phosphate phosphatidyl-transferase (C)	Catalyzes the formation of PG phosphoric acid (a step in PG synthesis) from CDP-diacylglycerol and glycerol-3-phosphate.	Same as described for ref. (C). Enzyme was assayed in lung homogenate.	No change in activity. However, previous studies show 50% increase 3 days after cortisol treatment at (24 days) earlier gestational age (see ref. (D)). In this study they did wait 3 days after hormone treatment and found increase in PG in lung lavage. Agrees with ref. (D).
(A)		Same as described for ref. (A). Assayed in homogenate.	No significant effect on specific activity in lung homogenate.
(D)		Same as described for ref. (D). Activity measured in cell free homogenate.	Enzyme showed approximately 1.5-fold increase in activity over controls.

Table 11. (Continued)

Enzyme	Role in Lipid Synthesis	Experimental Description	Effects of Hormone Treatment
CDP-diglyceride-inositol phosphatidyl-transferase (D)	Catalyzes formation of PI from CDP-diacylglycerol + inositol (Lehninger, 1976).	Same procedure as for ref. (D). Enzyme measured in cell free homogenate.	No significant change from control levels after cortisol treatment.
Lysophosphatidylcholine acyl-transferase (B)	Inserts a FA in 1-acylglycerol-3-phosphocholine at position 2. May be specifically involved in DPPC synthesis.	Same as described for ref. (B). Assayed in microsomal fraction.	Almost 2-fold increase in activity over controls on day 17 and 1.5-fold increase in activity over controls on day 18.
(C)		Same as for ref. (C). Enzyme assayed in the microsomal fraction.	Small significant increase in activity with cortisol treatment.
(D)		Same as for ref. (D). Assayed in microsomal fraction.	No significant change from control levels.
Lysophosphatidylcholine: lysophosphatidylcholine acyl-transferase (B)	Involved in DPPC synthesis. Catalyzes the transesterification between 2 mol of 1-palmitoyl-sn-glycerol-3-phosphocholine.	Same as for ref. (B). Cytosolic assay.	No significant change from control levels.

Table 11. (Continued)

Enzyme	Role in Lipid Synthesis	Experimental Description	Effects of Hormone Treatment
Lysophosphatidic acid acyl-transferase (C)	Inserts a FA on the C-2 position of 1-acylglycerol-3-phosphate. May be specifically involved in DPPC synthesis.	Same procedure as for ref. (C). Enzyme assayed in microsomal fraction.	No significant change from control levels.
(D)		Same procedure as for ref. (D). Assayed in microsomal fraction.	(Same as above).
Acetyl CoA carboxylase + FA synthetase	Acetyl CoA carboxylase catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate. FA synthetase is actually a complex of 7 proteins involved in catalyzing the formation of palmitic acid from 1 acetyl-CoA group plus 7 malonyl-CoA groups. (Both enzymes occur in cytosol). (Lehninger, 1976)	See ref. (D)	Cortisol decreases activity of enzymes of FA synthesis in fetal rabbit lung (Rooney et al., 1974 - from ref. (D)). Thyroxine had no effect on these enzymes.

Refs.

- A - Brechier et al., 1977
 B - Oldenborg and Van Golde, 1977
 C - Rooney et al., 1976
 D - Rooney et al., 1975

phosphate cytidyltransferase, the second enzyme of the choline incorporation pathway, does not have a clearly defined response or lack of response to hormone treatment. The results of Oldenborg and Van Golde (1977) show that this enzyme does not show an increase or decrease from control levels in response to hormone treatment. However, Rooney et al. (1976) assaying fetal rabbit lung homogenates, found a small increase in activity over control levels. Of course, one cannot directly compare the results between these investigations because the experimental systems varied. The last enzyme in the choline incorporation pathway, phosphocholine transferase, has also shown varying results. Three of the experiments cited in the table agreed that there was no effect of corticosteroid treatment on the enzyme's activity. However, those experiments studied the enzyme activity in lung homogenates. Oldenborg and Van Golde (1977) studied phosphocholine transferase in the microsomal fraction of lung homogenates. They found a significant increase in enzyme activity in response to corticosteroid treatment. Their results agreed with those of Farrell and Zachman (1973 - from Farrell, 1977). Attempts have been made to resolve the differences between results showing no change in activity and those showing significant increases. Rooney et al. (1976) found that their surgical manipulation and saline injection caused an almost three-fold increase in activity of this enzyme. Thus, they suggested that the stimulation caused by their procedure may have "masked" any increase in activity due to administration of exogenous hormone. They further suggested that the "stress" of their surgical manipulations may have induced the release of endogenous cortisol which may have in turn "masked" the effects of added hormone. However, Brehier et al. (1977) administered corticosteroid intramuscularly, without any elaborate surgical procedures and they still found no change in phosphocholine transferase activity. Rooney et al. (1976) also tried to explain the differences in activity by using different substrates in the enzyme assay. They suggested that Farrell and Zachman (1973) may have been assaying enzyme activity in response to endogenous diacylglycerol rather than added dipalmitoylglycerol. Rooney et al. (1976) measured the enzyme activity without added substrate in control and cortisol-treated rabbits, in the presence of dipalmitoylglycerol, and in the presence of dioleoylglycerol. No matter which substrate was used, the enzyme activity in the control and cortisol-treated lung homogenates did not differ significantly (see Table IV from Rooney et al. (1976)). Investigators have found that exogenous dipalmitoylglycerol was hardly utilized by phosphocholine transferase (Oldenborg and Van Golde, 1976 - from Oldenborg and Van Golde, 1977) in assay emulsions. Here again the suggestion may be made that the activity of the enzyme toward endogenous diglycerides was being measured. However, looking back at the use of dioleoylglycerol by Rooney et al. (1976) and by Brehier et al. (1977) it can be seen that the activity of the enzyme toward this substrate increased by three-fold over control levels. Using this substrate, both investigations showed that there was still no difference between control levels of

Table IV (from Rooney et al., 1976)

TABLE IV

ACTIVITY OF FETAL RABBIT LUNG CHOLINEPHOSPHOTRANSFERASE TOWARD DIFFERENT DIACYLGLYCEROLS

Experimental details as in Table III. Results are expressed in pmol/min per mg protein.

Added diacylglycerol	n	Control	Cortisol-treated
None	6	51.0 $\pm$ 2.2	50.9 $\pm$ 5.9
1,2-dipalmitoyl-sn-glycerol-3-phosphate	6	65.6 $\pm$ 2.9	65.6 $\pm$ 6.3
1,2-dioleoyl-sn-glycerol-3-phosphate	6	120 $\pm$ 10	108 $\pm$ 9

activity in response to hormone treatment. It has been suggested by Rooney et al. (1975) that the hormone may act by increasing substrate availability. Brehier et al. (1977) suggested that the increase which Farrell and Zachman (1973) observed, may have resulted from an increase in the number of available endogenous diglycerides in response to an increase in PAPase activity. The controversy over these enzymes remains to be resolved.

Because PC is the primary component of pulmonary surfactant, many investigations have focused attention on PC synthesis. However, investigators have studied PG synthesis because it also makes up a significant portion of lung surfactant.

One of the enzymes involved in PG synthesis is glycerol-phosphate phosphatidyltransferase. Here again different studies cited in Table 11 used slightly different time periods. Brehier et al. (1977) injected rabbits with corticosteroid $\frac{1}{2}$, 1, or 2 days before being sacrificed on day 26 of gestation. Rooney et al. (1976) injected rabbits at 25 days of gestation and fetuses were delivered at 27 days. In a different experiment, Rooney et al. (1975) injected rabbits at 24 days and delivered fetuses at 27 days. The first two experiments cited showed no effect of hormone treatment on enzyme activity. However, the third experiment did show an increase in enzyme activity in response to hormone treatment. Rooney et al. (1976) suggested that the length of time following the hormone administration was the critical factor in seeing an increase in activity. They contend that the increase in activity would not be seen unless rabbit fetuses were delivered three days after injection on day 24 rather than delivering them two days after injection on day 24. Support for this theory came from analysis of lung lavage which had an increase in PG three days after hormone injection but no increase in PG after only two days following injection (Rooney et al., 1976). This could explain why the experiment by Brehier et al. (1977) showed no increase in enzyme activity following hormone administration. The specificity of corticosteroid stimulation of this enzyme is interesting to note, also. Rooney et al. (1975) found that cortisol only affected the enzyme activity in the lung and not the liver.

Hallman and Gluck (1975a - from Rooney et al., 1975) reported a decrease in synthesis of PI with the increase in PG synthesis during development. Rooney et al. (1975) demonstrated that the enzyme involved in PI synthesis, CDP-diglyceride-inositol phosphatidyltransferase was unaffected by cortisol treatment. In a later experiment by Rooney et al. (1976) they showed no effect of cortisol on the amount of PS + PI synthesized. A possible reason for the differences in results may be due to differences in sampling periods.

The studies discussed previously have dealt with those enzymes involved in the synthesis of certain classes of phospholipids. Enzymes thought to be involved in the synthesis of dipalmitoylphosphatidylcholine (DPPC), a specific phospholipid molecule which occurs almost entirely in the lung, have also been studied. Results from these experiments were not totally in agreement either.

Lysophosphatidylcholine acyltransferase, one of the enzymes thought to be specifically involved in DPPC synthesis has been shown to increase in activity upon corticosteroid treatment (Oldenborg and Van Golde, 1977; Rooney et al., 1976). Rooney et al. (1975) however assayed the enzyme activity in an earlier experiment and found no change in activity, which may have been due to determining its activity one day after the enzyme had reached its peak. Recall that the first Rooney et al. (1975) experiment cited assayed enzyme activity three days after injection at 24 days, and the second Rooney et al. (1976) experiment assayed enzyme activity two days after cortisol injection at 25 days. It could have been that within two days after hormone administration during this period of development, the enzyme reached its peak activity. Oldenborg and Van Golde (1977) demonstrated an increase in enzyme activity in mouse lungs which came from hormone treated pregnant mice. Direct comparisons between the mouse and rabbit experiments cannot be made, but there does appear to be some response of this enzyme to hormone treatment.

Two other enzymes thought to be involved in DPPC synthesis have been studied. Results from experiments on lysophosphatidylcholine:lysophosphatidylcholine acyltransferase show no response to corticosteroid treatment (Oldenborg and Van Golde, 1977). Results from the two Rooney et al. (1976, 1975) experiments on lysophosphatidic acid acyltransferase showed no response of this enzyme to hormone treatment either.

Rooney et al. (1974 - from Rooney et al., 1975) have demonstrated that cortisol decreases the activity of enzymes involved in fatty acid synthesis in the lung. This effect appears to be specific in that de novo FA synthesis is stimulated in the liver in response to cortisol administration, and the hormone has no effect on brain fatty acid synthesis (Gross et al., 1975a; Rooney et al., 1974 - from Rooney et al., 1975). Even though corticosteroids may inhibit de novo fatty acid synthesis in the lung, they may provide fatty

acids via a different mechanism. Hamosh et al. (1976 - from Farrell, 1977) have demonstrated that lipoprotein lipase activity is stimulated by corticosteroid treatment. Thus, hydrolysis of circulating triglycerides may provide fatty acids for phospholipid synthesis (Farrell, 1977).

Up to this point in the discussion of corticosteroid effects on fetal lung maturity, only biochemical, anatomical, and physiological changes have been described. The maturational effects have been assumed to be a direct result of corticosteroid influence on lung tissue itself. Evidence for the direct "action" of corticosteroid on lung tissue came from studies which demonstrated the presence of corticosteroid receptors in lung tissue (Ballard, 1977; Kauffman 1977a).

Ballard (1977) has reviewed the subject of glucocorticoid receptors. The following discussion comes mainly from his review. Glucocorticoid receptors have been studied mainly in fetal rabbit lungs (Ballard, 1977). These hormones appeared to bind to a single class of proteins (Ballard and Ballard, 1969; Giannopoulos, 1973; Giannopoulos et al., 1972; Toft and Chytil, 1973 - from Ballard, 1977). The specificity of binding has been indicated by the high affinity of binding and relatively low number of binding sites (Ballard, 1977). Glucocorticoid receptors have shown a higher affinity for several synthetic glucocorticoids than they have for the endogenous glucocorticoids, cortisol and corticosterone. The receptors showed little affinity for androgens or estrogens (Ballard, 1977). Glucocorticoid receptors have been shown to appear early in development. Receptors have been detected as early as 18 days in the fetal rabbit (term is 31 days), and as early as 12 weeks of gestation in humans (Ballard, 1977). Ballard (1977) has suggested that because these receptors appear early in gestation their development and presence probably does not play a regulatory role in tissue response to hormone treatment. The receptor site also appears to have some sulphydryl groups. Loss of binding capacity is prevented by the presence of sulphydryl compounds (Ballard, 1977). Binding sites in lung cytosol have been stabilized by the presence of glucocorticoid (Giannopoulos, 1973; Toft and Chytil, 1973; Granberg and Ballard, 1977 - from Ballard, 1977), and dithiothreitol (DTT) did not affect the stability of the receptor-steroid complex (Granberg and Ballard, 1977). In discussing the properties of lung glucocorticoid receptor sites, it is interesting to note that these receptors were inactivated by dephosphorylation in studies on mouse fibroblasts and rat liver preparations (Nielsen et al., 1977). Nielsen et al. (1977) have suggested a phosphorylation-dephosphorylation mechanism which may be responsible for determining the level of active receptors in the cell. Perhaps oxidation of sulphydryl groups and dephosphorylation of receptors or some other cellular component affecting the receptor may be regulatory factors in glucocorticoid effects on cells.

To continue with the discussion of glucocorticoid receptor properties, a study on various adult rat tissues

revealed that the thymus had the highest binding capacity for dexamethasone and lung tissue the next highest binding capacity in the presence of DTT (Ballard, 1977). However, as Ballard (1977) pointed out, these studies were done on whole tissue preparations and may not have provided an accurate picture of the receptor capacity in each type of glucocorticoid target cell. Experiments have been done on different types of lung cells or lung derived cell lines. Using L-2 cells, a cell line that apparently originated from a type II epithelial cell of adult rat lung (Douglas and Kaighn, 1974 - from Ballard, 1977), it has been found that L-2 cells had a higher binding capacity for dexamethasone than cultures enriched for type II cells (see Table 1 from Ballard (1977)). In addition to these experiments, studies have been done on dispersed rat lung cells from adult rats. Results demonstrated that 50-75% of the bound dexamethasone was found in the nuclear fraction when the reaction was carried out at 36°C for 30 minutes. However, when the same experiment was carried out at 2°C, there was little accumulation of nuclear bound hormone even after overnight incubation (Ballard, 1977). Ballard (1977) suggested that the temperature dependence for nuclear transfer may have reflected the "activation" of receptor-steroid complex, a process required for nuclear binding both in vivo and with isolated nuclei in vitro (Cake and Litwack, 1975 - from Ballard, 1977).

The results from experiments discussed in this section clearly support the hypothesis that corticosteroids can have a direct effect on lung tissue development. In the final portion of the discussion on the effects of corticosteroids on lung development, there will be a brief description of the model for glucocorticoid-receptor interaction.

The Figure 1 from Ballard (1977) shows the current model for glucocorticoid-receptor interaction. The initial steps of the model resemble the well characterized estrogen model of steroid-receptor interaction. According to Ballard (1977) studies in other tissues which respond to glucocorticoids have shown that nuclear interaction influences the rate of mRNA synthesis for specific proteins (Cake and Litwack, 1975; Rousseau, 1975 - from Ballard, 1977). Additional studies on the molecular responses of specific types of lung cells, such as the alveolar type II cells, need to be performed in order to support or disprove this model. As noted earlier, the same hormone can elicit different responses in different tissue types. Therefore, once the basics of the model are worked out, refinement must include those controlling factors in the cell which stimulate synthesis of certain phospholipids, inhibit de novo fatty acid synthesis, trigger secretion of surfactant, and etc.

In summary, it appears as though corticosteroids accelerate lung maturation including the development of the surfactant system. The microscopic appearance of lungs from fetuses treated with hormone resemble those of more mature lungs. Anatomical examination includes an increase in the proportion

Table 1. (from Ballard, 1977)

TABLE 1. Binding of dexamethasone by intact cells of rat lung

Cell type	Specific nuclear binding	
	Sites/cell	K_D , nM
Dispersed adult lung cells		
Freshly isolated	7,500	6.9
Cultured 1-2 wk	26,500	7.9
Isolated type II cells (80-90%)	5,700	8.2
Organotypic cultures of fetal lung (90% Type II cells)	13,150	10.2
Cultured L-2 cells	58,200	8.0

Intact cells were incubated in serum-free Hams F-12K or Swim's S-77 medium with various concentrations of [3 H]dexamethasone with or without $3 \mu\text{M}$ nonlabeled dexamethasone for 30 min at 37°C , and specific binding was determined in a nuclear fraction after washing as described in ref 25. The dispersion and culture of lung cells has been reported (25). Isolation of a population enriched for Type II epithelial cells was carried out by R. M. Mason (32), and the L-2 (passage 36-51) and organotypic cells (cultured 1-2 wk) were generously provided by W. H. Douglas (15, 16). Values are the mean of 3-10 experiments.

Figure 1. (from Ballard, 1977)

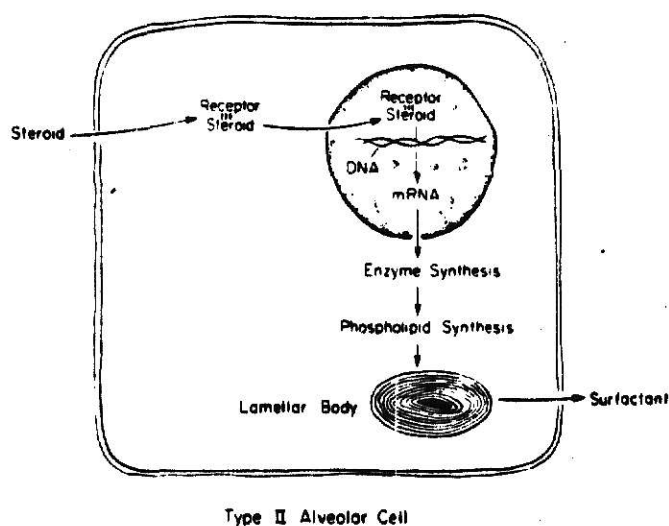


Figure 1. Proposed mechanism of glucocorticoid effects in the type II alveolar epithelial cell. In this model glucocorticoids freely cross the cell membrane to combine with specific receptors in the cytoplasm. The receptor-steroid complex is then translocated to the nucleus where it binds to chromatin, initiating the synthesis of specific messenger RNA's for new protein synthesis in the cytoplasm. The inducible proteins include enzymes involved in the synthesis of phospholipid, which is stored in lamellar bodies and secreted as surface active material into the alveolar space.

of epithelial cells having OIB's. Glucocorticoid treatment also accelerated the appearance of SAM, increased the volume density of air space, and enhanced deflation stability. The presence of certain surfactant phospholipids were also increased with hormone treatment during fetal development. The synthesis of PC via the CDP-choline pathway was increased by corticosteroid treatment of rabbit fetuses. Some enzymes involved in the CDP-choline pathway (PAPase, cholinephosphate cytidyltransferase, and phosphocholine transferase) appear to increase in activity in response to hormone treatment in some investigations. (PAPase also produces diacylglycerols used in synthesis of other phospholipids). Glycerolphosphate phosphatidyltransferase also appears to increase in activity after hormone treatment depending on the timing of the hormone treatment and eventual assay (see text). In the experiment on CDP-diglyceride-inositol phosphatidyltransferase cited in the table (11) cortisol did not appear to affect this enzyme. One enzyme possibly involved in DPPC synthesis (lyso-PC acyltransferase) was stimulated by hormone in some experiments. The activity of enzymes involved in FA synthesis were decreased after hormone treatment, but lipoprotein lipase was apparently stimulated. Finally, glucocorticoid receptors have been found in the lung, and L-2 cells (clonal type II cells) appear to have the highest binding capacity compared to other lung cell preparations.

As was mentioned in the introduction to this section, several agents besides corticosteroids have been studied for their effect on surfactant production or secretion. One agent associated with an apparent increase in secretion of surfactant is pilocarpine. Pilocarpine is a muscarine alkaloid which has been shown to directly stimulate effector cells in a manner similar to that of postganglionic cholinergic nerve stimulation (Koelle, 1975 - from Yoneda, 1977). That is why it can be used as a parasympathomimetic (Merck, 1968). Pilocarpine has been shown to stimulate secretion in the bronchiolar Clara cell (Yoneda, 1977) and in the type II alveolar epithelial cell (Florey et al., 1932; Goldenberg et al., 1969; Morgan and Morgan, 1973 - from Yoneda, 1977). According to Yoneda (1977) pilocarpine stimulation of secretion and secretory processes in the pancreas show similarities to pilocarpine stimulation of Clara cell secretion. It is now thought that the Clara cell secretes protein and not surfactant (Yoneda, 1977). Thus, attention will be focused on evidence that pilocarpine stimulates surfactant secretion in type II alveolar epithelial cells. There is increasing evidence that the type II cell is the site of surfactant production (Goerke, 1974).

In a study by Pysher et al. (1977), the effects of hydrocortisone and pilocarpine on rat fetal lung explants were determined. They found the 19 day old fetal lung explants to be the most responsive to hormone treatment. Therefore, this was the age at which their pilocarpine studies were conducted.

Pysher et al. (1977) analyzed the media for incorporation

of ^{14}C -palmitic acid into PC after hydrocortisone and pilocarpine treatment. Explants were preincubated with 10^{-6}M hydrocortisone for 24 hours, then washed in new media with or without 10^{-6}M pilocarpine. Three hours of incubation were needed before they obtained a significant increase, over control levels, of labeled PC in the culture media. A decrease in labeled explant PC was found over the same time interval. They performed volume proportion studies and differential cell counts on explants primed with hydrocortisone, and treated with pilocarpine for $\frac{1}{2}$ hour. They found no differences between hydrocortisone/pilocarpine treated explants, and those explants treated for 24 hours with hormone only. Because it took three hours before the effects of pilocarpine were noted as an increase in labeled PC in the media, it would have been a good idea to have done morphological examinations of explant tissue at that point. Perhaps $\frac{1}{2}$ hour was not a long enough period of time for pilocarpine to have a detectable effect (Pysher et al., 1977).

Although the labeled PC was not directly traced back to the surface-active layer, there was evidence that surface-active material was released. Pysher et al. (1977) used the foam stability test to measure surfactant. In essence this test involved the dilution of a sample containing surface-active material to see how much the sample could be diluted and still obtain a ring of bubbles greater than 50% of the meniscus after agitation. They found that the media from hydrocortisone/pilocarpine treated explants could be diluted further than controls (hormone treatment only) and still produce positive foam stability tests.

Based upon the evidence presented, 1) pilocarpine stimulates an increase of labeled PC in the culture media; 2) there is a concurrent decrease in labeled PC of explants; and 3) more positive foam stability tests are obtained with pilocarpine, it appears as though pilocarpine stimulates surfactant secretion in lung explants. The mechanism of pilocarpine action is not known. However, it appears to have a nonspecific effect on the process of secretion in that it stimulates secretion in the Clara cell, pancreatic cells, and type II alveolar epithelial cells, all of which secrete different products. Pilocarpine may be useful in studying the mechanism by which surfactant is secreted.

It is important to note the inference made by those pilocarpine studies. Pilocarpine can act as a parasympathomimetic, plus it apparently stimulates secretion in various cell types. One hypothesis which can be drawn from these observations is that secretion, specifically surfactant secretion in this case, may be regulated by effectors of the nervous system such as acetylcholine or perhaps norepinephrine (released by sympathetic postganglionic axons (Anthony and Kolthoff, 1971)). (Recall the study on the A549 cell line in the cellular aspects section which demonstrated an increase in saturated PC secretion in response to cholinergic and adrenergic agents).

Studies on lung tissue and lung cells using various cholinergic and adrenergic agents has led to speculation that perhaps secretory activity of the type II alveolar cell is under sympathetic and/or parasympathetic nerve control. The innervation of the lung has been studied in detail but is beyond the scope of this paper and this author's knowledge. However, a few observations made in the literature do indicate that nerve cells may be involved in regulation of type II cell secretory activity. According to Thurlbeck and Wang (1974) the innervation of "alveolar walls" is less certain than innervation of the airways.

In an abstract published in 1972, Hung et al. reported the presence of different nerve endings on the type I and type II cells. "Nerve terminals on type I pneumocytes are located under the epithelial cell body or its processes in the alveolar duct areas and the alveolar walls." A second "type of nerve ending forms a cell-shaped swelling with its wide base in close contact with the lateral surface of the type II pneumocyte." They postulate that the nerve terminals located "at type I pneumocytes are sensory endings and those at type II are autonomic motor endings."

In a chapter on lung structure (Thurlbeck and Wang, 1974) three studies were reported which "challenged" the view that nerve fibers "could only be seen in alveolar walls which were adjacent to vessels or airways, and that the nerves were supplying the latter structures." The first study showed nerves, thought to be cholinergic and noradrenergic, in relation to alveolar capillaries. The second study reported the presence of nerves in "alveolar walls which were a considerable distance from airways and vessels." These were thought to be sensory fibers. The third study discussed was the one by Hung et al. (1972) cited above. However, it was stated that the "granules in the motor endings described by Hung et al. (1972) are not those associated with cholinergic nerves." Thus, their results were in apparent conflict with a pilocarpine study showing stimulation of MLB secretion (Thurlbeck and Wang, 1974). The presence of nerves has been demonstrated in alveoli, but their activity in regulation of type II cell secretory activity has not been proven.

In addition to corticosteroids, thyroxine is another hormone which has been tested for its effect on surfactant production in the lung. Thyroxine is a tetraiodinated derivative of the amino acid tyrosine. A deficiency or excess of thyroid hormone is responsible for a number of diseases (Lehninger, 1976, pg. 824) including one developmental disorder called cretinism in humans (Filburn and Wyatt, 1974). Thyroid hormones (including thyroxine (L-3,5,3',5'-tetraiodo-thyronine) and L-3,5,3'-triiodothyronine) also influence the development of skeletal and reproductive systems (Filburn and Wyatt, 1974). One of the most well known examples of thyroid hormones' role in development has to do with amphibian metamorphosis (Filburn and Wyatt, 1974; Lehninger, 1976, pp. 825-826). Thyroid hormone also appears to help regulate the

basal metabolic rate of an organism (Lehninger, 1976, pp. 824-825).

The results from thyroxine treatment on lung surfactant production were controversial as was noted in the discussion on corticosteroid studies. In Table 12, three different experiments using thyroxine in studying lung development were outlined. The experiments by Mason et al. (1972) and Redding et al. (1972) differed in their results. Mason et al. (1972) found no difference in the amount of phospholipid or disaturated PC when thyroxine concentration was varied by thyroidectomy or by simple thyroxine injection. In contrast, Redding et al. (1972) found that the amount of lung surfactant increased with exogenous thyroxine treatment, and the surfactant yield decreased when rats had undergone a thyroidectomy. The reason for differences between these two studies may have been due to differences in procedure. Mason et al. (1972) injected rats with 1 mg of thyroxine per kg of body weight for 7 days which was similar to the injection procedure followed by Redding et al. (1972). However, Mason et al. (1972) rinsed the lung with cold saline after removing it, then homogenized the tissue and extracted lipids from the homogenate. Redding et al. (1972) lavaged the lungs after removing them, then separated out the surfactant from the lung wash. This was done by differentially centrifuging the wash fluid at 600g, then ultracentrifugation of the pellet obtained from the differential centrifugation. They obtained one band from their sucrose density gradient which they said was highly surface-active and used it in their analysis. The quantity of surfactant was expressed as mg of lyophilized surfactant per gram of wet weight of fresh lung.

The main difference between the two studies cited above was that one assayed phospholipids from the entire lung, and the other assayed surfactant from lung lavage. Also, the saline rinse of lung tissue by Mason et al. (1972) may have rinsed out surface lipids. Both studies could be improved upon. A better experiment may have been to quantitate total PC and disaturated PC in both lung tissue and lavage fluid in response to thyroxine treatment. Thyroxine could have been injected into the amniotic sacs of fetal rats, then their lungs analyzed for total PC and disaturated PC. Of course thyroxine has been shown to affect several different tissues, so that would have to be considered in analysis of the results. More detailed biochemical analyses of lung phospholipids in response to thyroxine treatment need to be performed before conclusions can be drawn about the influence of thyroxine on surfactant production.

Rooney et al. (1975) have studied the enzymes of phospholipid and fatty acid synthesis in response to thyroxine (see Table 12). They found that some of the enzymes involved in PG, PC, PI, dipalmitoyl PC, or in fatty acid synthesis were not affected by thyroxine treatment. The process of fatty acid elongation was also unaffected. Of course it was possible that these enzymes did not show any response in the

Table 11. Thyroxine Effects

Ref.	Method	Results
A	Expt. 1: 4 groups of rats were tested - 1)sham-operated controls; 2)sham-operated thyroxine treated; 3)thyroidectomized controls; 4)thyroidectomized thyroxine-treated. Two weeks after operation - animals received 7 daily subcutaneous injections of 1 mg of L-thyroxine per kg or an equal vol. of the "vehicle." Expt. 2: 2 groups of rats tested - 1)received thyroxine; 2)received the vehicle using same treatment procedure as in Expt. 1. In both expts. assayed for 1)serum protein bound iodine (PBI), 2)total phospholipid, 3)disaturated PC.	1) Average serum PBI was much greater in animals which were injected with thyroxine. 2) Expt. 1: No significant difference between total phospholipid or disaturated lecithins in comparing sham operated controls and sham operated thyroxine-treated rats, or in comparing thyroidectomy control and thyroxine-treated rats. 3) Expt. 2: No significant difference between total phospholipid or disaturated lecithins in comparing nonoperated rats which received thyroxine or did not receive it.
B	5 groups of rats were tested: 1)hyperthyroid (received 1 mg of L-thyroxine (T4)/kg for 6 days), 2)hyperthyroid (received 1 mg of T4/kg for 15 days), 3)littermates of group 1 injected with saline only; littermates of group 2 injected with saline only, 4)hypothyroid rats killed 6-8 wks. following thyroidectomy - 5)these were compared with sham operated littermates. (Continued)	Morphological: 1) Only type II alveolar cells showed marked changes in response to increasing thyroxine concentrations. 2) In lungs from hyperthyroid rats, type II cell diameter was larger, no. of lamellar bodies greater, and lamellar body diameter was greater than control values. 3) In lungs from thyroidectomized rats, type II cell diameter was smaller, no. of lamellar bodies fewer, and lamellar body diameter was smaller than control values. 4) Nuclear chromatin clumped in type II cells from hypothyroid rats - suggesting inactivity 5) Clara cells, ciliated epithelial cells of terminal bronchioles, and capillary endothelial cells were (Continued)

Table 12. (Continued)

Ref.	Method	Results
B	Lungs from each group were washed with saline and lavage fluids were analyzed for surfactant. Lungs from each group were also analyzed under the electron microscope.	5) (cont'd.) unaffected by varying thyroxine. Surfactant Analysis: (Mean phosphorus per mg surfactant same in all groups). 1) In hyperthyroid rats, amount of lung surfactant increased over control values - rats treated for 15 days with T4 produced more surfactant 2) Hypothyroid (thyroidectomized) rats had less surfactant than controls or sham operated controls 3) Control and sham operated controls had same amount of surfactant.
C	Fetal rats of 24 days gestation were injected with 1 μg of thyroxine and similar amount injected into amniotic sac. Fetuses delivered at 27 days.	Morphological: Thinner alveolar septa and wider air spaces than controls and type II cells had less glycogen and more OIB's than controls - lungs more mature than controls. Biochemical: No effect on activity of glycerol-phosphate phosphatidyltransferase, choline phosphotransferase, CDP-diglyceride-inositol phosphatidyltransferase, lysophosphatidic acid acyltransferase, lysolecithin acyltransferase, FA synthetase, acetyl-CoA carboxylase or FA elongation.

Refs.:

- A - Mason et al., 1972
- B - Redding et al., 1972
- C - Rooney et al., 1975

particular time period selected, but may have shown changes had the time of injection and sampling been different. The only conclusion which can be drawn from this study is that thyroxine did not appear to play a role in surfactant production via a change in activity of certain enzymes involved in phospholipid or fatty acid synthesis over the particular time period selected. It is possible, as Mason et al. (1972) suggested, that thyroxine may induce a change in the "distribution" of phospholipids within the lung rather than in phospholipid synthesis.

Evidence for the concept that thyroxine may influence the distribution of lung phospholipids came from morphological studies. Rooney et al. (1975) demonstrated that thyroxine treatment accelerated lung maturation. They found that type II alveolar cells from thyroxine treated fetuses contained less glycogen and more OIB's than type II alveolar cells from controls of the same age. They also found thinner alveolar septa and wider air spaces in fetal lungs from thyroxine treated fetuses than in control fetal lungs. Redding et al. (1972) found that type II cells from adult rats made hyperthyroid also had a greater number of OIB's and these OIB's appeared to be larger than in lungs from hypothyroid or control rats. They found that the only cell which appeared to be affected by thyroxine was the type II alveolar cell. These observations were supported by Wu et al. (1971 - from Redding et al., 1972) who learned that surface activity and the appearance of OIB's in type II alveolar cells occurred earlier in development when rabbit fetuses were treated with thyroxine. Because osmiophilic lamellar bodies of type II cells have been thought of as the site of surfactant storage (Goerke, 1974), thyroxine may be stimulating storage of surfactant. Results from Redding et al. (1972) and Wu et al. (1971) which showed an increase in lavage surfactant and earlier appearance of surface activity in response to thyroxine, respectively, infer that thyroxine may have been stimulating surfactant secretion. Thus, thyroxine may have induced surfactant secretion and storage in these studies but it was not shown that this hormone affected surfactant synthesis.

The next step in experimentation on thyroxine's role in lung development should be to determine whether or not thyroxine directly affects lung tissues. This could involve in vitro incubations of lung tissue in the presence of thyroxine with subsequent analysis of media and tissue for PC (including disaturated PC), and morphological examination of lung tissue. Lung tissue could also be analyzed for the presence of thyroxine receptors. Of course, thyroxine may stimulate another tissue which could in turn affect lung development. Profiles of normal thyroxine secretion in developing animals could be compared with profiles of lung development in the same animal to see if there may be correlations between thyroxine secretion and developmental events in the lung. Clinical use of thyroxine as a preventive measure or in treatment of RDS in infants may not be practical because addition of thyroxine to the fetus may have deleterious effects on its normal development. However, it would be

important to know if and how thyroxine regulates lung maturation.

Because various components of carbohydrate metabolism are incorporated into lipids, investigators are studying different agents which affect carbohydrate metabolism in relation to their effects on lipid production in the lung. The relationship between glycogen metabolism and phospholipid synthesis in the lung has been reviewed in the section on synthesis. According to a review by Gross (1977), the fetus of a diabetic mother is exposed to high levels of endogenous insulin due to glucose stimulation of the fetal pancreas. Glucose from the hyperglycemic mother crosses the placenta (insulin does not) and stimulates insulin production by the fetal pancreas. Babies born to diabetic mothers have a greater tendency to exhibit respiratory distress syndrome (RDS) of the newborn. Robert et al. (1976 - from Gross, 1977) have found that the susceptibility of these infants to RDS is not due only to the fact that these infants tend to be born prematurely or by Caesarean section. Insulin, a hormone which promotes glucose uptake and glycogen synthesis in target cells (Lehninger, 1976, pp. 820-821) has an apparent effect on surfactant production as will be seen in the experiments discussed below. (See Fig. 1 from Gross (1977) showing the importance of glucose to phospholipid synthesis in the alveolar type II cell). Proinsulin, the protein precursor molecule of insulin, is synthesized in the β cells of islets of Langerhans in the pancreas, then cleaved by proteases to form insulin which is stored in vesicles until the cell releases it in response to a rise in blood glucose (Lehninger, 1976, pp. 817-822). Diabetes, as induced by two agents, alloxan or streptozotocin is known to influence phospholipid synthesis as well.

In a recent experiment, Moxley and Longmore (1977), demonstrated that induced diabetes in rats apparently caused a decrease in the amount of glucose incorporated into certain lipids. Adult rats were treated with alloxan or streptozotocin to induce diabetes. Lungs from diabetic animals were then removed and perfused with medium containing radioactively labeled and unlabeled glucose. They found that glucose incorporation into neutral lipids and phospholipids was lower in lungs from alloxan and streptozotocin treated animals than in normal animals. A reduction in labeled glucose incorporated into neutral lipids and phospholipids from the residual (nonsurfactant) fraction was also seen in lungs from diabetic animals. More specifically, there was a reduction in glucose incorporation into PC and PG seen in the surfactant and residual fractions in alloxan and streptozotocin diabetic animals with the exception of PG in the residual fraction from streptozotocin treated animals. They speculated that this lack of change in labeled PG may have been due to errors in isolating a lipid (PG) which constitutes only 2-3% of the lipid in the residual fraction (Sanders and Longmore, 1974 - from Moxley and Longmore, 1977). The incorporation of [U- 14 C]glucose into palmitic acid and glycerophosphocholine portions of PC isolated from alloxan-diabetic rats was reduced compared to

Figure 1. (from Gross, 1977)

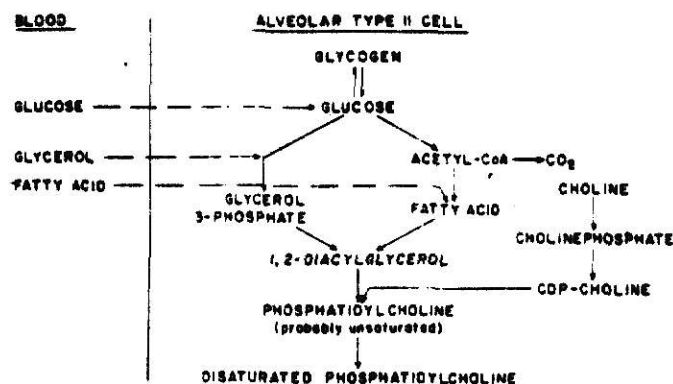


Figure 1. The interaction of substrates in lung phosphatidylcholine synthesis.

normal values in both surfactant and residual fractions. Labeled glucose incorporation into PC, PG, and palmitic acid was increased over control values when rats were treated with insulin 15 or 30 minutes prior to perfusion. In an earlier experiment, Moxley and Longmore (1975) found that insulin treatment given to alloxan-diabetic rats restored the ability of their lungs to incorporate labeled glucose into phospholipids and neutral lipids at a rate similar to or exceeding that found in normal lungs. They also found that exogenous insulin treatment (insulin contained in perfusion media) had no effect on the incorporation of glucose into lipids of lung from alloxan-diabetic or normal rats (Moxley and Longmore, 1975).

From Moxley and Longmore's (1977, 1975) results, it appeared as though insulin and/or the diabetic state of an animal had an effect on its ability to incorporate glucose into lung lipids. Just how this particular phenomenon may be involved in regulation of overall lipid synthesis in the lung, has not been determined. It would have been helpful if residual and surfactant concentrations of lung PC and total phospholipids had been determined. Stratta-Monga et al. (1973 - from Moxley and Longmore, 1975) have found that alloxan diabetes caused an increase in lung phospholipid and a decrease in triglyceride concentration, but insulin caused a smaller increase in phospholipids and no change in triglycerides. Das and Kumar (1975 - from Moxley and Longmore, 1975) reported that streptozotocin diabetes altered the level of acetyl-CoA carboxylase and fatty acid synthetase, thus reducing the lung's lipid synthesizing capabilities. Activity of these enzymes was returned to normal levels 12 hours after diabetic animals were treated with insulin. Thus, it appeared as though pathways of lipid and fatty acid synthesis which incorporate glucose as a precursor molecule may be stimulated by insulin. The apparent contradiction of Stratta-Monga et al. (1973) results may have been due to the possibility that less glucose was being stored as glycogen in lungs of diabetic animals and was thus readily

available for phospholipid synthesis. However, this would not resolve the contradiction fully.

Although the mechanism of insulin action on lung cell phospholipid synthesis is not known, experiments showing the opposing effects of insulin and corticosteroids may provide a clue to insulin's action. Smith et al. (1975 - from Gross, 1977) have observed that insulin appears to oppose the action of cortisol on monolayer cultures of mixed fetal rabbit lung cells. The addition of cortisol results in increased incorporation of choline into PC. When insulin is added to the culture in the presence of cortisol, these effects are nullified. However, when insulin alone is added to the culture medium, incorporation of choline into PC is slightly stimulated.

In experiments on fetal rat lungs in organ culture, Gross and Smith (1977 - from Gross, 1977) have found that insulin had an effect opposite to that of corticosteroid on lung glycogen content. In their culture system no cortisol or serum (which may contain hormones) was added, yet explants continued to mature. They suggested that this may have been due to exposure to corticosteroids or other hormones before being placed in culture. Explants from 19-day fetal rat lung (term = 22 days) cultured for 24 hours showed decreased glycogen content and an increased number of lamellar bodies compared to uncultured lung. When insulin was added to the culture medium, explants had fewer lamellar bodies and more cytoplasmic glycogen than control cultures. They also observed that the lamellar bodies of the alveolar type II cells from insulin treated cultures were usually earlier forms. Gross (1977) concluded that insulin delayed the maturation of fetal lungs in culture as gauged by morphologic evidence.

Gross (1977) suggested that insulin may act by inhibiting glycogenolysis and thus deprive the lung of substrate for phospholipid synthesis or it may act by stimulating glycogen synthesis, thereby storing glucose rather than allowing it to be used in phospholipid synthesis. Because insulin increased the glycogen content and decreased the number of lamellar bodies, and corticosteroids had the opposite effect (Kikkawa et al., 1971; Wang et al., 1971 - from Gross, 1977), Gross (1977) suggested that surfactant synthesis may be under control of several agents whose balance affects surfactant production.

The findings of Moxley and Longmore (1977) seem to conflict with the morphological observations made by Gross and Smith (1977 - from Gross, 1977). Moxley and Longmore (1977) showed an increase in the rate of glucose incorporation into phospholipids with insulin treatment. It should be noted that their system used adult rat lungs. Gross and Smith (1977) used fetal rat lungs and showed fewer lamellar bodies and more cytoplasmic glycogen with insulin treatment. Recall that Smith et al. (1975 - from Gross, 1977) found a slight increase of choline incorporation into PC when insulin alone was added to culture media. Perhaps there is some age

dependence for insulin effects. It could be that the monolayer culture of Smith et al. (1975) and the adult rat lung are missing a factor which makes insulin a "retarding" influence on development. The "retardaion" effect of insulin was noted by Smith et al. (1975) in which accelerated maturation induced by corticosteroid was halted by insulin, and noted by Gross and Smith (1977) who found morphological evidence of slowed development in the presence of insulin. The mechanism by which insulin causes changes in the adult or fetal lung has not been determined.

As to the mechanism of insulin action, Moxley and Longmore (1977) have suggested that new enzyme synthesis is probably not involved in insulin's action on the lung. It took as few as 15 minutes following insulin injection to see an increase in glucose incorporation into PC, PG, and palmitic acid. This did not appear to be enough time for new enzyme synthesis according to them. Regarding the mechanism of insulin action, it is important to note that insulin inhibits lipolysis (Lehninger, 1976, pg. 821). Also, a diabetic has a greatly decreased capacity to convert glucose into fatty acids (Lehninger, 1976, pg. 851). Thus, it is possible that insulin's action on lungs may involve inhibition of lipolysis. Demonstration of the presence or lack of insulin receptors in lung cells would be a helpful measure in working out the mechanism of insulin action.

Dibutyryl cyclic AMP (cAMP) and agents which affect cAMP concentration have been studied with regard to their influence on surfactant production and secretion. Theophylline, a cAMP phosphodiesterase inhibitor (Gross, 1977) and aminophylline (a derivative of theophylline which also inhibits phosphodiesterase (Gross and Rooney, 1977)) have been tested for their effects on lung surfactant production.

Production of certain lung phospholipids appears to be stimulated by aminophylline, theophylline, and cAMP. Gross and Rooney (1977) have demonstrated an 88% increase in choline incorporation into PC in explants of 19-day fetal rat lung after they were exposed to 1.0 mM aminophylline for 48 hours. They found an increase of choline incorporation into SP, but the rate of choline incorporation into PC was thirty times greater than that into SP. Varying the dose from 0.5 to 5.0 mM had little affect on the rate of choline incorporation. They also found a slight increase in the amount of disaturated PC from aminophylline treated cultures. In an experiment using pregnant rabbits, Barret et al. (1976 - from Gross, 1977) showed an increase in cAMP and stimulation of choline incorporation into PC in fetal lungs after pregnant rabbits were treated with aminophylline. In a similar experiment, Karotkin et al. (1976 - from Gross, 1977) found that when pregnant rabbits were injected with aminophylline, there was an increase in lung lavage phospholipid. They also found that aminophylline did not increase the number of lamellar bodies in the type II cell. Gross and Rooney (1977) demonstrated an age dependence for the effects of aminophylline. At 19 days of gestation, aminophylline had its

maximum effect on choline incorporation into PC and SP in the fetal rat lung explants. At 18 days there was less stimulation than at 19 days, and on days 20 and 21, the effect of aminophylline decreased. Gross and Rooney (1977) looked at the effects of aminophylline on two enzymes involved in PC synthesis. After a 48 hour exposure to this agent, they showed no effect on the activity of choline kinase or cholinephosphotransferase. Shapiro et al. (1977 - from Gross, 1977) using human alveolar tumor cells in monolayer culture found that cholinephosphotransferase activity was not affected by theophylline, and that choline kinase activity may be increased by theophylline. (See Table 3 from Gross (1977) summarizing the effects of theophylline. Note that many of the observations made about aminophylline are also true of theophylline).

The influence of dibutyryl cAMP on lung tissue phospholipid synthesis is similar to the effects of aminophylline or theophylline on lung tissue. Gross and Rooney (1977) observed a 77% increase in choline incorporation into PC and a 53% increase into SP after fetal lung explants were exposed to 0.2 mM dibutyryl cAMP for 48 hours. Using a different system, Hallman (1977) has studied the effects of cAMP on phospholipid synthesis and on the distribution of phospholipids. Hallman (1977) always used theophylline along with cAMP, and used PG as the main index for change because of PG's functional importance to surfactant. Lung slices from perinatal rabbits which were treated with cAMP showed an increase in the rate of labeled palmitic acid and labeled glycerol incorporation into PG as compared to controls. The rate of palmitate incorporation showed a greater increase with cAMP treatment than glycerol incorporation. Hallman (1977) also observed an age dependence for the effects of cAMP. Lung samples were taken from 26, 28, and 30 day old fetuses, at the time of birth, and from 10 day old rabbits. The most significant effects of cAMP on incorporation into PG occurred between the 28th and 30th fetal days. When 28 and 30 day old fetuses were removed from the uterus and kept in room air at 30°C for one hour before incorporation studies were done on the lung slices, the 28 day old fetal lung slices showed a significant increase in glycerol and palmitate incorporation into PG after cAMP treatment. Thirty-day old fetal lung slices did not show a significant increase in incorporation. Hallman (1977) also studied glycerol and palmitate incorporation into PC and PI in response to cAMP treatment. Cyclic AMP treatment appeared to cause a decrease in incorporation into PI (see Table 2 from Hallman (1977)). According to Hallman's comments in the body of the paper, palmitate incorporation into PC significantly increased between the 26th and 30th fetal days with cAMP treatment. However, Table 2 (Hallman, 1977) showed this to be true of glycerol, not labeled palmitate. Thus, these results did give some indication that perhaps PC synthesis was enhanced by cAMP treatment as Gross and Rooney (1977) have found.

Table 3. (from Gross, 1977)

TABLE 3. Comparison of the known effects of corticosteroid and theophylline on lung phospholipid synthesis

	Steroid	Theophylline	References
Lung phosphatidylcholine synthesis	Increased	Increased	2, 5
Lung lavage total phospholipid content	Increased	Increased	15, 22
Lung lavage phosphatidylcholine:			
sphingomyelin ratio	Increased		22
Alveolar type II cell lamellar bodies	Increased	No change	15, 16, 28
Lung surface tension	Decreased	Decreased	15, 17
In culture:			
Phosphatidylcholine synthesis	Increased	Increased	10, 24, 26
Sphingomyelin synthesis	No effect	Increased	10, 24, 26
Enzyme activities:			
Choline kinase	No effect	(?) increased	10, 24, 22
Cholinephosphate			
cytidyltransferase	Increased		22
Cholinephosphotransferase	(?) increased	No effect	1, 5, 10, 22, 24
Lysolcithin acyltransferase	Increased		22
Phosphatidic acid phosphatase	Increased		1
Glycerolphosphate			
phosphatidyltransferase	Increased		23

Table 2. (from Hallman, 1977)

Table 2. Incorporation of [^3H] glycerol and [^{14}C] palmitate into lecithin and phosphatidylinositol in lung slices from rabbits of different ages

	Dibutyryl cyclic AMP	DPM/nmoles total phospholipid			
		Lecithin		Phosphatidylinositol	
		^3H	^{14}C	^3H	^{14}C
Fetus, 26 days	-	164 $\pm$ 12 (6)	320 $\pm$ 10	76 $\pm$ 3	25 $\pm$ 1
	+	181 $\pm$ 10	343 $\pm$ 17	68 $\pm$ 5	24 $\pm$ 1
Fetus, 28 days	-	136 $\pm$ 9 (10)	676 $\pm$ 34	99 $\pm$ 8	59 $\pm$ 4
	+	152 $\pm$ 8	631 $\pm$ 29	80 $\pm$ 4*	49 $\pm$ 4*
Fetus, 30 days	-	155 $\pm$ 7 (6)	481 $\pm$ 20	76 $\pm$ 2	52 $\pm$ 2
	+	165 $\pm$ 9	466 $\pm$ 17	71 $\pm$ 3	46 $\pm$ 1*
Newborn, 10 days	-	89 $\pm$ 2 (4)	426 $\pm$ 31	62 $\pm$ 3	40 $\pm$ 3
	+	81 $\pm$ 6	480 $\pm$ 27	56 $\pm$ 2	33 $\pm$ 3

For experimental details see METHODS and Table 1.

*p < 0.1 as compared to that without cyclic AMP.

Hallman (1977) has also reported some important observations regarding the distribution of surfactant in response to cAMP. Twenty-eight day old premature fetal rabbits were injected with cAMP intraperitoneally at birth. These rabbits were allowed to remain in room air for one hour. During the first hour after delivery, controls had retractions and cyanosis, but rabbits which had received cAMP were acyanotic and had no apparent respiratory distress. One hour after cAMP treatment, 10.2% of the total lung phospholipids were found in the alveolar wash whereas only 6.5% was found in alveolar wash from controls. There was a higher PC/SP ratio in the alveolar wash than in the residual lung in both controls and treated animals. Hallman (1977) reported a very low SP content in wash and residual lung, and that 49% of the total PC from controls and 54% of PC from treated animals was disaturated. This apparently indicated that the material recovered from the lavages was mostly surfactant. PG in alveolar lavage was detected one hour after cAMP treatment but this phospholipid was almost absent in the controls. The percent distribution of other phospholipids (such as PI, PE, and PS) in alveolar wash and in residual lung did not show a significant change with cAMP treatment. Hallman (1977) also found that PG appeared in the alveolar wash even if 0.5 mg of puromycin was given 5 minutes before cAMP injection. Thus, cAMP appeared to stimulate secretion of surfactant even in the absence of new protein synthesis.

From the studies discussed above, the following conclusions can be drawn: 1) aminophylline (AM), theophylline (TH), and cAMP appear to stimulate PC synthesis and SP synthesis; 2) AM increases lung lavage phospholipid; 3) AM does not affect the number of lamellar bodies of type II cells; 4) there is an age dependence for the effects of AM and cAMP; 5) AM does not appear to affect choline kinase or cholinephosphotransferase activity, but TH may affect choline kinase; 6) cAMP appears to stimulate PG synthesis; 7) cAMP appears to stimulate secretion of surfactant lipids; and 8) cAMP can stimulate secretion of surfactant lipids in the absence of new protein synthesis.

In comparison with corticosteroids, it can be seen that corticosteroids share the ability to stimulate PC synthesis, lack the ability to stimulate choline kinase activity (Rooney et al., 1976; Oldenberg and Van Golde, 1977), and lack the ability to stimulate cholinephosphotransferase (Brehier et al., 1977; Rooney et al., 1976; Rooney et al., 1975) (according to Oldenberg and Van Golde (1977) this enzyme can be stimulated by dexamethasone). Recall in the section on corticosteroids that there was an age dependence for their effects. The apparent stimulation of PG synthesis and secretion in response to cAMP is comparable to the increase in PG found in fetal rabbit lung lavage after corticosteroid treatment by Rooney et al. (1976). Rooney et al. (1975) also found an increase in glycerophosphate phosphatidyltransferase in fetal rabbit lungs when fetuses were injected with corticosteroid. (This is the rate limiting enzyme in PG

synthesis (Hallman and Gluck, 1975b; Kiyasu et al., 1963 - from Hallman, 1977)). In contrast to cAMP studies, corticosteroids do not stimulate SP synthesis (Smith et al., 1974 - from Gross, 1977), and corticosteroids cause an increase in the number of lamellar bodies in type II alveolar cells (Gross, 1977). Thus, it appears as though corticosteroids and AM, TH, and cAMP share some characteristics. However, AM seems to have a less specific effect on lung phospholipid synthesis by virtue of the fact that it stimulates both PC and SP synthesis (Gross, 1977). Normal maturation of the lung and development stimulated by corticosteroids shows an increase in the L/S ratio possibly due to increased PC synthesis but not SP (Rooney et al., 1976). Corticosteroid(s) and cAMP may work together in bringing about surfactant production in normal development. However, lung maturation is probably not only influenced by these two factors. Cyclic AMP activities in response to other hormones suggest a more complex system of development.

Cyclic AMP is the mediator of several hormones including glucagon and epinephrine (Lehninger, 1976, pg. 811). Formation of cAMP is the first step in stimulation of glycogen breakdown by epinephrine and glucagon in liver cells (Lehninger, 1976, pp. 814-816). It is possible that cAMP promotes glycogenolysis in lung cells, perhaps in response to these hormones or some other agent. One observation made by investigators is that there appears to be less glycogen storage in lung cells and more lamellar bodies as the lung matures (Gross et al., 1977 - from Gross and Rooney, 1977). Therefore, it is possible that cAMP promotes glycogen breakdown in the lung cell, thus making more glucose available to go into formation of lipids (Gross, 1977). Cyclic AMP activates certain enzymes in the glycogen breakdown pathway in response to glucagon or epinephrine (Lehninger, 1976, pp. 814-816). That may be cAMP's mechanism of action in lung cells.

The insulin-cAMP balance in lung tissue may have an effect on regulation of lipid synthesis and degradation. Insulin promotes glycogen synthesis and cAMP promotes glycogenolysis. The balance of these two agents may control the amount of glucose available as substrate for lipid synthesis. Moxley and Longmore (1977) suggest that new enzyme synthesis is probably not responsible for insulin's action on lung cells. Similarly, Hallman (1977) suggests that new protein synthesis is not responsible for cAMP's effects. These observations indicate that cAMP and insulin may be related in their mechanism of action.

The effect of insulin on lipolysis may be related to cAMP content, also (Lehninger, 1976, pg. 821). Epinephrine, glucagon, and other hormones are known to stimulate lipase activity in adipose tissue by causing an increase in cAMP in that cell (Lehninger, 1976, pg. 817). The presence of insulin causes a decrease in cAMP and an increase in cGMP in fat cells (Lehninger, 1976, pg. 822). Insulin is also known to inhibit lipolysis (Lehninger, 1976, pg. 821). Cyclic AMP may stimulate lipase activity, thus providing additional fatty

acids and glycerol for phospholipid synthesis in lung cells. If lung cells contain insulin receptors, then insulin bound cells may have a decreased cAMP content, lower lipolysis, and less phospholipid synthesis. It is also possible that insulin may act by lowering the cAMP level in fat cells, thus reducing lipase activity and the amount of free fatty acids transported into the bloodstream to be made available for phospholipid synthesis. However, this would not entirely explain results from in vitro experiments. Aside from this, the balance between insulin and cAMP may be involved in regulation of surfactant production.

Up to this point, it appears as though corticosteroids, insulin, and cAMP content may be involved in regulation of surfactant production with some degree of relatedness. Some corticosteroid and cAMP effects parallel each other. Insulin opposes the action of corticosteroids (see section on insulin). Insulin and cAMP have opposing effects. In the next section, another possible mode for cAMP action will be proposed in relation to yet another agent.

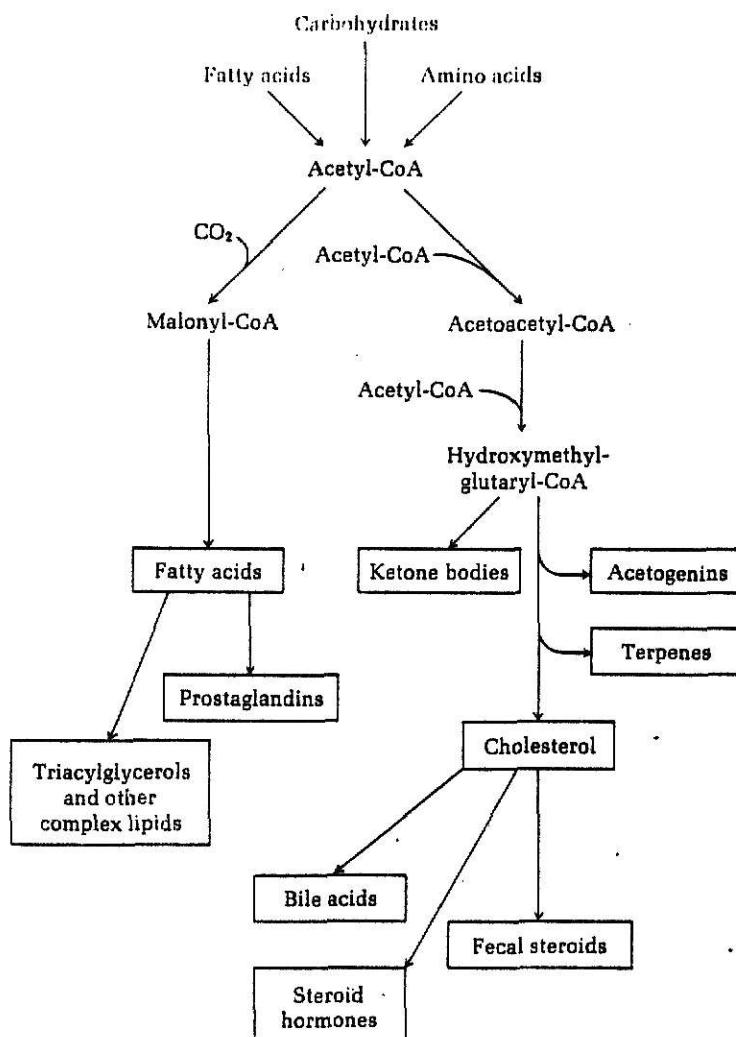
Prostaglandins are a class of molecules which have many diverse functions. These molecules are cyclic derivatives of certain unsaturated fatty acids having 20 carbon atoms (Lehninger, 1976, pg. 686). Figure 24-33 from Lehninger (1976, pg. 688) shows where prostaglandins lie in relation to various other lipids whose precursor is acetyl-CoA. Figure 2 from Mathe' et al. (1977) shows the principal pathways of prostaglandin synthesis and catabolism.

Much of the research involving these molecules has dealt with their stimulation of vasodilation or vasoconstriction. However, looking at the effects of various prostaglandins on cAMP concentrations and data showing the prostaglandin profiles, some testable hypotheses can be made about the effects of prostaglandins on surfactant production.

Pace-Asciak (1977) along with his co-worker Rangaraj (1977, 1978) have done much research in this area. In recent years they have developed profiles of various prostaglandins in organs of different animals. Also, Pace-Asciak (1977) has done research on the metabolism of different prostaglandins in fetal sheep lungs. He studied PGE₂ and PGF_{2α}, the main prostaglandins produced in sheep lungs and various tissues (Pace-Asciak and Rangaraj, 1978). He studied these two prostaglandins in fetal sheep ranging in age from 40 days gestation to term (140 days gestation). In general, he found that the total PGE₂ and PGF_{2α} (PGE₂ + PGF_{2α}) concentrations increased with increasing gestation. The 40 day lung formed more PGF_{2α} than PGE₂, but production of PGE₂ continued to increase with greater age until at term both prostaglandins were being produced in almost equal amounts. He also found that "PGF_{2α} catabolism occurred mainly by the PG15-hydroxy dehydrogenase pathway." The activity of this enzyme remained constant up to about 80 days. At 90 days there was a sharp increase in enzyme activity until term when it decreased back to the 40 day level

Figure 24-33 (from Lehninger, 1976)

Figure 24-33
Acetyl-CoA as the key precursor in biosynthesis of many different lipids.



of activity.

Pace-Asciak (1977) suggested that the increase in prostaglandin catabolism around 90 - 116 days may have been due to 1) an increased requirement to inactivate circulating prostaglandins (one of the functions of adult lung according to a reference which he cited and Mathe' et al. (1977)) because more blood flows through the lungs at that stage of development, or, 2) an increased requirement to inactivate and control levels of prostaglandins originating within the lung because these might be crucial to maintain pulmonary vessels in the relaxed state. PGE₂ may function as a potent vasodilator because the ratio of PGE₂ to PGF_{2α} shifts toward PGE₂ when the proportion of cardiac output increases to the lungs.

Figure 2. (from Mathé et al., 1977)

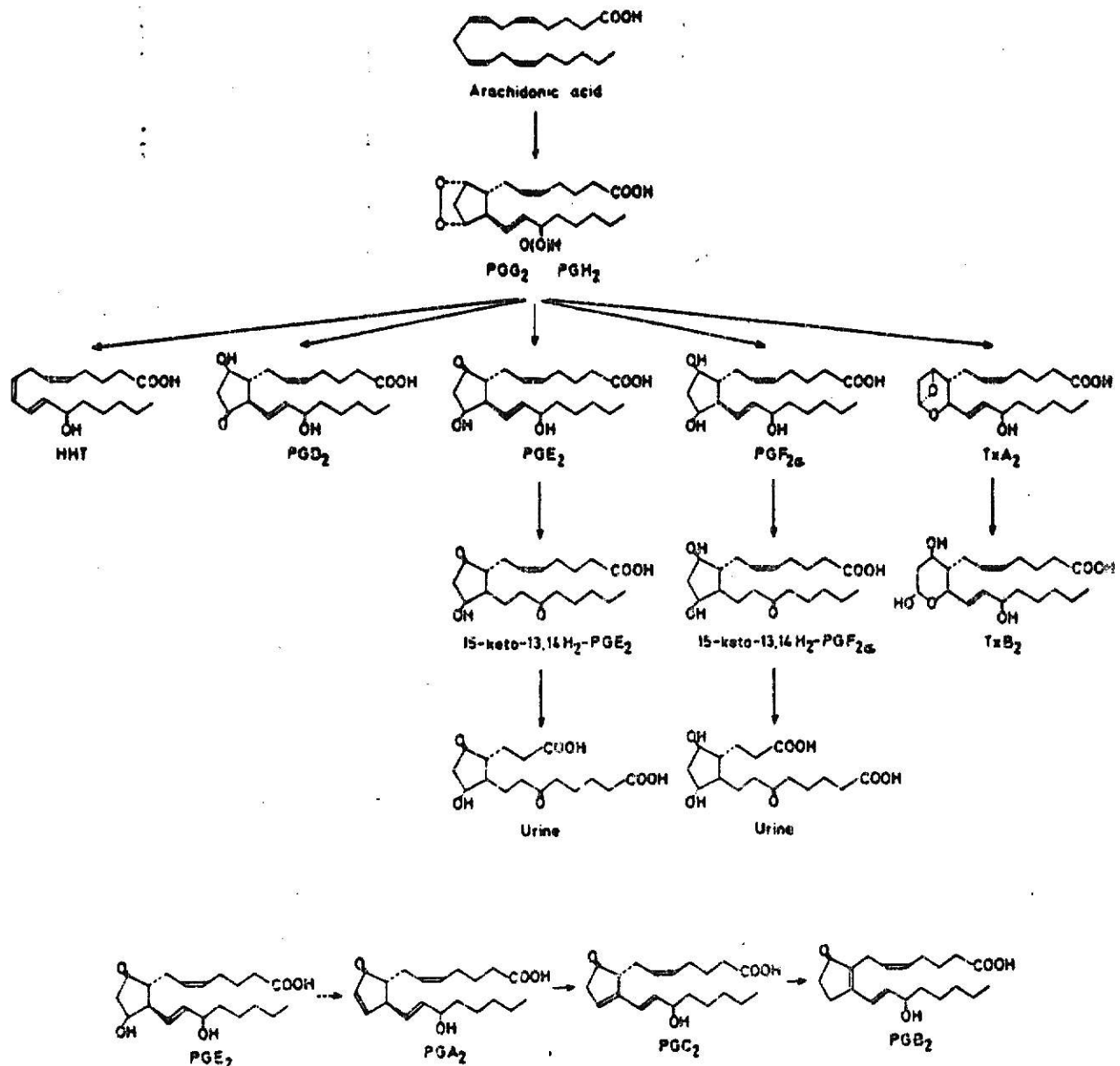


Figure 2. Principal Pathways of Prostaglandin Synthesis and Catabolism.

At approximately 110 - 116 days sheep lung begins canalicular development, and type II alveolar cells begin to differentiate at about 110 days in preparation for surfactant production. Recall that there is a rise in PGE₂ formation and in prostaglandin catabolic activity at this stage of development. Pace-Asciak (1977) proposes that if PGE₂ is important to type II cell differentiation and to pulmonary "hemodynamics," then one might expect the "half-life" of PGE₂ to decrease as target sites for PGE₂ action might become overly sensitive to this compound. (Increased sensitivity to other vasodilators has been reported at about 90-100 days (Pace-Asciak, 1977)). Corticosteroids are known to accelerate

lung maturation and to inhibit prostaglandin synthesis. Pace-Asciak (1977) suggests that corticosteroids may mimic the action of prostaglandin catabolic enzymes by maintaining low levels of prostaglandins during this period of development. Mathe' et al. (1977) suggest that corticosteroids may act to decrease prostaglandin release by stabilizing membrane phospholipids, thereby decreasing the supply of available substrate for prostaglandin synthesis. It is possible that maintenance of the proper level of prostaglandins, especially PGE₂, may help to regulate type II cell development.

Now that the proposition concerning a possible role of prostaglandins in surfactant production has been made, further speculation may be made on a mechanism of action. One mechanism which seems plausible is the possibility that prostaglandins partially regulate surfactant production by controlling the level of cAMP in the cell. The relationship between cAMP and prostaglandins has been demonstrated in several ways. PGE₁ is thought to serve as an intermediate messenger between the cell surface hormone receptor and the membrane-bound adenylate cyclase in some target cells (Lehninger, 1976, pg. 817). PGA₁ inhibits adenylate cyclase of intestinal cells by binding to specific sites on the enzyme (Lehninger, 1976, pg. 817).

Now that the groundwork showing an apparent link between prostaglandins and cAMP has been laid, a proposed mechanism can be discussed. The concentration of prostaglandins (particularly PGE) in the lung may increase at approximately the time of alveolar type II cell differentiation. This increase in concentration may be due to more prostaglandins carried into the lung by greater pulmonary circulation, or due to increased synthesis of prostaglandins (prostaglandins are not stored in the lung (Mathe' et al., 1977)) in the lung. This increase in PGE may cause an increase in adenylate cyclase activity in the type II alveolar cell. The resulting increase in cAMP concentration may then stimulate glycogen breakdown in the cell (see previous discussion), thus providing additional substrate for surfactant production, and a stimulus for surfactant secretion. Recall that cAMP apparently stimulates surfactant production and secretion (Gross and Rooney, 1977; Hallman, 1977). A slight variation of the above mechanism may provide for hormonal influence. The increase in PGE concentration may provide a greater number of messengers between the cell surface hormone receptor and membrane-bound adenylate cyclase in the type II cell. As was mentioned previously, PGE₁ apparently has this ability (Lehninger, 1976, pg. 817). Corticosteroids may act to control the level of prostaglandins by increasing catabolism as proposed by Pace-Asciak (1977). In monkey lung, 15-PGDH (15-hydroxy prostaglandin dehydrogenase) was not affected by steroids, calcium and cAMP, but was activated by thyroid hormones (Mathe' et al., 1977). Thus, it is possible that thyroid hormones act to control prostaglandin levels by stimulating their catabolism.

At this point, evidence for a role played by prostaglandins

in surfactant production and/or secretion is highly speculative. Prostaglandins have diverse effects and act on several types of cells. Studies need to be performed to show whether or not specific types of prostaglandins affect surfactant production and/or alveolar type II cell development. Perfusion of fetal lungs with specific types of prostaglandins followed by biochemical analysis for disaturated PC and morphologic examination of the type II cell, would provide preliminary evidence for or against prostaglandin involvement in surfactant production. Assaying cAMP concentration after prostaglandin treatment would indicate whether or not the proposed mechanism is plausible. A direct correlation between the presence of prostaglandins and surfactant production, and/or secretion, should be demonstrated before any further speculation or conclusions can be made.

In previous sections, the effects of different hormones or other agents on lung development have been discussed. This section will include discussion of how manipulation of an animal's dietary intake may affect surfactant production. These studies will be grouped according to the age of the animal tested.

Gross (1977) reviewed this topic and provided some of the information to follow. Pregnant animals have been fasted or given a nutritionally limited diet in order to see what changes would result in fetal lung development. Faridy (1975 - from Gross, 1977) found a decrease in PC content of fetal rat lungs (expressed per unit lung or DNA) when pregnant rats were fasted for four days or given a low protein diet for longer periods. He also observed that the minimum surface tension of lung extracts was increased in undernourished animals. If newborn rats were allowed to breathe for 10 hours, these changes disappeared. Hawrylewicz et al. (1975 - from Gross, 1977) showed a decrease in cholinephosphotransferase activity in fetal rat lungs when the mother was deprived of protein. A decrease in PC such as that seen by Faridy (1975) might be expected with a decrease in this enzyme's activity. However, Gross (1977) studied fasted pregnant rabbits and found no change in the rate of PC synthesis or in PC content of fetal lungs.

The results from experiments on pregnant animals provide two opposing views. One side says that there is a decrease in PC and the other says that there is no change in PC when pregnant animals were fasted. Differences in the species of animals tested may explain differences in results. There may be some sort of biochemical factor which is not shared by both species which would account for these results. A more plausible explanation may be derived from differences in the length of gestation. The rat has a shorter gestation period than the rabbit. Short periods of fasting may bring about magnified changes in the rat, but not in the rabbit. Differences in experimental method may also account for the opposing views.

The effects of fasting have also been studied in newborn

animals. Gross et al. (1978) studied changes in phospholipid synthesis brought about by fasting newborn rabbits. Thirty-day-old rabbit fetuses were delivered by Caesarian section, then placed in an incubator designed for human newborn infants, and kept there for three days following birth. These rabbits were fed periodically by a gavage technique, or were fasted during this time. The results from their experiments are summarized in Table 13.

The observation that fasting had no effect on total phospholipid or on PC or disaturated PC from lung lavage or lung tissue when results were expressed per mg protein may be misleading. Gross et al. (1978) found a 10% decrease in lung protein from lung tissue after 72 hours of fasting. A decrease in lung phospholipids may not be seen if there had been a concomitant decrease in protein. DNA content was found to be the same in fasted and control animals after fasting for 72 hours. When results were expressed per lung or per DNA, a decrease in lung tissue phospholipid was revealed in fasted animals.

Whether results were expressed per mg protein, per lung, or per DNA, there was no apparent change in the phospholipid content of lung lavage in fasted animals. The lack of change may have been due to a delay in the appearance of newly synthesized PC in lung lavage as suggested by Gross et al. (1978). Newborn rabbits were injected with labeled glycerol to follow PC turnover. Specific activity of lung tissue PC reached a peak at six hours then declined during the rest of the 72 hour period. Specific activity of lung lavage reached a peak at 24 hours, and slowly declined over the rest of the 72 hour period. There was no significant difference between curves for control and fasted animals. Glycerol is rapidly metabolized by the lung, so the delay in lavage PC appearance was probably not due to recycling of glycerol (Gross et al., 1978). The delay in lavage PC appearance was probably due to the time needed for new PC to go into lamellar bodies of type II cells then be secreted. The absence of a change in lavage PC content in fasted animals probably has to do with the fact that it took at least 48 hours of fasting to see a decrease in the rate of PC synthesis, and that there was an additional delay in the appearance of new lavage PC. (Newborn rabbits did not survive more than 72 hours of fasting).

From these results, Gross et al. (1978) concluded that fasting did not appear to decrease alveolar surfactant in the newborn rabbit. By the time a major change in surfactant could occur, the animal would probably be dead. This supported the findings of Gross (1977) in which he found no change in the rate of PC synthesis or in PC content of fetal rabbit lungs in experiments with fasted pregnant rabbits. Their results differed from Faridy's (1975 - from Gross, 1977) studies in which he found a decrease in PC content of fetal rabbit lungs when pregnant rats were fasted.

Studies regarding the effects of fasting on adult lung

Table 13. Summary of Results from Gross et al. (1978)

A) Weight

Fed Control - Did not show a significant increase in wt. until after three days of incubation

Fasted - Continuous decrease from birth wt. from 1 to 3 days of incubation

B) Choline Incorporation

Fed Control - Incorporation into PC and SP decreased over the 3 days of incubation

Fasted - Incorporation into PC and SP decreased over the 3 days of incubation; significantly lower rate of incorporation compared to control values after 2 days of incubation

C)		Per mg Protein Over 3 Days		Per DNA or Lung at 72 Hours	
		Lung Tissue	Lung Lavage	Lung Tissue	Lung Lavage
Fed Control	Total Phospholipid	Slight increase over 72 hrs.	Increased for 48 hrs. then reached plateau	-----	-----
	Total PC and DSPC	Slight increase over 72 hrs.	Similar to above	-----	-----
Fasted	Total Phospholipid	No significant change compared to controls	No change compared to controls	Decrease from control values	No change from controls
	Total PC and DSPC	No significant change compared to controls	No change compared to controls	Decrease from control values	No change from controls

D) When these studies were done with 29 day old premature rabbits, they obtained virtually the same results.

indicate a decrease in surfactant content. Garbagni et al. (1968 - from Gross, 1977) fasted adult rabbits for four days and found a decrease in the bubble stability ratio (sometimes used as a measure of lung surfactant). Faridy (1970 - from Gross, 1977) studied the effects of fasting on adult rats and found increased surface tension in lung extracts. He also found a decrease in deflation stability of lungs from fasted rats. When results were expressed per unit DNA, he discovered a decrease in lung PC. Kyriakides et al. (1976 - from Gross, 1977) determined that there was a lower percentage of disaturated PC in lung tissue and lavage but no change in total lung PC when rats were fed a diet lacking essential fatty acids. These changes were reversed when rats were fed

a diet including these nutrients. Gross et al. (1976, 1975b - from Gross, 1977) have studied the effects of nutrition on phospholipid synthesis in weanling rats (21 days old). Rats fasted for 60 hours showed 33% decrease in lung protein but no change in DNA. After 60 hours of fasting or feeding of pure glucose, pure fat, or pure protein, there was a decrease in both lung tissue and lung lavage PC when results were expressed per lung or per unit DNA, but not when expressed per unit protein or weight. The activity of cholinephosphotransferase and acetyl-CoA carboxylase was decreased in fasted animals, but normal in glucose-fed animals. Fatty acid synthetase activity was decreased in fasted and glucose-fed animals. From these results Gross (1977) concluded that fasting causes a decrease in cell size but not cell number, and the PC content of lung tissue and lavage appears to decrease per cell but not per unit mass.

From the above studies on fetal, newborn, and adult lungs and the effects of fasting, no definite conclusions can be drawn. Experiments performed with pregnant rats indicate that a deficient diet decreases surfactant PC and the activity of cholinephosphotransferase in fetal rat lungs, and causes a decrease in surface tension of lung extracts. However, Gross (1977) found no change in the rate of PC synthesis or in PC content in fetal rabbit lungs when the mother was fasted. There does appear to be an effect of fasting on lung surfactant production in fetal rats, and it is possible that changes occur in fetal rabbits whose mothers are fasted but that Gross (1977) simply did not detect it. The preliminary report which Gross (1977) cited as giving the above results on fasted pregnant rabbits was not further referenced or detailed in his text.

The study on fasted newborn rabbits by Gross et al. (1978) revealed only a slight decrease in phospholipid of lung tissue but no change in lavage when results were expressed per unit DNA or lung. They found a decrease in choline incorporation into PC and SP but an increase in total PC and disaturated PC (expressed per mg protein) in lung lavage 48 hours after birth in both control and fasted animals. Gross et al. (1978) concluded that fasting had no effect on lung surfactant content in newborn rabbits.

Studies on the adult lung and effects of deficient diets reveal that a lack of nutritional balance affects the phospholipid content of the lung and an apparent deficiency in lung surfactant as evidenced by greater surface tension of lung extracts, decreased deflation stability and a decreased bubble stability ratio. Also, the activity of enzymes involved in phospholipid synthesis were decreased in fasted animals. This is similar to the results of Hawrylewicz et al. (1975 - from Gross, 1977) who found a decrease in cholinephosphotransferase activity in fetal lungs when pregnant rats were fed a protein deficient diet.

The studies discussed above do indicate that nutritional variation plays a role in pulmonary surfactant production.

It appears as though the most fruitful studies would involve a nutritionally restricted diet rather than total fasting. A study such as this would allow experiments to cover longer time periods. Also, other phospholipids making up surfactant should be analyzed for changes due to nutritional variation. Because PG is functionally important to surfactant, this phospholipid should be included in such an analysis. Much more research into nutritional variation must be done before the importance of this factor in surfactant production can be fully realized.

To summarize this section on factors affecting surfactant only the most significant points will be repeated. (This discussion will only include those factors mentioned after corticosteroids). Pilocarpine was shown to stimulate secretion of surfactant in fetal lung explants. This plus other studies suggests some neurohumoral influence on surfactant secretion. Thyroxine appears to have an effect by accelerating the development of type II cells (i.e. as indicated by their morphological appearance), and apparently increasing surfactant secretion, but it was not shown to have an effect on surfactant synthesis. The induced diabetic state was shown to decrease the amount of glucose incorporated into certain lung lipids, including PC and PG. Insulin restored the ability of diabetic rat lungs to incorporate glucose into lung phospholipids. Insulin was also shown to have an opposite effect from corticosteroids in various lung cell preparations. Six conclusions were drawn from the AM, TH, and cAMP studies. These included an apparent stimulation of PC and SP synthesis by these agents, stimulation of PG synthesis by cAMP, and apparent stimulation of surfactant lipid secretion by cAMP. It was proposed that cAMP may have an effect by promoting glycogenolysis and thereby making more glucose available for lipid production. The insulin-cAMP balance in terms of a possible regulatory effect was discussed. It was proposed that certain prostaglandins may play a role in type II cell differentiation based mainly on coincidental development of type II cells and prostaglandin presence plus catabolic activity of prostaglandin. Some speculation was offered on a possible link between prostaglandins and cAMP in the development of the surfactant system. Finally, nutritional effects on the surfactant system were found to differ between investigations. In some cases, nutritional deprivation appears to have a negative effect on the surfactant system.

References for Influence of Hormones and Other Factors on
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VI. PROPOSED RESEARCH

The final section of this report will outline several proposed in vitro experiments. With the techniques presently available, detailed studies may be performed on in vitro models to test their validity and demonstrate possible lung cell functions. These are the basic aims of the following experiments. Three basic experiments will be outlined, but they are essentially variations on the same theme. This is meant to be more of a rough outline of experiments including references which may be useful in carrying them out.

Experiment A. Surfactant Activity in the Type II Cells of Developing Lung

The purpose of this experiment is to establish the "normal behavior" of fetal and newborn type II cells upon isolation and in primary culture. Different assays for surfactant production will be described. The normal activity of type II fetal lung cells will be an important standard against which to measure the validity of other lung cell culture models. It will also reveal the changes in this population of cells during development. To this author's knowledge, few studies have been published on fetal type II cell populations.

Materials and Methods:

Animals: Rabbits are the animals of choice for this experiment. It must be kept in mind that fetal lung does not contain a high percentage of type II cells until late gestation, and fetal rat or mouse lungs would contain a small quantity of these cells by virtue of their size. Thus, in order to optimize the number of cells available for isolation, the rabbit should be used. This animal has been used in many surfactant experiments, therefore results may be readily compared.

Time of Sampling and Mode of Sacrifice: Pregnant rabbits whose time of conception is known within a few hours should be anesthetized with intravenous sodium pentobarbital and the fetuses delivered by Caesarian section (Gross et al., 1978) when fetuses are 26, 27, 28, 29, 30, and 31 days old (term = 31 days). Newborn rabbits from 1, 2, 3, 5, 7, and 10 days old should be sacrificed by the method of Mason et al. (1977) using sodium heparin and sodium pentobarbital prior to sacrifice (see that study for details - they used rats). This study begins at day 26 of gestation because few type II cells exist any earlier. According to Wang et al. (1971) at 24 days' gestation, only 6.8% of the cuboidal epithelial cells display OIB's and by 27 days, 16.5% have OIB's. A later time for beginning these experiments may be necessary if the yield of type II cells is not adequate to conduct biochemical experiments.

The uteri should be removed from the anesthetized pregnant rabbit and fetuses delivered following the procedure of

Hamosh and Hamosh (1977) to prevent breathing. Delivery of fetuses and all subsequent steps in cell isolation and culture should be done using aseptic technique in a "sterile" room or under a sterile hood.

Removal and Isolation of Lung Cells: Lungs should be removed from the newborn rabbits using the procedure of Mason et al. (1977) which includes perfusion of ventilated lungs. Their procedures for lavage, heavy-particle macrophage uptake, trypsin digestion, mincing, and isolation of type II cells by discontinuous gradient centrifugation should also be followed.

For lung removal and type II cell isolation from fetal lungs the same basic procedure used by Mason et al. (1977) may be used with some modifications. Lungs may be perfused with the buffered salt solution they describe but without lung ventilation. (Note that heparin treatment was used by Mason et al. (1977) in sacrificing animals and this technique was used in sacrificing newborn rabbits. It may be necessary to treat fetuses with heparin in order to take full advantage of their cell isolation technique). Lavage of the lungs and all further steps in type II cell isolation may follow Mason et al. (1977). (Note: The procedures outlined by Mason et al. (1977) were for isolation of type II cells from adult rats, therefore some modifications may need to be made in volumes of solutions used and possibly in concentrations).

Purification: The type II cell population obtained at the interface between 1.040 and 1.080 density solutions using the Mason et al. (1977) procedure are not pure. For cells to be studied in culture, differential adherence in primary culture as described by Mason et al. (1976) may be used to purify this population. (See more details below on plating and maintenance). For studying freshly isolated populations of type II cells, the procedure of Kikkawa and Yoneda (1974) may be used. (Note: They obtained a 95% type II cell fraction after barium sulfate treatment of tissue blocks, suspension, discontinuous gradient centrifugation, and simple centrifugation. They did not perform biochemical studies on these cells. This author does not know if the population would be large enough for biochemical characterization. If not, then one may use the Mason et al. (1977) procedure for obtaining a freshly isolated cell population of type II cells even though it is not entirely pure). Purity will be determined as described by Mason et al. (1977). Viability will be determined by trypan blue exclusion (Phillips, 1973).

Culturing: Type II cell cultures will be plated and maintained as described by Diglio and Kikkawa (1977) using Dulbecco's modified Eagle's Minimum Essential Medium and 10% FCS supplemented with gentamicin and fungizone. Diglio and Kikkawa (1977) found that high density plating ($>3 \times 10^5$ viable cells per sq. cm) and attachment for 48 hours showed greater attachment efficiency than lower density seeding during shorter time periods. Their table on plating level

can be used to determine seeding levels.

Experimental Analyses: The following analyses will be performed on freshly isolated type II cells at each age sampled and on 1, 2, 3, 4, and 5 day type II cell cultures from each age sampled. (There is a five day limit on the cultures under the conditions of Diglio and Kikkawa (1977)).

The lipid composition of cells and lamellar bodies will be determined as well as lipid synthetic activity using labeled precursors. Purified fractions of freshly isolated type II cells will be extracted according to Folch et al. (1957) as described by Rooney et al. (1974). Lipids will be analyzed by the procedures outlined by Rooney et al. (1977).

Cultured cells will be harvested by using trypsin then sedimented, resuspended, and sonicated as described for A549 cells in culture (Shapiro et al., 1978). Lipids from these cells will then be extracted and analyzed according to the procedures cited above.

Isolated purified fractions of type II cells, and cultures of type II cells (harvested according to Shapiro et al. (1978)) may be suspended in a buffered sucrose solution and homogenized as described by Shapiro et al. (1978). These homogenates may then be used for isolation of lamellar bodies according to the process of Gil and Reiss (1973) on discontinuous sucrose gradients. These lamellar bodies will then be extracted with chloroform and methanol using Bligh and Dyer's (1959) method. The lipids may be analyzed by the procedures outlined by Rooney et al. (1977).

Lipid synthesis in cultured type II cells may be followed by using radioactive precursors. PC synthesis in type II cell cultures may be assayed by using [^3H]choline. The paper by Smith (1977) on the A549 cell line may be used as a guide for the labeling and assay procedure. Using the methods he outlined, DSPC and total PC may be quantitated, also. However, he demonstrated continuous uptake of the label for 24 hours. It would be better to pulse these cells one hour prior to sampling then chase them with unlabeled choline. The cells may then be assayed for label uptake every hour for four hours to establish a rate of label uptake.

The incorporation of labeled glycerol may also be followed in order to look at the synthesis of the primary phospholipids of these cultured cells. This author has not been able to locate a good paper showing this label being used for lipid synthesis in cell culture. Different amounts of this label around the range used by Smith (1977) may be tried for one hour periods to see which concentration is best. After the optimal labelling amount (μCi) has been established, then the same basic procedure as outlined above for choline may be used. However, other phospholipids than PC may be assayed for activity using the procedures of Rooney et al. (1977) for isolation, and then using liquid scintillation counting of these isolated, quantified lipids.

If a sufficiently high number of freshly isolated cells can be obtained (which may require freezing cells to get enough), then the same basic procedures as outlined and cited above may be used. However, cells will be suspended in medium with the label and shaken in a 37°C water bath for periods of incubation. After this, they will have to be pelleted and rinsed with "cold" medium at least twice, then reincubated with cold medium until time for lipid extraction and analysis as outlined above for the different labels. Extraction should be done on a medium-free pellet of these cells.

Various enzymes involved in the PC, DPPC, and PG synthetic pathways will be assayed in the freshly isolated type II cells and in the cell cultures.

Choline kinase activity will be assayed in the cell homogenate. Isolated purified fractions of type II cells, and type II cell cultures (harvested by the method of Shapiro et al. (1978)) may be suspended in a buffered sucrose solution and homogenized by the procedure of Shapiro et al. (1978). Choline kinase activity may then be assayed by the method of Rooney et al. (1976) based upon Weinhold et al. (1973) techniques using liver. The assay involves the incorporation of [methyl-¹⁴C]choline into phosphocholine. The assay will probably need to be scaled down because Rooney et al. (1976) were working with homogenates of whole rabbit fetal lungs.

Cholinephosphate cytidyltransferase will be assayed in the soluble fraction of the homogenates obtained as above for both cell cultures and isolated cells. Fractionation of these homogenates to obtain a soluble fraction will follow the methods of Rooney et al. (1975b) for differential centrifugation. The assay itself will follow the procedures used by Rooney et al. (1976). They followed the incorporation of phospho-[methyl-¹⁴C]choline into CDPcholine. Here again, the assay was used for whole fetal rabbit lungs, so it will need to be scaled down.

PAPase activity will be measured in the cell homogenates as obtained above for both isolated and cultured cells. The assay will follow the amount of inorganic phosphate released from phosphatidic acid. The procedure of Maniscalco et al. (1978) (a modification of the Garcia et al. (1976) technique) will be followed.

Cholinephosphotransferase will be measured in the microsomal fraction of isolated and cultured cell homogenates obtained as outlined above. The microsomal fraction will be isolated by the method of Verekeyen et al. (1972) using differential centrifugation. The assay for this enzyme will involve following the incorporation of labeled choline (CDP-[Me-¹⁴C]choline) into 1,2-diacyl-sn-glycerols. Oldenborg and Van Golde (1976) appear to have overcome difficulties in finding the optimal substrate and conditions for this enzyme, so their assay method will be used. Several additional references were cited by them in discussing their technique

which will need to be obtained in order to complete the assay. In brief, they synthesized 1,2-diacyl-sn-glycerols from egg yolk PC, mouse lung PC, and synthetic 1,2-dipalmitoyl PC using phospholipase C. This was the substrate for their assay. They used a filter-disc method of measuring incorporation Goldfine (1966). (To check their results, lipids were extracted then separated from this reaction mixture and it was determined that PC was the only phosphoglyceride labeled).

Phospholipase A₂ will be assayed using a modification of the techniques employed by Longmore et al. (1979). First type II cell cultures will be labeled with [1-¹⁴C]palmitate, [1-¹⁴C]oleate, or [1-¹⁴C]linoleate for one hour. Incubation of isolated type II cells with labels will be for one hour in suspension. After the cells are labeled, they will be homogenized by the technique cited above by Shapiro et al. (1978). After homogenization, the microsomal fraction of the respective cell homogenates may be obtained by the method of Vereyken et al. (1972) as cited above. Activity of the phospholipase A₂ enzyme on endogenous substrate may then be followed using the method of Longmore et al. (1979).

LysoPC:lysoPC acyltransferase will be assayed in the 105,000 X g supernatant (soluble fraction) obtained by the methods of Oldenburg and Van Golde (1976) and Vereyken et al. (1972) from isolated and cultured cell homogenates, prepared as above. The soluble fraction will be incubated with 1-[1-¹⁴C]palmitoyl-sn-glycero-3-phosphocholine and PC will be assayed for this label according to the procedures of Oldenburg and Van Golde (1976).

Lyso-PC acyltransferase will be assayed in the microsomal fraction obtained by the methods of Oldenburg and Van Golde (1976) and Vereyken et al. (1972) from isolated and cultured cell homogenates, prepared as above. The microsomal fraction may then be incubated with [9,10-³H₂]-palmitoyl-CoA plus other compounds, and PC assayed for this label as described by Oldenburg and Van Golde (1976).

Glycerolphosphate phosphatidyltransferase will be measured in the homogenate of isolated and cultured type II cells obtained as above. The assay will be carried out according to the procedure of Rooney et al. (1975a). It will follow the incorporation of L-[¹⁴C]glycerol 3-phosphate into lipid.

Electron microscopic and immunologic examination will be made at the different developmental stages sampled. At each stage of development sampled, cultured cells will be fixed, embedded, and stained according to the procedure of Douglas and Teel (1976) (who used organotypic cultures) for electron microscopic examination. Cultured cells will be fixed and embedded in situ. This method was chosen due to the good preservation of TMS and other cell characteristics. Isolated cells will be fixed, stained, and embedded according to the procedure of Mason et al. (1977) at each stage of development for electron microscopy.

At each stage of development studied, type II cell primary cultures will be "stained" with antibodies to rabbit surfactant protein and examined under the electron microscope. The procedure to be followed is outlined by Sueishi et al. (1977). However, this procedure will be modified slightly because cells and not tissue are being labeled with antibody. Basically this procedure involves injection of a fraction characterized as surfactant from lavage fluid into ducks to obtain antibodies to SAM. This antiserum is then absorbed with rabbit serum, and Fab' fragments of the anti-SAM 7S γ -globulin are prepared. The Fab' fragments are labeled with electron dense horseradish peroxidase. After this, the tissue is fixed and "stained" with conjugated Fab' fragments. The modification of their procedure will involve fixing the type II cell cultures in situ using a shorter fixation time (about one hour or less - the time used by Douglas and Teel (1976) using 2.5% glutaraldehyde). After fixation the cells may be washed immediately without cryostat sectioning, then incubated with specific conjugate (anti-SAM Fab'-horseradish peroxidase labeled fragments). The rest of the procedure may be followed as described in their (Sueishi et al., 1977) paper.

Finally, a RIA may be performed to quantitate the developmental increases in lung surfactant apoprotein in the isolated and cultured type II cells. The methods followed will be those of King et al. (1974). The main difference in this experiment is that rabbit lung surfactant protein, not sheep will be studied. Anti-rabbit SAM antibodies may be obtained in the same way outlined by Sueishi et al. (1977). The rest of the procedure may be followed pretty much as described by King et al. (1974) however, amounts may vary due to the fact that one is dealing with homogenized cells and not tissue. For cultured cells, the method of Shapiro et al. (1978) should be used to harvest cells. Cells may be homogenized using basically the same procedure of King et al. (1974) for tissue homogenization.

One must obtain an apoprotein from rabbit (or rat in the experiment to follow) in order to perform this assay, and it must be tested for purity. The methods of isolation using sheep lung surfactant (King et al., 1974) may be followed for the rabbit and rat. However, it should be stressed that the uniqueness and purity of such a protein must be demonstrated before the RIA is performed. A number of ways exist to demonstrate these characteristics. The methods used by King et al. (1974) may be used for this purpose. SDS-PAGE may be used to demonstrate a single band and this will reveal the MW of the protein. Double-diffusion immunoprecipitation in 0.75% agarose gels with apoprotein, and purified surfactant against purified antisurfactant antiserum will also demonstrate purity.

Experiment B. Characterization of Adult Lung Type II Cells, A549 Cell Line, Clonal Cell Line

The main purpose of this experiment is to obtain results

which may be compared to developing lung type II cells and to cells grown under altered conditions. This experiment should also establish the validity of cell lines for use as models of normal type II cell activity. The basic techniques will be very similar to those used in the first experiment.

Materials and Methods:

Animals: The adult rat will be made use of in this study. It will have an adequate yield of type II cells, may be readily compared with other studies with this animal, and would seem to cost less to use than rabbits. In some ways they may be easier to work with than rabbits.

Time of Sampling and Mode of Sacrifice: Adult rat lung type II cells will be studied as freshly isolated and as primary cultured cells. Adult rats will be sacrificed according to the procedure of Mason et al. (1977) using sodium heparin and sodium pentobarbital followed by transection of the aorta.

Cell line A549 cultures will be studied for a period of 10 days after initiation of the subculture for comparison with Smith (1977).

Clonal type II cell cultures will be obtained from adult rats sacrificed as above.

Removal and Isolation of Lung Cells, Purification, Culturing:

Freshly isolated type II cells and cells for further culturing will be obtained by the method of Mason et al. (1977). Purification of these cells may be performed as outlined in experiment A. Culturing of these purified type II cells may be performed by the methods cited in experiment A.

Cell line A549 may be plated at 1.5×10^6 cells in 75 cm² culture flasks in minimum essential medium, and maintained as described by Smith (1977). FCS will be added as well as antimicrobial drugs (Smith, 1977) unless otherwise stated.

Clonal type II cell cultures will be obtained according to the procedures outlined by Douglas and Kaighn (1974) and Douglas et al. (1976). Lungs will be removed from rats after sacrifice without being ventilated and perfused because Douglas and Kaighn (1974) did not specify such treatment. Tissue will be washed, minced, and further prepared according to their procedures. FCS and antibiotics will be added to the medium unless otherwise specified. This author could not find out how long these cultures could be maintained before they needed to be passed. It appears that these subcultures reach confluence every 2 days (Douglas and Kaighn, 1974). For purposes of comparison, these cultures will be maintained for 5 days if possible.

Experimental Analyses: The same basic procedures outlined in experiment A will be followed here.

Lipid analysis will be performed on freshly isolated type II cells and on cultured type II cells from adult rats.

The A549 and clonal type II cells may also be used in this analysis.

Analysis of freshly isolated type II cells and 1, 2, 3, 4, and 5 day cultures of type II cells will be performed according to procedures cited in experiment A for lipid composition.

Cells of the A549 cell line will be assayed for lipid composition before culturing according to the procedure for isolated type II cells in experiment A. These cells will be assayed every 24 hours for lipid composition for 10 days according to procedures cited in experiment A for type II cell cultures.

Clonal type II cells may be assayed before and after culturing as above for the A549 cell line only they will be assayed at 1, 2, 3, 4, and 5 days after subculturing from the confluent clone growth.

Lamellar bodies from isolated type II cells, A549 cells, and clonal type II cells prior to subculturing will be obtained and analyzed according to the procedures cited for isolated type II cells in experiment A.

Lamellar bodies from cultured type II cells, A549, and clonal type II cells will be obtained and analyzed according to procedures outlined for cultured type II cells in experiment A at their respective time for harvest.

Labeling of isolated type II cells, A549 cells, and clonal type II cells before subculturing with [^3H]choline and labeled glycerol may be done as described for isolated cells in experiment A.

The cultured type II cells, A549 cells, and clonal cells in culture may be labeled for one hour prior to sampling time, then assayed every hour for four hours for uptake into PC (for choline) and into other lipids (for glycerol) as described in experiment A. (Douglas has studied uptake of choline into L-2 cells (Douglas et al. (1976)).

Enzyme activities will also be looked at in these cells. Activities of the enzymes listed in experiment A may be assayed using the same procedures outlined in that experiment. Enzyme activities in freshly isolated, purified type II cells, A549 and clonal cells prior to subculturing may be assayed using the procedures outlined for isolated type II cells in experiment A. Enzyme activities in cultured type II cells, A549, and clonal type II cells may be assayed at their given sampling times using the methods cited in experiment A for cultured type II cells. (Note: Douglas et al. (1976) studied the activities of the CDP-choline pathway enzymes in L-2 cells).

Electron microscopic and immunologic examination will be performed with these cells. Freshly isolated cells, A549, and clonal type II cells will be fixed, stained, and embedded

prior to subculturing using the procedure of Mason et al. (1977).

At each time point sampled, cultured type II cells, A549, and clonal type II cells will be fixed, stained, and embedded in situ using the procedure of Douglas and Teel (1976).

For antibody "staining" the same basic procedure as outlined in experiment A using the methods of Sueishi et al. (1977) may be used here on cultured type II cells, A549, and clonal cultured cells at each time point sampled. However, in this case antibodies will be prepared to rat surfactant. Adult rats may be sacrificed according to the procedures of Mason et al. (1977), then lungs lavaged with saline using the methods of Colacicco et al. (1973) for rabbit as cited by Sueishi et al. (1977). This SAM may then be used to immunize a duck, and the rest of the procedure as outlined in experiment A may be followed.

Conjugated, antirabbit Fab' fragments may be used on the rat type II cells, A549, and clonal type II cells to see if there is some immunologic cross-reactivity in the surfactant protein. The conjugated antirat Fab' fragments may also be used on rabbit fetal and newborn cultures in experiment A.

The RIA will reveal changes in the quantity of surfactant antigen with different culture times and will quantitate the amount of surfactant antigen in adult and type II derived cell line cultures. The difference between the procedure outlined by King et al. (1974) and the procedure to be used here is the fact that antirat SAM antibodies will be used. These antibodies may be obtained according to the procedures outlined above after Sueishi et al. (1977). Isolated cells prior to culturing and cultured cells (harvested as in Shapiro et al. (1978)) will be homogenized using the same basic methods as King et al. (1974) for tissue. The rest of the procedure may be followed as described by King et al. (1974).

Experiment C. Effects of Hormonal and Other Agents on Developing Type II Cell Cultures, Adult Type II Cells, A549, and Clonal Cell Cultures

The first two experiments described here provide a basis for the knowledge of how culturing may change cell characteristics (i.e. differences between isolated or non-subcultured cells and the cultured "versions" of these same populations). They also establish the "normal" behavior of these cells under the conditions of isolation and culture used here. In addition, differences between cells taken from developing animals and from cell lines or adult animals can be compared. The developmental changes in cells are studied, and differences between cells of different species and cell lines are compared.

Having obtained this data, mechanisms regulating the

functions of these cells may now be probed. An earlier portion of this paper was devoted to the effects of hormones and other agents on lung. Very little work has been done with such agents using cell culture models. The purpose of this experiment is to explore the mechanisms which may control type II cell functions.

Materials and Methods:

The same cell cultures and assays described in the first two experiments will be used here. The difference between this experiment and the first two experiments is the use of hormones and other agents on surfactant related activities in these cells.

Cell Cultures: Fetal and newborn type II cell cultures will be established and maintained by using the methods outlined in experiment A. Adult type II cell cultures, A549 cell cultures, and clonal cell cultures will be established and maintained as outlined in experiment B. There will be changes in the composition of the incubation media used to maintain these cultures as will be noted later.

Hormones and Other Agents: Six different hormones (including cAMP and aminophylline), and four different "agents" capable of inducing surfactant secretion will be studied alone and in combination. These compounds will be listed here along with some guidelines for their use. Figures 19 and 20 show how these hormones and secretory agents will be combined.

Cortisol (C) has been shown to accelerate lung maturation as well as stimulate choline incorporation into saturated PC (Smith, 1977). Following the guide of Smith (1977), this hormone will be used in a 10^{-6} M concentration in all cell cultures. A competitive inhibitor of glucocorticoid, 11-deoxycortisol (10^{-5} M) will be added to media containing cortisol to see if the effects seen were indeed due to cortisol stimulation (Smith, 1977).

As was discussed in the section on hormones and other agents, thyroxine appears to accelerate type II cell development and possibly surfactant secretion. Wu et al. (1973) found as they expected that thyroxine did not have an effect on lung maturation in fetal rabbits when administered to the mother (most thyroxine apparently does not cross the placenta). However, direct injection of the fetus and amniotic sac with this hormone did accelerate lung alveolar maturation and apparent surfactant secretion. Taylor et al. (1979) used thyroxine on organotypic fetal rat lung cultures to see if it affected prostaglandin production. The concentration used in that study was 10^{-6} M. Therefore, 10^{-6} M L-thyroxine (Hamosh et al., 1976 specified this in rat lung) will be used in this study.

The role of insulin in lung maturation is not known. As discussed in the section on hormones and other agents, contradicting findings have been made as to whether or not

insulin enhances or inhibits alveolar maturation. Smith et al. (1975) have shown that insulin antagonizes cortisol action in cultured fetal lung cells (used a monolayer culture of trypsin-dispersed lung cells (Smith et al., 1974)). Insulin had a slight positive effect on PC synthesis between 10 and 100 μ U/ml, but reduced PC synthesis in cortisol treated cultures. Smith et al. (1975) used from 1 to 100 μ U/ml of insulin to demonstrate an effect. Taylor et al. (1979) used 0.1 units/ml in organotypic cultures to look at prostaglandin synthesis. This study will use 0.1 units/ml as used by Taylor et al. (1979).

Hamosh and Hamosh (1977) have shown that prolactin injected into rabbit fetuses apparently stimulated total phospholipid, PC, and DPPC content in fetal rabbit lung, but did not affect overall body size, lung weight, DNA content of lung, or protein content of lung. Taylor et al. (1979) used this hormone in a dosage of 10 μ g/ml in their experiment on prostaglandin production in organotypic fetal rat lung culture, therefore, this study will employ the same concentration.

The cAMP phosphodiesterase inhibitor, aminophylline, has been shown to stimulate PC and SP synthesis in fetal rat lung organ culture with a slight increase in DSPC synthesis (Gross and Rooney, 1977). They used 1.0 mM aminophylline, however, 0.7 mM concentration will be used in cell cultures of this experiment. Dibutyryl cAMP in these organ cultures caused similar results. The cAMP was used in a concentration of 0.2 mM. Hallman (1977), using a similar concentration (0.25 mM) of cAMP in the presence of theophylline, demonstrated an increase in PG synthesis in lung slices from fetal rabbits. Therefore, this study will utilize the 0.2 mM concentration of dibutyryl cAMP.

Acetylcholine, carbachol, isoproterenol, and pilocarpine have been shown to increase secretion of surfactant phospholipids. The following is a list of concentrations to be used along with references for papers from which the concentrations were derived:

- acetylcholine 10^{-4} M (in presence of 10^{-3} M physostigmine, an acetylcholinesterase inhibitor) (Smith, 1977)
- atropine 10^{-4} M (acetylcholine inhibitor - will be used with acetylcholine to demonstrate that effects are due to that compound) (Smith, 1977)
- carbachol 10^{-6} M (atropine will also be added to demonstrate specificity)
- isoproterenol 10^{-6} M (will be used with propranolol to demonstrate specificity of agent) (Smith, 1977)
- propranolol 10^{-5} M (inhibits isoproterenol) (Smith, 1977)
- pilocarpine 10^{-6} M (same concentration used in fetal lung explants - will also be combined with atropine) (Pysher et al., 1977).

Experimental:

Three different experiments will be described here. The first one briefly tests the ability of hormones to replace

FCS. The second one tests the hormones and media conditions for effect on surfactant parameters. The third tests the effects of various secretory agents on surfactant related characteristics, plus it tests the combination of hormones plus secretory agents for their effects on the surfactant system.

Experiment a. FCS Replacement

In most every culture medium for growing cells, the presence of fetal calf serum (FCS) appears to be required at least to get the culture started. To this author's knowledge, the growth factor(s) of FCS which enable cells to grow in culture is not known. However, hormones are likely to be a part of this serum which may have effects on cultures. Therefore, to try and eliminate or reduce this unknown factor in these experiments, an experiment will be performed to test effects of hormones as possible FCS replacements.

The first part of this experiment will require adult rat lung type II cells to be grown in culture as described in experiment B. However, one of the hormones listed previously or aminophylline or cAMP may be added to the medium at the concentration specified above in the place of FCS. Every 24 hours these cells may be observed for attachment, then harvested by scraping with a rubber policeman and viable cells counted using trypan blue exclusion (Phillips, 1973). The cells may be maintained for 5 days (assayed every 24 hours) if possible.

The second part of this experiment repeats the first part only using a combination of hormones (including cAMP and aminophylline). Combinations of 2, 3, 4, 5, and finally all six hormones may be tried. Square A in Figure 19 shows some of the possible combinations and how others may be derived.

These experiments may provide a clue as to what is present in FCS that enhances growth of cultured cells, and may provide information on requirements for type II cell growth.

Experiment b. Type II Cell Culture Treatment with Hormones

Fetal and newborn cell cultures isolated at the ages specified in experiment A, adult type II cell cultures, clonal type II cell cultures, and A549 cell cultures will be established as outlined in experiments A and B. However, the timing and preparation for addition of hormone will somewhat follow the method of Taylor et al. (1979) who looked at the effect of various hormones on prostaglandin synthesis in organotypic type II cell cultures.

The first part of this experiment tests the effects of various culture conditions including the addition of a hormone on different surfactant parameters.

Cultures should be seeded such that they reach confluence at the third day in culture. An idea for seeding densities may be determined from results in experiment A.

Figure 19. Possible Combinations of Hormones, Aminophylline, and cAMP

(Note: Aminophylline and cAMP are often included the the term "hormone" in discussing these experiments but of course these are not actually hormones).

	C	T	I	P	A	cAMP	(A)
C		CT	CI	CP	CA	CcAMP	
T			TI	TP	TA	TcAMP	
I				IP	IA	IcAMP	
P					PA	PcAMP	
A						AcAMP	
cAMP							

	C	T	I	P	A	cAMP	(B)
CT			CTI	CTP	CTA	CTcAMP	
CI				CIP	CIA	CIcAMP	
CP					CPA	CPcAMP	
CA						CACAMP	
CcAMP							
TI				TIP	TIA	TIcAMP	
TP					TPA	TPcAMP	
TA						TAcAMP	
TcAMP							
IP					IPA	IPcAMP	
IA						IAcAMP	
IcAMP							
PA						PACAMP	
PcAMP							
AcAMP							

Three more "squares" would need to be set up to obtain all the permutations possible for combining these six compounds (C, T, I, P, A, cAMP). The pattern set by these two sample squares may be followed to obtain the other three squares.

C = cortisol
T = thyroxine
I = insulin
P = prolactin
A = aminophylline
cAMP = dibutyryl cAMP

On day three of culture, fresh medium will be fed to the cultures in the following scheme:

- complete medium → 10% FCS, antibiotics, medium cited in experiment A or B depending on culture
- hormone → complete medium + specified amount of hormone
(this step will be repeated for each hormone)
- FCS → complete medium minus FCS
- antibiotics → complete medium minus antibiotics
- FCS -antibiotics → complete medium minus FCS and antibiotics
- FCS +hormone → complete medium plus hormone and minus FCS
(this step will be repeated for each hormone)
- antibiotics +hormone → complete medium plus hormone and minus antibiotics (this step will be repeated for each hormone)
- FCS -antibiotics +hormone → complete medium minus FCS and antibiotics plus hormone
(this step will be repeated for each hormone).

These media will remain on the cultures for 36 hours. At the end of that time the medium will be replaced as above only without hormone added (i.e. -FCS +hormone media will be replaced with -FCS medium) for another 36 hours, then cultures will be assayed for:

cellular lipid	electron microscopy
lamellar body lipid	antibody staining
lipid synthesis	amount of surfactant (RIA)
enzyme activities	secretion (to be described)

according to procedures outlined in experiments A and B depending on the culture being tested.

In addition to the hormones already discussed, the effects of 11-deoxycortisol (10^{-5} M) on cortisol treatment will be performed. Cortisol plus 11-deoxycortisol will be co-incubated. As a control, 11-deoxycortisol will be incubated alone, instead of hormone. The assays above should be done on the control cells and cells incubated with both hormone and inhibitor.

The assay for secretion by cells treated according to the above scheme is somewhat derived from Smith (1977). For 24 hours prior to addition of hormones (i.e. during the day two of culture) cells will have 1 μ Ci/ml of [3 H]choline added to the media (see above scheme). At the end of that time, cultures should be washed three times with the media to be added, and finally incubated with the media as outlined above. At the end of the incubation period, cells and media will be assayed for saturated PC labeled with [3 H]choline according to the procedures of Smith (1977).

The second part of this experiment will essentially repeat the first with the exception that hormones used in the above media will be combined as outlined in Figure 19. For example, cortisol (10^{-6} M) plus thyroxine (10^{-6} M) may

be added in combination. After that, combinations of 3, 4, 5, and finally all 6 hormones will be added to the same culture. (The addition of 11-deoxycortisol may be eliminated in these subsequent experiments).

Experiment C. Secretory Agents

This experiment has been broken down into four parts. The first part deals with the effects of these agents on various surfactant parameters. The second deals with the secretory effects of several of these agents. The third part involves the combined effects of hormones and secretory agents on surfactant parameters and secretory activity. The fourth part outlines how pilocarpine may be used alone and in combination with hormones to study surfactant parameters and secretion.

The first part of this experiment involves the use of acetylcholine, atropine, carbachol, isoproterenol, and propranolol according to the procedure of Smith (1977) as modified here.

Smith (1977) allowed only 15 minute incubation periods using the above agents for expression of labeled PC secretion. Because most of the assays performed in experiments A and B are not as sensitive (not all involve labeled compounds) as Smith's assay for secretion, the length of time following addition of these agents will be lengthened.

Cell cultures will be grown to confluence in 25 cm² flasks in complete medium (the growth medium specified for that culture in experiments A and B). The media will then be replaced with media minus serum and antimicrobial drugs for 48 hours. Cells will then be treated with the following agents for 15 minutes in Hank's BSS after washing the cells three times with Hank's BSS:

- acetylcholine (+physostigmine)
- acetylcholine (+physostigmine) + atropine
- atropine
- carbachol
- carbachol + atropine
- isoproterenol
- isoproterenol + propranolol
- propranolol.

Serum and antimicrobial drug free medium will be replaced after washing the cells three times with Hank's BSS. Some cells will be used in the assays outlined in experiments A and B for lipid composition, lipid synthesis, morphology, and presence of surfactant protein. Other cells will be maintained in the above serum and drug free media and assayed every 30 minutes for a total of three hours after the end of agent treatment.

The second part of this experiment follows Smith's (1977) assay for evidence of secretion. The effect that each of these agents, listed alone or in combination with an inhibitor above, on secretion will be tested exactly as outlined

by Smith (1977) with the modification that the basic medium used will be that specified in experiments A or B for that culture type. Serum and antimicrobial drugs will be eliminated from these media as outlined by Smith (1977).

The third part of this experiment essentially repeats the first two parts with the addition of hormone(s) as the only modification. The first part of the experiment described previously will be followed here with a few changes. After the 48 hour incubation period with serum and antimicrobial drug free medium, cells will be treated with C, T, I, P, A, or cAMP in the medium (serum and drug free) for 24 hours before addition of secretory agents.

The second part of the experiment will be followed as outlined above (Smith, 1977) only C, T, I, P, A, or cAMP, will be added during the labeling period. The combinations of hormones and secretory agents to be used in this third part can be found in Figure 20.

The fourth part of this experiment repeats the first three only with pilocarpine. This author has not located an article which tells how long pilocarpine may take to have an effect on isolated cells, or if it even does have an effect. Therefore, the basic outline of the first two parts of this experiment will be followed for pilocarpine. Part one will be followed as outlined only pilocarpine ($10^{-6}M$), and pilocarpine plus atropine ($10^{-4}M$) will be added to the cultures. Incubation times with pilocarpine will be for 15, 30, 60, and 90 minutes. Part two will follow the procedure of Smith (1977) as cited above. Of course pilocarpine, and pilocarpine plus atropine will be used in the secretion study. Fifteen minutes may not be enough time to see the secretory activity elicited by this compound, therefore incubation times of 15, 30, 60, and 90 minutes with pilocarpine and pilocarpine plus atropine will be used. Part three will be followed as outlined above for effects of hormones plus secretory agents only pilocarpine will be used. Both portions of part three will be carried out according to the procedure outlined above only pilocarpine incubations will be for 15, 30, 60, and 90 minutes.

These are just a few experiments possible using cell culture techniques to study surfactant synthesis. Many variations of the experiments outlined here are possible. Some other agents which could be tested are the A23187 ionophore, colchicine, and vinblastine. The ionophore would yield information on the role of Ca^{2+} in lipid synthesis and/or secretion. Colchicine and vinblastine may be used to see what role MT's may play in surfactant synthesis and/or secretion. Protein synthesis inhibitors (such as puromycin, actinomycin D, and cycloheximide) and metabolic inhibitors (such as cyanide) are a few examples of the types of agents which may be tested for their effects on surfactant synthesis and/or secretion in these cell cultures. Prostaglandins may also be tested.

Figure 20. Possible Combinations of Individual Hormones
with Individual Secretory Agents

	C	T	I	P	A	cAMP
AC	AC/C	AC/T	AC/I	AC/P	AC/A	AC/cAMP
CA	CA/C	CA/T	CA/I	CA/P	CA/A	CA/cAMP
IS	IS/C	IS/T	IS/I	IS/P	IS/A	IS/cAMP
PI	PI/C	PI/T	PI/I	PI/P	PI/A	PI/cAMP

AC = acetylcholine {cholinergic (Smith, 1977))
 CA = carbachol {cholinergic (Smith, 1977))
 IS = isoproterenol {adrenergic (Smith, 1977))
 PI = pilocarpine {cholinergic (see hormone + agent section)

Two other culture systems which may be quite useful for further in vitro study are fetal lung organ cultures and organotypic culture of fetal lung cells (Douglas and Teel, 1976). The problem with these cultures is that the populations are not pure. Naturally the organ culture has different cell types, however, the organotypic culture appears to be significantly enriched for type II cells. Once results from the isolated type II cell cultures are obtained, then the organ and organotypic models may also be tested to see if similar results are obtained before making further in vivo tests.

One important aspect to keep in mind when working with cells in culture is that their responses may not be unique. In several studies which Douglas has been involved in have utilized other cell types under the same culture conditions as the lung cells. Such "checks" will help to differentiate between unique cell behavior and those characteristics due to culture conditions.

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VII. FUTURE DIRECTIONS

The purpose of this report has been to give an overview of the lung surfactant system. The approach used was that of trying to provide examples of the types of studies and results obtained in looking at this system. Due to the large volume of literature on this topic, such an approach would naturally result in gaps of information. It is felt, however, that some of the most important points about this subject have been made.

Because each section of this report contained a summary of pertinent points, they will not be reiterated here. It may be useful instead to try and identify areas where basic research on this topic may be headed.

Two areas stand out in this author's mind as where the bulk of basic research may be centered for the next several years. First, research on the protein-surfactant association seems to be a growing interest. As was stated in that section, this area seems to be less well defined. One problem which needs to be resolved is the specificity of protein in surfactant (i.e. is this protein synthesized and secreted only for use in the surfactant matrix). The immunological studies indicate that protein (other than serum protein) is found in surfactant, but there exists the possibility that their antibodies are formed to a protein found on lung cell surfaces (perhaps in the surface membrane) which is easily removed during lavage and may have no functional role in surfactant. Of course the lipid-protein association in surface-active fractions and antibody labeling studies discussed in the protein section lend support for a specific interaction, but the results of the last two studies presented in that section cast doubt on this. One way in which specificity might be indicated is if a specific lipid-protein complex could be found in material secreted by isolated type II cells. If a specific interaction is proven, then the next step would be to try to define the function of that protein in surfactant. It would also be useful as a marker for following surfactant synthesis.

The second thrust of research appears to be toward cellular models of this system. The main advantages here are that one is working with a "pure" population of cells and the various experimental parameters are more easily defined than in a whole tissue or in an organism. Ultimately results from experiments on these cells will lead to experiments in whole organisms to see if clinically viable treatments may be devised. Innumerable experiments using cell models may be designed. The most obvious are investigations using various hormones and neurohumoral substances as proposed in the research section of this paper. As was alluded to earlier, perhaps more attention could be focused on the protein synthetic and secretory activities of these cells as a means of solving the controversy over specific protein-lipid interactions.

These are just a few ideas on where research may be headed. The ultimate goal of research on the surfactant system is to come up with a means of clinically treating and possibly preventing RDS in newborn infants. Such research has already contributed toward this end. Once this system is better understood, perhaps better methods of treatment may be developed.

A REPORT ON THE SURFACTANT SYSTEM OF THE LUNG

by

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AN ABSTRACT OF A MASTER'S REPORT

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1979

This paper is a report on the surfactant system of the lung. It has been broken down into seven parts. The first two sections are introductory. They include reasons for studying this system and some brief notes on lung development, respectively. The third section of this report defines the surfactant system. It is comprised of five subsections which include discussions on the phospholipid, protein, and carbohydrate composition of surfactant. The last two subsections cover the structure, and synthesis of surfactant. The fourth section of this report primarily deals with the alveolar type II epithelial cell and model systems for studying these cells. Section five includes a discussion on different hormones and other factors which may affect the surfactant system. A rough outline of three experiments constitutes the sixth section. A brief identification of two directions in which research on this system appears to be headed makes up the concluding portion of this report.